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Food safety practices in catering during the coronavirus COVID-19 pandemic

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Abstract:

On January 30, 2020, the Director-General of the World Health Organization declared the outbreak of COVID-19 a Public Health Emergency of International Concern. There is hardly a country in the world that is not currently facing this problem. The number of cases is constantly growing, patients and carriers being the main mode of transmission. The economies of all countries are at stake. However, people need essential goods and food, regardless of the situation. In this respect, agriculture, food industry, food market, and catering have become priority industries. A continuous operation of food service enterprises (FSE) is crucial for the uninterrupted food supply in the period of preventive measures. The paper describes how pathogen makes its way into FSEs, spreads, and infects people. This information makes it possible to assess the probability of coronavirus infection and to reduce its spread, thus ensuring the safe operation of the enterprise. There are three transmission routes the coronavirus can take at a FSE: (1) aerial transmission from clients to staff or from employee to employee via direct or indirect contact, and (3) transmission via contaminated surfaces, e.g. packaging, furniture, equipment, etc. FSEs have to follow the recommendations published by the federal and/or local authorities, which may vary depending on the COVID-19 incidence rate in the area. These recommendations are based on the probability of the public health risk associated with person-to-person transmission, rather than on food safety.

Keywords: Coronavirus COVID-19, containment, food service enterprises, preventive measures, recommendations

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INTRODUCTION

An outbreak of coronavirus infection in Wuhan, China, has led to a global epidemic declared a Public Health Emergency of International Concern by the World Health Organization [1]. The emergence of a new human coronavirus has become a global public health problem. The virus has demonstrated a variety of forms, health effects, and incubation periods. COVID-19 is resistant to environmental factors, has a high penetration ability, and may be lethal. The challenge lies in the new form of the virus and the lack of experience in combating it, as well as in the absence of an effective medicine. An infected patient has practically no chance of recovery without medical intervention. Children under 14 and 65-plusers are at risk [2]. Since the current trajectory of the COVID-19 outbreak is unknown, authorities have to develop public health preventive measures to curb the spread and gain experience that could be transformed into treatment recommendations.

The COVID-19 pandemic has affected the whole world and all spheres of human life. Italy, Spain, France, Great Britain, USA, and China have suffered the most damage. The situation in Russia remains noncritical, which can be explained by its territorial features, longterm experience in anti-infection preventive measures, and their timely implementation. Risk awareness motivates people to adopt preventative behavior. The growing incidence and fatal outcomes abroad demonstrated Russians the need to follow official recommendations, both at home and at work.

COVID-19 is a global problem, and all global economies are going through hard times. However, there are vital industries that require priority measures, food supply being one of them. The US food and agricultural sector is 100% privately owned. It includes 2.1 million farms, 935 000 restaurants, and more than 200 000 enterprises of food production, processing, and

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storage. This sector accounts for about 20% of economic activity. The American Food Industry Association (FMI) promptly issued a Coronavirus Preparedness Checklist. According to the FMI, a pandemic will require the mutual efforts of many related spheres, including health, supply chain, logistics, food safety, labor, emergency management, and the media.

The food sector is one of the strategic sectors of Russian economy. It owes its strategic implication to the fact that every citizen is a consumer of food products. The national security demands that citizens should have access to essential food products of domestic origin, regardless of imports. The food sector is represented by agriculture, food industry, food processing industry, and public food service. It is a backbone sphere of Russian economy. The food sector forms the agri-food market and ensures food security. Agriculture employs 4.346 million people, food and processing industry accounts for two million jobs, and one million people are engaged in public catering. Together, this is more than 10% of the total number of people employed in the economy. Russian food industry is represented by more than 50 000 enterprises, while public catering includes 187 000 enterprises [3]. Therefore, the national food sector is important, especially in emergency situations.

The Federal Service for Supervision of Consumer Rights Protection and Human Security responded to the epidemic as early as in February 2020. It developed Recommendations for the Prevention and Disinfection Measures against the Spread of New Coronavirus Infection in Catering Enterprises and Food Storage of Educational Organizations [4].

The coronavirus outbreak exposed serious problems in all sectors of human life, including food security. The research objective was an analysis and synthesis of the available information in order to bring it to the scientific community and the population engaged in the food sector, thus facilitating the adaptation of food service enterprises to the extreme conditions and preparing them for a possible worst-case scenario.

The practical application of the article is that it can inspire further studies of this urgent problem and set goals for the future scientific research. This seems to be the case when the development does not go "from science to practice" but "from practice to science".

RESULTS AND DISCUSSION

Canteens and refectories expose staff and clients to pathogenic microorganisms, e.g. viruses or bacteria. Workers in the production and service areas should know the so-called sanitary control points and be able to control them so as not to endanger the health of colleagues and clients.

The work of food service enterprises (FSE) during the COVID-19 pandemic is fraught with extreme conditions. At a FSE, the pathogen can spread via the following routes: - by inhalation of microorganisms that can stay suspended in the air for a long time;

- by direct contact with oral fluids or other bodily materials that enter the FSE premises with clients;

- when the mucous membrane of the nose, mouth, and eyes is exposed to droplets and aerosols that contain microorganisms formed in the infected person and spread over short distances with coughing, sneezing, or talking without a face mask; and

- by indirect contact with contaminated tools and/or surfaces [5–10].

During the outbreak of COVID-19, the infection can spread by any of these routes, each of which is associated with an infected person visiting public places, including FSEs.

Hotels, catering and tourism incur huge losses. During the quarantine period, most enterprises were closed as high COVID-19 incidence is associated with crowded places. Therefore, it is extremely important to prevent a further spread of the virus in public places [11, 12]. Social distancing is one of the recommended preventive measures. Social distancing is most effective in all scenarios, e.g. airborne contamination when the microorganism stays viable in the air for a long time, or in cases of aerial transmission by coughing or sneezing, as well as in cases of direct or indirect physical contact, e.g. via contaminated surfaces, etc.

Adequate measures to combat the pandemic in crowded places are possible only with respect to a full understanding of the transmission mechanism and viability of the virus. Given the current global situation, the mechanism of transmission of the COVID-19 virus at FSEs requires an urgent and thorough research. Such research could help analyze and adapt measures aimed at COVID-19 risk reduction.

Food service employees run into danger of the COVID-19 infection. They communicate with clients face to face and have to deal with cutlery and table surfaces. Moreover, they are exposed to potentially dangerous biological material, e.g. saliva droplets on napkins and tableware. Therefore, FSE employees must know and follow the necessary safety rules, which may play a great role in preventing the spread of COVID-19 [5].

There is currently no evidence that COVID-19 can be transmitted via food or food packaging. Nevertheless, a person can get COVID-19 by touching a contaminated surface or object and then touching their mouth, nose, or eyes. However, this is not the main way the virus spreads. The US Centers for Disease Control and Prevention claim that COVID-19 does not live long on surface areas. Therefore, the risk of virus transmission via food or packaging is very low. It takes a few days or weeks to deliver food products and goods to FSEs. The person-to-person route is more likely, e.g. via close contact with a patient or carrier.

We performed a brief analysis of scientific literature that revealed the following COVID-19 transmission routes:



Figure 1 Transmission routes of 2019-nCoV at food service enterprises

- a person-to-person direct transmission through coughing, sneezing, inhalation of droplets, contact with oral, nasal, or ophthalmic mucous membranes, or indirect transmission via saliva [5, 11, 13, 14];

- from surfaces to humans;
- from animals to humans; and
- asymptomatic transmission [15].

There is currently no evidence that COVID-19 can be transmitted through food products. Yet, this fact does not mean that there is no risk. To assess the chance of coronavirus infection in FSE conditions, let us describe the mechanisms by which the pathogen enters a FSE, how it is transported and transmitted to humans.

Aerial transmission. This mechanism of COVID-19 transmission has been described in several studies [5, 7-8, 11, 15-18]. The pathogen can stay airborne and viable for a long time. It can spread via direct contact with oral fluids or other bodily substances. The conjunctiva of nasal or oral mucosa may contact with droplets and aerosols that contain microorganisms generated by an infected person and set in motion by coughing or talking at a close distance with no face mask on. Finally, the virus can spread through indirect contact with contaminated tools, dishes, or surfaces [7-10]. If an infected person is present on the FSE premises, the infection can follow any of these paths (Fig. 1).

Food production and catering practices show that many production processes result in droplets or suspensions that hang in the air, e.g. washing the dishes, equipment, and tools, or processing raw materials, etc. These droplets move freely in the air stream. As a result, there is a chance that they can capture the virus and carry it on [17].

Community transmission. The waitperson comes into direct or indirect contact with the bodily liquids (biological media) of the clients through table surfaces, dishes, cutlery, napkins, toothpicks, etc. Such exposure is likely to facilitate the COVID-19 spread [9].

Transmission via contaminated surfaces. The coronavirus remains viable on such surfaces as glass, plastic, wood, or metal [7, 18]. Therefore, all surfaces that food workers have to deal with are potential sources of COVID-19 transmission. There is a practice

of detecting droplets and aerosols from infected people who can contaminate surfaces while visiting public places. At FSEs, the list of potentially contaminated surfaces includes tables, chairs, furniture, door handles, etc. The packaging surfaces of products and goods supplies are another potential source of the coronavirus infection. At the stage of the incoming goods inspection, the employees touch the surfaces that may contain traces of the viral infection. Thus, the virus can enter a FSE via supply transportation or simply via contact with external environment and infected people who contaminate the surfaces around them. Some types of coronavirus are known to remain viable at room temperature from two hours to nine days. Several studies showed that the virus has a better chance of survival at 50% of relative humidity than at 30%. According to Russian regulatory documents, the recommended relative air humidity at FSEs should be 60–40% in the cold season and 40–60% in the warm season. Therefore, maintaining a clean and dry environment at FSEs can help reduce the COVID-19 viability [2, 19, 20].

Popova, the Chief Sanitary Doctor of the Russian Federation, issued a monitoring system and a number of decrees that control the spread of the coronavirus infection in Russia. The decrees contain preventive measures against the COVID-19 infection. As the threat of importation and spread did not cease, Federal Law No. 53 On the Sanitary and Epidemiological Safety of the Population was issued on March 2, 2020. It introduced some further preventive measures. Additional recommendations on the COVID-19 prevention were developed for employees and heads of organizations, regardless of the legal forms of ownership.

FSEs had to limit public events during the pandemic. As for refectories and canteens, they received the following recommendations, depending on the specific conditions at the enterprise they serve:

- if the enterprise has a canteen, it is to be provided with disposable tableware. After using, the utensils are collected, disinfected, and destroyed in the prescribed manner. Reusable utensils are to be processed in specialized dishwashers at $\geq 65^{\circ}$ C for 90 min, or manually at $\geq 65^{\circ}$ C with disinfectants, as stated in the related sanitary standards;

cooking process should involve high-level heat treatment technologies;

- sale or consumption of raw or insufficiently thermally processed products of animal origin is forbidden;

- butchers should observe personal hygiene rules, which include frequent hand washing after contact with raw materials and products of animal origin;

 expired raw materials can cause food poisoning; therefore, feedstock volume should be planned taking into account the decreasing amount of produced products;

- canteen and refectories should give preference to foods with a high nutritional value;

- the incoming goods inspection is to ensure a strict quality control of the incoming raw materials and the accompanying documents;

- if the enterprise has no canteen, employees are not allowed to eat at workplaces: they can have their meals only in a specially designated meal room; and

- should there be no meal room, employees are to be provided with a specially allocated meal area with a sink for hand washing and disinfectants.

The above recommendations also determine the list of food service enterprises that can be potentially dangerous:

 – crowded places, e.g. food courts and canteens, with a high circulation of people, which increases the risk of encountering coronavirus patients and virus carriers;

- FSEs that sell finished products in reusable utensils are to ensure their proper sanitization with the recommended concentrations of disinfectants;

- self-service enterprises, e.g. self-service buffets, salad bars, etc.

FSEs that deliver finished culinary products have certain advantages in the current situation. Disposable tableware and shipping containers, e.g. thermal bags, reduce the risk of contamination. However, in this case, the risk zone shifts towards the person-to-person transmission route: the infection can be transmitted via airborne droplets from the delivery person to the consumer or vice versa. The surfaces the delivery person contacted with have to undergo additional disinfection, while both the employee and the client are to wear face masks and disposable gloves.

FSEs unlicensed to deliver finished products cannot operate in full during the quarantine. However, they can sell takeaway meals in disposable packaging, on condition they follow all recommended preventive measures concerning the seller – client communication [7].

Recommendations for restaurants offering takeaway services include the following points:

- FSEs can only accept online or phone orders, without face-to-face communication on the FSE premises, of which consumers should be informed via traditional advertising means;

the delivery time should be individual for each client,
i.e. they must not enter the premises until their order is ready;

- spontaneous clients are be advised to leave the premises to place their order by telephone or online and return at the appointed time to receive it;

 customers whose orders are ready must enter the site one at a time to collect their orders and make payments; and

- employees are to prevent crowding outside by using queuing systems to maintain the recommended twometer distance.

In many Russian cities, including Moscow, public and leisure events consisting of 50 people or more were banned as early as in mid-March. Cafes, restaurants, and other FSEs fell under these restrictions, since the number of personnel and clients combined is likely to exceed 50 people. An exception was made only for cafes, restaurants, and FSEs that provide delivery service. In the Kuzbass, the flow of clients to cafes and restaurants decreased by an average of 50% in less than a week. During the quarantine, most consumers prefer to eat at home or take home-cooked food to work. On March 17, 2020, all FSEs received Recommendations for the Prevention of the New Coronavirus COVID-19 Infection and the Protection of Citizens in Trade and Public Catering Organizations from the Ministry of Industry and Trade of the Russian Federation. The recommendations set forth immediate preventive measures to protect citizens in public catering and trade organizations.

However, not all FSEs can switch to the takeaway mode. This type of activity has its own specific features associated with the quality and safety of remotely sold products. Food delivery imposes extra obligations on the businesses, i.e. appropriate permits from regulatory authorities for the delivery of finished catering products.

In Russia, regulatory documents that control food safety issues include Sanitary Regulations and Standards, Technical Regulations of Customs Union, etc. For instance, the On Food Safety Technical Regulation of Customs Union 021/2011 indicates the mandatory presence of a safety management system in the food industry^I. According to Article 10 (Clause 2), food production processes must be based on the principles of Hazard Analysis and Critical Control Points. Otherwise, FSEs cannot fully ensure the production and sale safety, especially during the coronavirus pandemic. The basic systems for ensuring the quality and safety of food products in industrial practice include Good Manufacture Practice (GMP) and Good Hygiene Practice (GHP). Maintaining these systems can minimize the possibility of surface contamination or eliminate it. This factor is important for the safe operation of FSEs, both in the current situation and in the future.

Infection control at FSEs. Catering personnel and managers should be aware of coronavirus transmission routes, symptoms, and preventive measures.

Identification of potentially infected clients. All catering staff should be prepared to identify and report a client suspected to be infected. Ideally, COVID-19 patients are not allowed to visit public places. However, FSE employees ought to maintain COVID-19 alert and refuse to provide service to any client with symptoms. They must immediately inform the manager of possible infection, as well as appropriate authorities as instructed.

Protocol for assessment of FSE staff. The epidemiological safety of a FSE is the responsibility

¹ TR TS 021/2011. Tekhnicheskiy reglament Tamozhennogo soyuza "O bezopasnosti pishchevoy produktsii" [TR CU 021/2011. Technical regulations of the Customs Union "On food safety"]. 2011.

of its managers. They are to monitor the possible incidence among the employees every day. Managers must keep their staff updated on the situation and make sure that they take the situation seriously. Managers are to collect data that can be used as the primary method for identifying potential COVID-19 carriers. In other words, they are to measure the body temperature of their employees with a non-contact forehead thermometer.

A simple survey is another useful precaution. The list of questions may include the following: Have you been abroad in the past 14 days? If yes, what country did you visit? Have you had a fever in the past 14 days? Have you participated in gatherings, meetings, or had contact with large groups of strangers? Have you had contact with a patient with confirmed coronavirus infection? Do you have breathing problems? etc. If the employee gives a positive answer to any of the questions, he/she is suppoused to be self-isolated and guarantined, and the management must inform the appropriate authorities as instructed [5, 22]. If an employee proved COVID-19positive, employers should inform the staff about the possibility of COVID-19 infection without revealing the identity of the infected employee. The latter is to be denied access to the premises until officially reported safe to return to work by health authorities.

Hand hygiene. Foreign sources reported cases of fecal-oral route of COVID-19 transmission, which makes the issue of hand hygiene even more important for FSE staff. Despite the fact that hand hygiene is mandatory in food production as part of sanitary and hygienic requirements, the level of compliance still leaves much to be desired. During the coronavirus pandemic, hand hygiene should be the golden safety rule and an essential element of personal hygiene. Hand washing is obligatory before commencing work; whenever your hands become dirty; after every trip to the toilet; after touching raw materials or packaging; between process operations, e.g. when proceeding from raw materials to finished products; after touching your hair, nose, ears, and eyes; after smoking or eating; after handling garbage, chemicals, or cleaning tools, etc. Proper hand washing is of particular importance for waitpersons, administrators, and cashiers, i.e. those who touch banknotes or various surfaces in the retail space. Any unprotected contact with environment and equipment without proper disinfection afterwards can be dangerous if one subsequently touches one's oral, nasal, or ophthalmic mucous membrane or damaged skin. Visitor service area staff should be especially careful [24].

Personal protective equipment for employees. There are currently no specific COVID-19 infection protection measures for catering staff. Taking into account the transmission route by airborne droplets, all employees are to wear goggles, face masks, gloves, protective clothing, and mop caps. Disposable protective equipment should be changed every 2–3 h [22, 23].

Disinfection. FSEs must take strict and effective disinfection measures according to the current sanitary

standards. Public, industrial, storage, and utility areas should be washed and disinfected on a regular basis, including door handles, chairs, and tables. The same procedure holds for elevators. Employees that have to deal with cash and plastic cards, e.g. managers, cashiers, accountants, couriers, etc., should avoid touching any objects of shared use, e.g. terminals, cash desks, etc., after direct contact with the specified items [25, 26].

The list of scientifically approved anti-coronavirus surface disinfectants includes a 62–71% ethanol solution and a 0.1% sodium hypochlorite solution [23, 24, 27, 28]. Disinfectants that contain even small doses of these substances in the required concentration proved most effective. The Research Institute of Disinfectology of the Federal Service for Supervision of Consumer Rights Protection and Human Welfare published a longer list of registered disinfectants.

Every FSE should have a sufficient supply of disinfectants. Disinfection procedures require protection of skin, eyes, and breathing organs. The personnel responsible for disinfection should wear protective equipment, i.e. face masks or respirators, goggles, and gloves, according to the specific application instructions.

All in all, FSE personnel should constantly monitor the existing risk assessments and safe working systems. Managers are to update the personnel responsible for human safety on any relevant official information. Managers should follow updates on the epidemic in other countries to be aware of possible new transmission routes. For instance, FSEs purchase raw materials of animal and fish origin to produce catering products. The World Health Organization published recommendations that are aimed at transmission risk reduction from animals to humans at the market. Visitors of live animal markets, seafood markets, or animal products markets should practice general hygiene measures. They include regular hand washing with soap and water after touching animals and animal products, avoiding touching eyes, nose, or mouth, and avoiding contact with sick animals or spoiled animal products. Buyers should also avoid contact with potentially contaminated livestock wastes or spilt liquids in stores and market facilities. Raw or undercooked animal products can be dangerous for consumption. According to food safety practices, raw meat, milk, and animal organs should be handled carefully to avoid cross-contamination.

CONCLUSION

The outbreak of COVID-19 has become a clinical threat to people around the world. The World Health Organization declared COVID-19 a global pandemic. The situation crippled healthcare, production of first priority goods, and service industry. Their employees cannot avoid face-to-face contact with clients while having to perform their professional duties even during epidemics. In spite of the fact that all countries are doing their best to solve the problem, our current knowledge about the new virus remains limited. Scenarios for antiviral therapy and vaccination are still being developed. As a result, preventive and infection control measures remain the most effective instrument to combat the current spread of COVID-19. Scientists and pandemic experts are studying epidemics of the past to find options for urgent prevention and treatment of severe acute respiratory infections caused by COVID-19.

The rapidly growing number of person-to-person transmission cases delivered a hard blow to the catering industry. In conditions when self-isolation has become the main preventive measure, most food service enterprises switched to delivery or takeaway sales. However, a complete isolation of food service enterprises does not seem possible. A significant part of the population needs food service while at home or at work. By learning the mechanism of COVID-19 transmission, managers of food service enterprises can develop measures to reduce the risks. A further analysis and synthesis of methods should take into account the national, cultural, economic, and climatic features of specific countries. Many catering establishments will draw a lesson from their pandemic experience. On the one hand, they will understand that failure to comply with the Hazard Analysis and Critical Control Points system ruins production safety and increases the risk of infection. On the other hand, the negative experience can become a new growth point for the food service enterprises when they emerge from the crisis.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests related to the publication of this article.

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Influence of coronavirus crisis on food industry economy

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Abstract:

Recently, there has been a very rapid accumulation of empirical data of economic indicators of the food crisis associated with the coronavirus pandemic. The purpose of this article was to develop a set of measures aimed at minimizing the negative economic impact of the coronavirus crisis (CVC) both at the national and international levels. All these presupposed solving the following tasks. Having studied the nature of the crisis, we found out that it had the greatest impact on such components of food sector as transport, logistic chains, and human resources. We revealed that, first of all, such areas of the economy as fisheries and agricultural production of fruits and vegetables were affected by the crisis. It is obvious that it will also adversely affect small farmers, seasonal and migrant workers, etc. The work proposes the most effective public measures against the negative impact of CVC on the global economy. In particular, we examined the scenario of (possibly temporary) nationalization of operators of critical infrastructure to produce planned critical goods or services. The developed set of measures included limitation of the workers' rights and freedoms by imposing to them labor duties, or replacing them with military ones, limiting the price of strategic goods, and organizing their consumption and distribution. Possible triggers for the implementation of such a scenario in the agricultural sector are offered, as well as mechanisms of protecting and supporting groups of economic agents affected by CVC are proposed.

Keywords: Coronavirus crisis, food crisis, pandemic, food, financing, agricultural products, economy, food security, crisis management

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INTRODUCTION

It is obvious that the outbreak of COVID-19 (CV) in the Chinese province of Hubei, which took the character of a global pandemic in recent months, will have a negative impact on a global food security.

The following world organizations are involved in food, trade and health issues – the United Nations Food and Agricultural Organization (FAO), the World Trade Organization (WTO) and the World Health Organization (WHO). Their leaders – Che Dongyuy (FAO), T. A. Gebreyesus (WHO) and R. Azevedo (WTO) – made an official report on the risk of the food crisis caused by the pandemic of the new coronavirus [1]. They said that there was currently a danger of "food shortage" in the global market due to disruptions in the supply chain in the trading industry.

"This is a very tough test," explains M. Torero Cullen, FAO Chief Economist. - For the first time we

witness such a sharp drop in supply and demand. The supply fell due to a reduction in labor, while a drop in demand is determined by a recession. We must help to improve coordination between the authorities of different countries. Our main task is to ensure the "vitality" of the production and distribution chain, applying all the necessary safety standards" [2].

Currently, there is a problem of a large amount of empirical data of economic aspects of the food crisis with a simultaneous lack of scientific analytical data.

The purpose of this article was to develop an effective set of public measures to minimize the negative economic effect of CVC at the national and international levels.

To do this, it was necessary to determine the following aspects:

- a type of crisis;

- the main damaging factors;

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- areas of the economy, communities and social groups which are the object of the negative impact of the crisis; and

- search and justification of the most effective public measures to minimize the negative economic impact of the coronavirus crisis (CVC) at the national and international levels.

STUDY OBJECTS AND METHODS

The object of our study was a system of economic relations taking shape at the level of the aggregate of stakeholders in the national economies of states affected by the CV pandemic.

Our analysis covered the following aspects:

- Functional - production, distribution, exchange, consumption of material goods (food and related services);

- Territorial - developed countries of the "golden billion" (including Russia) and countries of the "third world";

 Decompositional – all levels of economic interactions: micro-, meso-, and macrolevel;

- Industrial. At the level of the national economy the analysis was made of the supply and demand for food (in various sectors of agriculture and food industry, in the restaurant business, in the transport industry), as well as in the labor market. At the global economy level, the market for basic commodities (rice, wheat, soybean) was investigated;

- Interdisciplinary. In this aspect, the study involved various aspects of the following sections of economic theory: employment theory, pricing theory, agricultural economics, transport economics, the world economy and international economic relations, and public administration theory.

The research materials were analytical works and speeches of the leaders of such world organizations as the FAO, WHO and WTO, expert materials of economists, sociologists, and doctors on the subject of our study, as well as the data from practitioners of the markets studied.

The methodology of the study is a combination of modern methods of retrospective, expert and scenario analyses. The research methodology is based on a systemic analysis, which ensures its integrity and comprehensiveness.

RESULTS AND DISCUSSION

Coronavirus crisis nature. To achieve the main goal of the work, we set the task to determine the type of crisis: what kind of economic crisis is observed in connection with the CV pandemic?

One of the approaches is the collection and analysis of information. In special literature we see the priority of purely "native" ones, i.e. medical, epidemiological, aspects of CVC.

As noted in the FAO Report "Agricultural Food Markets and Trade Policy at the time of

COVID-19", disease outbreaks can affect food supply and demand. They can lead to a reduction in the workforce (including seasonal and labor affecting the preparation migrants), of land. planting, maintenance of crops and harvesting [3]. They also affect employment in labor-intensive industries, household incomes and food security. During the outbreak of Ebola in West Africa in 2014, the following facts were noticed: disruptions in the supply chain of agricultural products at critical times of the season; a reduced access of workers to the farmland and, as a result, a decrease in their salaries and the area of cultivated land; as well as restrictions in the transportation of goods to processing enterprises and markets. In Liberia, during the same outbreak period, 47% of farmers reported the presence of uncultivated farmland.

However, the analysis of the WHO and WTO information data, local information sources, as well as the data on previous epidemics at the beginning of the 21st century do not provide a clear answer why exactly in the case of the CV pandemic the global economic phenomena and consequences are so different.

Another approach is to prioritize the impact of the crisis on food demand.

At a first glance, this makes sense. The 2008 crisis analysis showed what happened when lower incomes and uncertainty made people spend less. This led to: a reduction in demand, a decrease in sales, and a decline in production. In addition, the most affected entities were forced to apply negative strategies to solve the problems, such as selling productive assets, less varied food, and overfishing to compensate for limited incomes [4].

Food demand is generally inelastic. But in poor countries, it is more related to the size of incomes, and here the loss of income opportunities can affect consumption. Fear of infection can reduce the number of visits to markets; and habits in the field of food purchase and consumption are also changing: a decrease in restaurant traffic, an increase in the supply of e-commerce, and food consumption at home.

However, it remains impossible to consider demand as the basic factor of CVC in isolation from supply, which we will discuss further.

Consumer panic is yet another "candidate" for the role of the main factor of CVC.

At the beginning of 2020, many sources reported that at the beginning of the CV outbreak, there was a significant increase in food demand. However, the CV pandemic cannot be the cause of food shortage, at least wheat, rice, or other products of mass consumption. However, the deficit may be caused by excessive stockpiling for the future. If some economic agents unnecessarily buy too much food or sell too little of their produce for fear that it will not be enough, others lose. In other words, fear of scarcity can be a self-fulfilling prophecy (a term coined by sociologist Robert Merton in 1948). It is rational to stock up food if you expect others to do so [5].

Coronavirus highlights two of the most important aspects of this extravagance: food waste, i.e., deliberate lack of edible products due to the behavior of companies and individuals, and "food loss" (according to the 2013 FAO report, 14% of the world products is lost or wasted after the harvest even before it goes to retail), as a result of supply chain inefficiencies, mainly due to the lack of infrastructure and poor procedures [6].

At the same time, there are sufficient reserves for the main commodities, the prospect of their harvest in 2020 is favorable. This means that this factor of the crisis is not decisive.

According to FAO Chief Economist B. Abassin, this crisis is not a production one, but, first of all, is that of transport and logistics. In our opinion, this is exactly half of the "guess": one of the two most important components of the CVC is correctly identified.

As we noted earlier [7], the first economic consequences of CVC were:

"- blockade, isolation, autarkization (economic independence) of geographical areas, countries and entire regions;

- decline, up to a complete collapse, of production systems of goods and services that require the physical presence of workers in the team;

suspension of processes, destruction of supply chains;
ruin and bankruptcy in a number of industries...".

To contain the CV pandemic, world leaders have taken measures to drastically reduce the volume of goods transported by land, sea and air, as well as to reduce labor migration at the national and international levels. These factors led to a general disruption in the logistics of food supply chains, creating obstacles to the transport of food and agricultural resources.

Logistics efficiency is critical in the agricultural sector, especially during the crisis. Failures can have a negative impact on the quality and safety of food (for example, with the prohibition of cargo transportation, the delay at the borders of containers with goods, the supply of perishable expensive goods, such as fresh fruit, vegetables, fish and seafood, is especially affected), and it can also reduce their availability (for example, due to the closure of farmers' markets in cities).

Most agricultural activities are fairly systematic, tied to specific seasons, weather, timing, processes. Delay can affect the entire production process, yield and output: for example, seeds, fertilizers, pesticides, veterinary drugs, machine oils and diesel fuel, etc. are not delivered due to transportation restrictions.

Failures in the supply chain logistics have already taken place. For example, Rosario in Argentina is a major center of grain export and soybean production. Argentina is the world largest exporter of soy flour, livestock feed. Dozens of municipal authorities around Rosario blocked the entry and exit of grain trucks into their cities to slow the spread of the virus [8]. This is contrary to the decision of the country authorities to unblock the roads, but it meets the anti-epidemic rhetoric. Soy is not imported to crushing plants, which affects its export. Similar problems arise in Brazil, another key exporter of basic commodities. If the large international port of Santos in Brazil or Rosario in Argentina closes, this could mean a disaster for world trade.

The second, equally important, basic factor of CVC, which we noticed, is related to human resources.

Measures affecting the free movement of people, mainly seasonal workers and migrants, greatly affect food production.

Labor-intensive agricultural production, such as fruit and vegetable ones, largely depends on temporary or seasonal workers, especially during planting, weeding, harvesting, processing or transporting crops to markets. With the closure of borders due to CV, farmers from developed countries need workers from other countries: the USA – Hispanics, Spain – North Africans to pick strawberries, Germany – agricultural workers from Eastern Europe in asparagus fields, etc. [1]. Slowing down the rotation of workers when they become ill or cannot come to work due to lockdown blocks many western farms. Agricultural workers in the informal labor sector will be seriously affected by the loss of jobs and income.

In turn, the absence or delay in the supply of products affects citizens working in the informal sector, who earn their living by selling agricultural products [9].

Small farmers, cattlemen and fishermen are also very vulnerable, their business may be hindered by CVC, depriving them of access to markets due to quarantine measures. Agricultural processing enterprises are also labor-intensive. Currently, most sorting and packaging lines do not meet the requirements of social distance. A separate category is children who, as a result of CVC, have lost access to school meals. In Latin America and the Caribbean alone, FAO school meals programs supported 85 million children (10 million of them had it as the main source of nutrition), which were suspended due to the pandemic.

Third world: a threat to economy. Developing countries are at risk particularly, since CV can lead to a labor force reduction, and affect the incomes of the population, depriving them of a part of their livelihoods, as well as labor-intensive forms of production (agriculture, fisheries/aquaculture).

According to [10], in 2018, about 820 million people live in conditions of chronic hunger daily, of which about 113 million people in 53 countries and territories in the world were in a state of food crisis. The food crisis involved, first of all, those areas where a significant proportion of the population experiences severe food shortage, and needs an urgent humanitarian assistance for food safety and nutrition as a result of significant shocks to cope with the consequences [10]. The three main food crisis drivers are conflicts and insecurity, weather disasters and natural hazards, economic turmoil.

Today, 44 countries of the world need an external food assistance. Any additional interruptions in access to food due to CV can lead them to tragic consequences. As a result of the 2007–2008 food crisis, due to the increase in world food prices, a number of undernourished people in the world increased from 848 million to 963 million people (by 14%) over 2 years [11]. The economic downturn correlated with a rising hunger in 65 of the 77 countries surveyed, as FAO and its partners warned in the world report "The State of Food Security and Nutrition" in 2019 [12].

The pandemic can have significant consequences for the delivery of humanitarian assistance (budget cuts due to redirecting resources to combat CV, the movement of equipment, cargo and personnel). The consequences of CVC will affect migrants (the threat of exploitation, poverty, hunger), as well as their families in countries of their origin due to a sharp reduction in a flow of coming financial resources.

From the experience of the food crisis 2007–2008, the inflationary effect of protectionist policies in the form of introducing import tariffs and export bans has increased the number of people who lost food safety around the world.

The overall impact of the pandemic on unemployment, household purchasing power, food prices and their availability in local markets can seriously jeopardize food access in the most vulnerable countries. Of particular concern are temporarily displaced persons (TDPs) and refugees, as well as communities already facing hunger or other crises: for example, the invasion of locusts in the Horn of Africa has led to an increase in the number of Ethiopian people in need of humanitarian assistance (more than 8 million out of 100 million people).

Risks associated with CV are well known in some poor countries. For example, quarantines and panic during the Ebola virus epidemic in Sierra Leone (2014– 2016) led to a sharp surge in hunger and malnutrition. The situation was aggravated because restrictions on movement led both to a shortage of labor during harvesting and to the inability to bring their products to the market. In 2008–2009 in parts of Asia, after a series of lean years, rising prices for rice and then for other crops led to "hunger riots".

In countries where migrant workers from rural areas lost their jobs in big cities because of the lockdown, more people can die from starvation than from CV. State food stocks can reassure consumers. But these reserves may be subject to corruption, or their management may be ineffective, according to a report from Nanyang University of Technology in Singapore [13]. Closing borders in Africa in the fight against the spread of CV raised fears of rising prices and a shortage of staple foods that Africa is forced to import due to population growth. On March 17, Cameroon announced the closure of its land, sea and air borders, while allowing the movement of goods trucks after the sanitary control of drivers. "We cannot close everything. The vast majority of what we consume comes from abroad," government spokesman J. Ecoga said [14].

In several African capitals, the people staged a massive purchase in supermarkets, stocking pasta, rice, oil, toilet paper, soap and other consumer goods. "In Morocco, the bazaars were stormed" [14]. However, the authorities of many countries reacted quickly, taking measures to calm the population and prevent speculation. The Ministry of Commerce of Côte d'Ivoire reported that the country had a supply of rice for seven months of consumption, tomatoes and sugar for five months, milk for four months and meat for three months, and urged the citizens do not change their consumer habits, playing into the hands of unscrupulous economic operators.

In Rwanda, authorities set a maximum price for basic foodstuffs in order to reduce price growth for rice, oil, and local fruits and vegetables imported from Tanzania. Lemon prices doubled, as many Rwandans tried to treat CV with it. In Morocco, authorities recalled that, on the eve of Ramadan, they had already formed food stocks. The authorities also allowed fruit and vegetable producers to sell crops directly to hypermarkets, without going through wholesale markets. In Algeria, a ban on food exports was introduced as well as price controls, and fines for violators.

The South African Department of Commerce announced price controls. The increase in prices should not exceed the increase in prices for raw materials or resources, the profit of operators should not be higher than in the period before the outbreak of CV. Retailers should limit the number of goods sold to one person. The list contains 22 products, including latex gloves and alcohol solutions. The president called on South Africans to "refrain from excessive and unnecessary purchases", given "constantly maintained stocks" [14]. In Madagascar, authorities promised to seize stocks of merchants who unfairly raise prices during the crisis. Large distributors have officially committed to comply with tariffs.

By April 2020, the crisis did not lead to inflation, which would primarily harm the poorest people. A quarter of Africans are already undernourished.

"Golden billion": change of consumer behavior. Food consumption throughout the developed world fell victim to social engineering, and temporarily succumbed to a consumer panic during the CVC:

"As the global coronavirus pandemic accelerates, a food panic continues across the country, affecting all outlets, and covering all categories of products (...). During the period of "social distancing", filling your closet and refrigerator with your favorite products becomes critically important. This is even more important for consumers who are accustomed to the fact that products do not contain gluten, sodium, lactose, sugar, GMO, and that vegetable meat, vegan (...) certified products are easily available. And these things are becoming very difficult to find" [15]. The author mentions an "unprecedented surge in demand" and, quite symptomatically, says of a "new consumer landscape".

This local surge occurs simultaneously with the fact that "the largest food brands in the world in recent years have been struggling to maintain their position and compete with innovative special products for [niche] consumers. This battle has been raging for almost ten years and the financial health of such large brands (...) has rapidly deteriorated, as has their liquidity (...). [Today] it will be extremely difficult for these large food producers to create additional burdens on their business by quickly placing orders and stimulating supply chains – especially during the next 4–6 critical weeks across the country. Maybe some of them will face potential bankruptcies..." [15].

In the United States, the situation with coronavirus initially increased the profits of some food manufacturers and retailers. According to Nielsen, in the last week of February, sales of powdered milk products increased by 84%. Sales of such products, as bread and eggs, along with rice, beans and frozen food, also increased.

Companies like Conagra Brands and Campbell Soup Co said they were ramping up a production of the most sought-after food. Some companies, including Coca Cola, by contrast, began to experience delays in February as a result of industrial disruptions from the spread of CV in China.

According to commodity exchanges, at the beginning of April 2020 there were no signs of panic buying up of wheat, corn, soy, pigs or cattle. The only agricultural product, the price of which increased, was rice. The wholesale price of coarse rice rose in March 30 on the Chicago Stock Exchange to 14.1 cents per pound, from 13 cents at the beginning of the year. This price is still much lower than that in April 2008 (24 cents per pound) [5].

The Consumer Brands Association, in a letter to the US Department of State and the US Sales Representative on March 15, expressed concern about a potential shortage of ingredients. According to Nielsen, Califia Farms, a long-shelf vegetable milk producer, increased sales by 323% during the last week of February. The head of the company, G. Shteltenpol, said he was building up supplies 3–4 weeks ahead: "What if our largest competitor suddenly breaks out of the factory? Then he may not be able to send orders at all within 3–4 weeks, so we should even be prepared for the fact

that our brand will occupy a large market share. This involves the adoption of entrepreneurial risks or risk management" [16].

With the workforce in the food industry, the situation in developed countries is not so good. In the UK, the "Landworkers' Alliance", representing more than 1000 British farmers and land owners, asked the Chancellor of the Treasury to create a "land army" of workers to fill the shortage of 60 000 foreign seasonal workers, and to create a reserve in case of illness for British workers, and provide a support package in the amount of £9.3 billion for its payment. A similar call to the authorities – to make it easier for people who became unemployed due to lockdowns to search for seasonal work on farms – was made by the "Country Land and Business Association", representing more than 30 thousand landowners and rural firms in the UK [17].

Russia: unequal influence on industries. The spread of CV in China has affected Russian food suppliers. Chinese food import from the Russian Federation in January–November 2019 increased by 23.7%, and amounted to \$2.72 billion, according to customs statistics provided by the Russian Export Center. Of this amount, 45% were deliveries of frozen fish, other large positions were crustaceans, sunflower oil, poultry and chocolate products [18].

By the end of January 2020, according to market participants, pollock prices fell by about 15%, a decrease in demand for crab in China led to a fall in prices from \$15–18 in 2019 to \$7 per kg in 2020 (a general estimate of possible losses in the long run exceeded \$150 million) [18]. The decrease in purchase prices for imported poultry meat due to CVC amounted to 10–15% and exacerbated the general decline in prices in this market.

Disruptions in the operation of transport infrastructure, as well as delays in Russian products supplied to Chinese processing plants were repeatedly noted. The president of the group of companies "Kabosh" D. Matveev noted that there were problems in animal husbandry due to lack of workers in the pandemic: "Even if we skip feeding or cancel one milking, we will receive not only one-time losses, but animal health problems and a significant drop in their productivity over a long period. It is also necessary to recover after a single failure of at least three weeks" [19].

In addition, a spring sowing season is approaching. One cannot cancel or reschedule the sowing time of fodder and grain crops. Due to the shortage of people, less feed will be prepared, which will lead to its higher prices. This means that it will be necessary to reduce the number of livestock or additional funds for feed. In addition, fewer crops will be sown and harvested. The cost of cereal will eventually increase. The result will be an increase in the cost of milk and all its processed products, of bread and all grain products [19].

In Russia in March 2020, after the introduction of a self-isolation regime to suppress the spread of CV, a consumer panic began. A rush of demand arose for household chemicals, personal hygiene and disinfection products, as well as for long-term storage products: canned food, flour, cereals, sunflower oil, sugar and salt. "SberMarket" product delivery service notified customers of an increase in delivery time "due to demand rise". The number of orders on March 13 and 14 increased by 104% throughout the country as compared to March 11 [20].

At the same time, there were no shortage of goods and empty warehouses. Production industries worked. The explanation can be a highly competitive environment. "According to our estimates, the top 10 FMCG retailers account for only 30% of the turnover of food and consumer goods. Being in a fierce competition, they cannot allow price speculation, because it will adversely affect sales," explained I. Fedyakov, general director of Infoline [20].

The impact of the coronavirus pandemic on the alcohol market was specific. According to V. Drobiz, Head of the Russian Center for Research of the Federal and Regional Alcohol Markets, during all previous crises (in 1998, 2008 and 2014) three trends were observed: an increase in the consumption of strong alcohol along with a decrease in the consumption of wine and beer products, a sharp increase in the market counterfeit goods, as well as a sharp decline in the consumption of imported products as a result of, as a rule, the fall in the exchange rate of the national currency.

However, the specifics of self-isolation, requiring a mass consumer to maintain working capacity during the day, made adjustments: a sharp increase in beer consumption compared with a more modest increase in strong drinks. At the same time, there is a well-founded fear that, in the context of a shortage of funds, a consumer has partially switched to illegal surrogate products [21].

A global aspect of the problem: international cooperation or protectionism. FAO Director-General Che Dongyu called for coordinated action by the governments of the world in connection with the CVC: "We know that deaths will be high and that enormous efforts to reverse this trend will entail high economic costs. To reduce the risk of even higher losses – food shortages for millions of people, even in rich countries – the world must immediately take action to minimize disruptions in food supply chains. (...) A coordinated and consistent global response is necessary" [22]. "No panic (...). There is enough food in the world to feed everyone. But we still have to ensure that food is available where it is needed" [22].

Che Dongyu noted that "uncertainty about the availability of food can encourage politicians to take restrictive trade measures to ensure food security at the national level."

The joint communiqué by FAO, WTO and WHO says that "uncertainty about food availability can cause

a flurry of export restrictions, which in turn can lead to a global market crisis" [1]: countries exporting major crops can hold it back for fear of scarcity, while at the other end of the global food chain, other countries will face serious difficulties. The communiqué calls to keep trade relations in order to avoid food problems, especially in the poorest countries. According to FAO experts, "export restrictions" usually cause hunger in other parts of the world.

After the financial crisis of 2007, rice producing countries such as India and Vietnam, worried about rising prices, imposed export restrictions which led to higher world prices and to famine unrest in some developing countries. Other grain exporters have also limited exports to protect their consumers from the initial increase in food prices. Food importing countries, in turn, reduced import tariffs on food, supporting the demand, and kept an upward pressure on world prices. As a result, instead of limiting price increase, these political measures only led to higher prices in the world market. Protectionist measures by national governments during the CVC may provoke food shortages around the world, as the FAO warned.

The experience of cascading export restrictions among the main exporters of food products (India, China, Vietnam and Pakistan for rice; Russia, Ukraine and Argentina for wheat) in 2007–2008 demonstrated that such a political chain reaction could destabilize international markets [23]. As more and more countries followed it, price increased as well as market volatility intensified. Overall quotes for rice grew by 52%, and for wheat and corn by 18% [24]. This caused a particular damage to poor countries dependent on import.

"The worst that can happen is if governments restrict the flow of food," FAO Chief Economist M. Torero told the Guardian. "All measures against free trade will be counterproductive. Now it is not the time for restrictions or introduction of trade barriers. Now it is the time to protect the flow of food around the world." "Trade barriers will create extreme volatility," Torero warned. "[They] will worsen the situation. This is what we observe during the food crisis."

World export is highly concentrated. Russia, the European Union, the USA, Canada and Ukraine will give 75% of all world wheat export most likely in 2019–2020. The rice market is also concentrated: 75% of export goes to the largest five exporters, and almost a quarter of it, to India. Vietnam's share in the world market is 16% [25].

Kazakhstan was the first to ban the export of wheat flour, being one of its largest exporters in the world, and imposed restrictions on buckwheat and vegetables, including onion, carrot and potato. Vietnam, the world third largest rice exporter, has temporarily suspended rice export contracts. The US position was uncertain, but it raised market concerns, due to Donald Trump's desire for a trade war with other commodity-supplying countries [17]. A number of experts rated the restrictions introduced as "completely unnecessary", since both countries produce much more than they consume and have enough reserves [5]. The Ministry of Agriculture of Kazakhstan on March 30 changed the course and announced the replacement of the ban by quotas for the export of wheat and flour.

Russia is the world largest exporter of wheat. At the end of March 2020, the ministers of economy and agriculture of the Russian Federation advocated limiting the export of Russian grain to seven million tons in April–June. And on April 1, pending approval by the government of this proposal, Russia decided to sell one million tons of wheat from state reserves in the domestic market in order to limit the increase in domestic prices.

The findings of experts from the International Food Policy Research Institute (IFPRI) are encouraging the data in [25]. At least in relation to staple food, such as rice, wheat, and corn.

The ratio of stocks to consumption is an indicator of the vulnerability of world food markets to shocks. According to IFPRI calculations, this indicator is close to the "normal value" (median level for 20 years) and significantly higher than in 2008, with the exception of China. A sufficient amount of stocks explains the price stability in the markets for basic goods. In China, rice and wheat reserves are enough for domestic consumption for a period of 10-13 months. The amount of rice stocks in India is 34% higher than its consumption. The prospects for the 2020 harvest are good. The US Department of Agriculture (USDA) predicts an increase in global wheat production by 5%, while rice production is projected to remain roughly the same as in 2019. The production of these products is unlikely to be affected by CVC, at least in large producing countries; since it is mechanized, does not require a large amount of labor, and is carried out in areas with dispersed rural populations. Disruptions in international transport and distribution are also unlikely: these dry bulk goods can be loaded and unloaded with minimal interaction between people.

"There is no global supply shortage today," European Commissioner for Trade Phil Hogan said during a conference telephone call to his colleagues in the G20 countries. Ministers promised to continue trade with each other, despite the pandemic, and "beware of behavior for the purpose of profit and unjustified price increases" [5].

An excellent mechanism for ensuring the transparency of global food markets is the Agricultural Market Information System (AMIS), an interagency platform created by the G20 in 2011 and hosted by the FAO to help coordinate political actions in the face of market uncertainty [26].

Due to the slowdown in supplies, the devaluation of currencies against dollar and the decrease in purchasing power, the vulnerability of countries dependent on food imports are increasing. Against the background of a prolonged lockdown, sharp fluctuations in food prices can occur. Fighting a pandemic through massive government spending, all countries in the world are interested in restricting inflation. In case of food price jumping they should carefully evaluate their fiscal and other responses, as well as their consequences in the medium and long term. By maintaining supply chains and strong international cooperation, the world can prevent food shortages and protect the most vulnerable population.

Recommended state policy measures. In FAO recommendations, the pandemic remains a top priority in food policy, including the use of isolation measures to slow the spread of CV. The second priority of state policy is the identification and meeting the needs of the most vulnerable economic groups of the population, since measures to restrain a pandemic are detrimental to the economy. Finally, the third task is to ensure the supply of food. These tasks should be considered in detail.

Fighting а pandemic. FAO recommended avoiding general restrictions on food import. This recommendation seems controversial, as crossborder movement of people and goods can exacerbate epidemiological problems. In the past, countries practiced epidemiological control by restricting trade and travel - for example, import bans from Peru during the 1991 cholera outbreak, from India during the 1994 plague outbreak, and from Guinea during the 2014 Ebola outbreak. However, FAO believes that, although in exceptional cases, these measures may be required to protect human, animal or plant health, they should be limited in time, minimize disruptions in international trade and ensure food availability and access to it [27]. Also, in order to avoid disruptions in the food supply chain, the creation of safe corridors for travel and trade in accordance with WHO recommendations is necessary.

"The party of the lockdown opponents" is numerous, there is just a few examples.

The authors of the FAO, WHO and WTO Communiqué emphasized the need to protect industry workers to "minimize the spread of the virus in the sector" and "maintain food chains." It was added that "while protecting health and well-being of citizens, it is necessary to ensure that the package of measures does not violate the food supply chain". "We must make sure that our response to the CV pandemic inadvertently does not create unnecessary obstacles to producing and does not exacerbate hunger and malnutrition" [1].

M. Torero warned: "Do not speculate with fear, because the consequence may be the introduction of embargo on exports. We must continue to ensure the movement of goods, because panic can have serious negative consequences" [2].

"Cargill" (USA), one of the largest food producers in the world, said that "suspending any new protectionist measures and removing existing barriers to trade will benefit farmers and consumers" [5].

Although social distance is necessary to break the chain of transmission of viruses, it should not be so extreme as to break the chain of food supplies, said T. Tienjens, chairman of the UN Committee on World Food Security, which coordinates food safety for governments, the private sector and NPOs. The authorities of many countries have already made adjustments to their strategy to combat CV. Thus, in the Philippines, farming and fishing were allowed again everywhere, except for the main island of Luzon, and food production and distribution companies can work using 50% of their labor. One of the recommendations of FAO is "giving priority to the health of consumers and workers in the value chains" [9]. This paragraph does not require commentary, since it mainly refers to WHO recommendations of a purely medical and antiepidemiological nature.

In particular, the authorities should take measures to ensure the safety of agricultural workers. If possible, workers should be tested for CV. Manufacturers of products and warehouses should ensure the absence of visitors and restructure the work taking into account standards of social distance. Physicians should measure the temperature of employees and ensure control over the use of masks, gloves and other protective equipment.

Identification and meeting the needs of the most vulnerable economic groups.

We can note among the specific recommendations, in particular, the expansion and improvement of emergency food assistance and social protection programs:

- the mobilization of food banks and local communities with the support of both authorities and private charitable organizations;

- the delivery of food packages to the elderly or people suffering from chronic diseases [8];

- the delivery of school meals to children at home, even after school closures;

- the use of mobile payment systems will prevent disruptions in the delivery of cash benefits due to restrictions on movement, while minimizing contacts between people for transferring cash;

- grants to micro-, small and medium-sized enterprises of the food industry, seasonal workers and hired employees temporarily left without work due to a lockdown in CV; and

- the exemption from taxes on basic food for families with schoolchildren, especially for workers in the most affected sectors of the economy [29].

Providing food.

Recommendations for this priority include the following measures to support farmers:

- the access to financing small farmers to continue their work;

- banks removal of fines and penalties for late payments to farmers and extension of payment terms;

- state purchases of agricultural products from small farmers to create strategic emergency reserves;

- accelerating the issuance of visas for migrant workers;

- the introduction of vouchers, subsidies for fuel, electricity, irrigation and fertilizers;

- the introduction of minimum announced prices for agricultural products;

 admission to the movement of seasonal workers and transport operators (for example, truck drivers) across domestic and international borders, while ensuring proper medical examination, testing and protective measures;

- the creation of special flights to help seasonal workers get to the place of work;

- the mobilization of unemployed or part-time workers in the absence of seasonal workers, a redistribution of workers from other areas on a temporary basis, a change in local public work programs [9];

- the promotion of regional trade; and

- securing international funding to support small farmers (the mechanisms for this are within the Global Program on Agriculture and Food Security, created after the food crisis of 2007–2008).

The measures taken by China and Italy to protect their smallholder farmers are particularly interesting [8].

During the lockdown, China used "the Basket of Vegetable" policy to reduce the impact of the virus on the lives of smallholders and to minimize food shortage. Back in the late 1980s this project expanded urban access to fresh food by extending vegetable farms in the suburbs and creating stocks. Under the same scheme, farmers and traders in nine provinces jointly supplied grain, oil, meat, vegetables, milk, eggs and seafood to Hubei Province, the epicenter of the epidemic. Some local governments centralized the purchase, slaughter of livestock and cold storage of county food cooperatives and fully subsidized storage costs. E-commerce platforms made it easier for farmers to trade. For example, the Chinese company "Alibaba", a giant in the field of e-commerce created a special fund to help farmers in finding markets for agricultural products and had a special "Green Channel" for this.

Chinese authorities allocated \$20 million in subsidies for the purchase of agricultural machinery and equipment. Loans with low interest rates and preferential rents are received by the companies developing innovative agricultural technologies, such as agricultural drones and other unmanned aerial vehicles, to reduce contacts with people while maintaining supply chains.

In Italy, the comprehensive program "Care Italy" includes a series of measures to support the agricultural sector. $\in 100$ million is allocated to support agricultural and fishing companies that had to suspend operations, and another $\in 100$ million, to finance them. Farmers are helped to receive advance payments from EU subsidies. The program also raises the EU budget for food distribution among the poor by $\in 50$ million and includes

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	POLICY OBJECTIVE	TYPICAL RESPONSE REACTIONS	SUGGESTED MEASURES
Proposal side	Adequate domestic supply	 Export restrictions 	 Avoiding proactive export restrictions
		 Expansion of domestic procurement 	Avoiding procurement expansion when
			inventory levels are already high
			 Promoting market transparency
	Food Safety Guarantee	Import bans	 Avoiding general import bans
			 Promoting transport and trade
			corridors as recommended by WHO
	Support for producers,	Subsidies to expand production	Direct transfers
	especially smallholders		 Avoiding excessive subsidies that
			could exacerbate market volatility
Demand side	Price containment	 Reduced import tariffs 	 Avoiding excessive accumulation
		 Regulation of domestic prices 	of imports
			 Encouraging lower import tariffs
			 Encouraging close price controls
			in partnership with the private sector
	Support for poor consumers	Cash transfers	The choice of one of the options,
		Grocery help, grocery sets	in accordance with the situation [32]

transfers of $\notin 600$ to agricultural workers with short-term contracts.

In [28], we considered a scenario that would be associated with the nationalization of the (possibly temporary) operators of the entire critical infrastructure or of its part. In our opinion, this would allow, for example, D. Trump to enact the Defense Production Act, adopted during the Korean War, to solve several problems at once:

- to establish planned production of goods or services critical for the nation;

- to limit the rights and freedom of workers of such enterprises by imputing them to labor duties, or replace them with military workers; and

- to limit the prices of strategic goods produced, to organize their consumption and distribution as efficiently as possible (cards, coupons, restriction of consumption "in one hand", etc.).

The application of this scenario to the food industry is unlikely; however, forecasts of a sharp increase in the deficit in this area, as the statement of the US Congressman T. Messi predicts that the country is a few weeks from the grave food crisis, can push the countries of "the golden billion" to this path" [30].

Another solution could be the zeroing of interest on consumer loans to farmers and CVC affected by other categories of the population (with the freezing of the loan body payments) and their exchange for tax exemptions of the same denomination, which creditor banks could pay to the budget or trade on the open market [28]. It is worth considering similar securitization options with respect to payments to farmers and food processing enterprises for taxes and contributions to social and health insurance funds. Russian practice of 1995–2000-s gave a number of examples of the application of similar measures regarding federal and regional budgets [31]. FAO recommendations for improving efficiency and reducing trade-related costs include:

 rejecting all measures restricting the mobility of goods and trade;

- reducing food waste and losses;

- a breakdown of bottlenecks in logistics;

- rejecting universal subsidies for food consumers;

- reducing restrictions on the use of stocks;

 reducing import tariffs in cases where the authorities want to minimize, for example, an increase in expenses due to the devaluation of their currencies and other restrictions;

- temporary reducing VAT and other taxes; and

- if necessary, revising the tax policy regarding imported goods to compensate for potential cost increases (as a result of currency devaluation) [8].

Another important measure, of course, is to facilitate the transfer of food trade online.

Political responses during the food crisis may exacerbate the situation and its market consequences, as was the case with the global food price crisis in 2007–2008. FAO analyzed the 2007–2008 food crisis experience, and authorities' actions during the Ebola epidemic in West Africa (2014), the acute respiratory syndrome (SARS) in East Asia (2003), HIV/AIDS in Africa (1990s, 2000s), plague in South Asia (1994) and cholera in Latin America (1991). The results of this analysis allowed us to draw a number of basic conclusions [32]:

1. Political measures should be aimed at eliminating actual, non-perceived, failures in supply and demand. Increasing market transparency and coordination among all stakeholders are critical;

2. The absence of trade restrictions can be no less important than the direct support of consumers and producers;

3. Compliance with international principles regarding the safety of transport and trade corridors can help preserve the supply chain of agricultural products.

The measures applied should be reasonable and appropriate to the context and the moment. Thus, excessive expansion of government procurements for stocks, especially if stocks are already large, can reduce the availability of foodstuff in international markets and put pressure on prices.

In 2007–2008 different countries decided the problem of excessive private food supplies by individuals differently. For example, in the Philippines, a target group was set up to search for food speculators, and in Ecuador police inspections were introduced throughout the supply chain [33].

When reducing tariffs and taxes on imported food products, their excessive accumulation must be avoided so as not to cause a world price increase. In 2007–2008, many countries reduced or removed duties on imported food products (India, Indonesia, Morocco, Nigeria, and Burkina Faso) and taxes (Brazil, Mongolia, Congo, Madagascar, Kenya, and Ethiopia).

In 2007–2008 many countries controlled prices at some or all stages of value produce (Sri Lanka, Senegal, Malawi, Malaysia, and Pakistan) [33]. Such a policy requires a large volume of products to meet the demand at fixed government prices, and financial potential for the purchase of grain and/or subsidies participants. It is important to introduce control over a small number of goods and for a short time, since low prices will stimulate the black market and impede domestic production. Such policy is applicable only in conditions of extreme price volatility.

The FAO proposed the creation of crisis committees in countries to analyze the impact of the outbreak of CV on food supply, including, in particular, representatives of ministries of agriculture, food industry, transport, economy, trade, etc. Crisis committees would be the most important mechanism for monitoring and developing strategies to minimize the impact of coronavirus on food safety [9]. It is important that crisis committees engage the private sector through a wider multilateral advisory committee, which would include representatives from all parts of the food supply chain. Then the measures proposed by them would fully meet the needs of the participants.

One of the important areas of work is "collecting the necessary information to coordinate reforms in the field of logistic policy and government intervention" namely [9]:

- conducting operational national and regional food stock assessments and yield forecasts;

- identifying any gaps or surpluses that may arise due to the prohibition or shortage of imports;

- studying the possibility of redistributing food stocks between different regions of the country;

- ensuring the availability of goods and preventing regional price spikes, etc.;

planning the dynamics of demand and the possibilities of adapting production, processing and distribution to it;
verification and monitoring of blocked transportation routes and workers who have left due to a lockdown.

CONCLUSION

Preventive measures are of paramount importance and will cost to the economies and governments less, which is especially relevant, given the growing expectations of a global recession. At the same time, when developing and subsequent practical implementation of a holistic concept of public policy measures to minimize the negative economic effect of CVC at the national and international levels, the imperative efforts should be given to measures neutralizing the negative economic factor of CVC in two key areas: logistics and human resources. It is necessary to ensure a coordinated approach of the participating countries to the development of a policy of counteracting CVC and to monitoring potential consequences.

CONTRIBUTION

Authors are equally related to the writing of the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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A quality index method for squid *Uroteuthis (Photololigo) chinensis* L. (Gray, 1849) preserved on ice

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Abstract:

Uroteuthis (Photololigo) chinensis L. is one of the most popular kind of seafood that accounts for about 20% of all cephalopod species caught in Vietnam. This study was aimed at developing a quality index method (QIM) scheme to assess the quality and freshness of Loliginid squids. The new method will be of benefit to consumers, fishers, seafood dealers, seafood industry businesses, and controlling entities. The QIM scheme for Loliginid squids was based on the changes registered for 10 sensory parameters, scoring from 0 to 28. The obtained equation for linear correlation with *P*-value < 0.05 during storage was $Y = 1.083 X_{tg} + 2.866$, with coefficient $R^2 = 0.99$. When preserved on ice, the Loliginid squids proved to have a shelf life of 10–12 days. The QIM program and the quality index equation provided a user-friendly, quick, and efficient scientific-based tool that can specify the storage time and estimate the remaining shelf life for Loliginid squids. The scheme can be combined with other chemical quality parameters of freshness to form a full quality assessment program for Loliginid squids.

Keywords: Squid, Uroteuthis (Photololigo) chinensis, sensory properties, QIM, ice storage

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INTRODUCTION

Uroteuthis (Photololigo) chinensis L. belongs to Cephalopods, one of the most popular seafoods in the world. Currently, the annual yield of Uroteuthis (Photololigo) chinensis in Vietnam is estimated at 110 000 tons, accounting for over 20% of total squids caught in Vietnam [1]. Cephalopods have no bones, which means that an average Loliginid squid has from 80 to 85% of edible parts, whereas teleosts have 40-75%, crustaceans – from 40-45%, and cartilaginous fish – only 25% [2]. Loliginid squids are associated with a wide range of dried, frozen, chilled, canned, and ready-made products.

Nowadays, consumers pay special attention to high quality and healthy food [3]. To ensure seafood safety, producers have to maintain the product quality on each step of the whole chain, from fishing net supply to fork [4]. The phase between fishing and processing facilities is of high importance because seafood is typically stored on ice, and its quality deteriorates quickly. Therefore, it is necessary to find a quick and effective method to evaluate the seafood freshness quality during this phase.

When seafood undergoes protein degradation during storage, the sequence of changes can be detected by human senses. Sensory evaluation is

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an easy and effective method of seafood freshness assessment for its economic properties. Sensory evaluation is a scientific method to measure, analyze, and explain the sensory responses as perceived by the senses of sight, smell, taste, touch, and hearing [5]. There are many modern sensory assessment methods to evaluate seafood quality, e.g. the EU-scheme, the quality index method (QIM), the Torry scale, and the quantitative descriptive analysis method [6]. In Vietnam, the sensory properties of Loliginid squids are typically evaluated using the descriptive method and scoring according to the Vietnamese National Standards No. TCVN 3215-79 and TCVN 5652-1992 [7-9]. There is a need to develop a compliance quality control to world standards to help the Vietnamese seafood standards reach the world-class level.

The QIM is a quick and reliable way to measure the freshness of cold storage seafood [10, 11]. The QIM method is based on the prespecified meaning of the seafood parameters, e.g. skin, mucus, eyes, belly, smell, etc. Each parameter scores from 0 to 3. The quality index is the total score. When the quality index approaches 0, the seafood is considered fresh, while a higher quality index means some degree of quality deterioration [5]. The quality index increases linearly during ice storage time and is used for quality control in seafood processing. The QIM is developed and applied individually to every seafood species [10, 12].

All squids demonstrate sensory changes during storage: they depend on the species, assessment method, fishing area, season, time of fishing, and storage conditions. There were several studies worldwide to evaluate the sensory changes of squids using QIM applied to individual kinds of squids, e.g. common cuttlefish (*Sepia officinalis* L.), southern shortfin squids (*Illex coindetii* L.), and common octopus (*Octopus vulgaris* L.) [13, 14]. Still, there have been no similar studies on Loliginid squids (*Uroteuthis (Photololigo) chinensis*).

Therefore, consumers, fishers, seafood traders, seafood industry businesses, and controlling entities need a reliable tool to determine the quality and freshness of Loliginid squids. This study proposes a sensory evaluation program based on the QIM scheme for Loliginid squids preserved on ice.

STUDY OBJECTS AND METHODS

The research involved fresh *Uroteuthis (Photololigo) chinensis* L. obtained from Cat Ba fishing port, Hai Phong City, Vietnam, in August 2019.

The squids were layered on a plastic tray with holes and a stainless steel cap. The set was put in an insulated icebox with the squid/ice ratio of 1:2 (w/w) and transported to the Laboratory of Research Institute for Marine Fisheries. In the laboratory, the insulated perforated bottom icebox was kept in the refrigerator at $0-4^{\circ}$ C. Ice was added to the boxes as required. The samples were taken every day, from day 0, when the

samples were at their freshest, to day 25, when they got spoilt.

The terms to describe the changes in the sensory parameters related to the texture, smell, and color came from direct observation of the samples, previous studies, and the Vietnamese National Standards No. TCVN 11182-2015 and TCVN 5652-1992 [7, 13, 14]. An expert committee selected the descriptive terms via discussion and agreement. The terms were short, clear, commonly used, and easy to understand. The members of the committee were chosen and trained according to TCVN 12388-2: 2018.

The establishing of the quality index method (QIM) scheme included three steps:

Step 1 – Establishing the initial scheme. Three to five experts observed all the changes in each of the following quality parameters: skin, flesh, and eye color; flesh structure; belly and mouth area smell; eye and tentacle status; and surface and mouth mucus. After that, they developed the terms for the initial scheme. Each property received a score from 0 to 3; a lower score indicated a better quality.

Step 2 – QIM scheme and committee training. The samples were stored at $0-4^{\circ}$ C and were evaluated daily during the 25-day period. The committee consisted of six experts. During this phase, the committee members were initially aware of the time of storage to be correlated with the changes of properties during storage. During further phases, the members received no information regarding the storage time of the sample until the results became accurate and reliable.

Step 3 – Applying the QIM scheme. Ten squid samples were evaluated using the QIM scheme established during step 2 (from Mo1 to Mo10). The correlation formula between storage time and quality index helped to estimate the storage time and the remaining shelf life. The estimation was then compared to reality.

The collected data were analyzed using the descriptive statistic method (average, standard deviation). ANOVA 1 factor (*P*-value < 0.05) made it possible to determine the difference of the factors in the experiments using the Statgraphic XV and MS Excel software. Each test was performed in triplicate and repeated three times with similar results.

RESULTS AND DISCUSSION

The aim of this step was to evaluate the changes in the sensory parameters of *Uroteuthis (Photololigo) chinensis* L. stored on ice. The parameters included color, texture, smell, etc. After that, the terms were selected to describe the changes to be used in the quality index method (QIM) scheme.

The color was assessed according to the state of the skin, meat, and eyes of the squids. Figs. 1–3 show the color changes in the skin, meat, and eyes of the squids.

The skin was translucent on day 0; translucent white, with black pigment spots on day 5; midly





Day 0

Day 5



Day 15



Day 20

Figure 1 Changes in the skin color during storage



Figire 2 Changes in meat color during storage

opalescent white, with pink spots, slightly reddish on day 15; and red, with blurry purple-red pigment spots on day 20 (Fig. 1). The meat was described as translucent white on day 0; mildly opalescent white on day 10; pearly white on day 15; and opalescent white, milky white on day 25 (Fig. 2). Changes in the eyes were as foolows: translucent on day 0; mildly opalescent on day 10; opalescent on day 20; and milky white with black fluid on day 25 (Fig. 3).

Smell: property changes and terms. The smell was assessed on the body and mouth area. The changes of the odour on the squid body were described as: seaweedy, fresh, or seafood smell (day 0); mildly fishy (day 10); strongly fishy, mildly sour (day 20); and heavily fishy, foul, spoiled (day 25). The changes of the smell on the mouth areas were seaweedy, fresh, seafood smell (day 0); seaweed, mildly fishy (day 5); strong fishy (day 10); strong fishy, mildly foul (day 15); and heavily fishy, foul, spoiled, respectively (day 25).

Texture and status: property changes and terms. The status or structure assessment was based on the structure of flesh, status of the eyes, texture of the mucus on the body, mouth, and condition of the tentacles.

The structure of the flesh was described as consistent, well elastic (day 0); consistent, elastic (day 5); less elastic, slightly soft (day 15); and flaccid, viscous (day 25).

The shape of the eyes was described as convex, round pupils (day 0); less convex, less round pupils (day 5); flat eyes, pupils not rounded (day 15); and eyes slightly concaved, pupil ruptured (day 20).

The textures of the mucus on the body were transparent (day 0); Transparen, viscous, thin layer (day 5); less sticky, watery (day 10); less sticky, watery (day 15), and none (day 20).



Figure 3 Changes in color and status of squid eyes during storage

The textures of the mucus on the mouth area were transparent (day 0); transparent, stick (day 5); and little and yellowish (day 20).

The tentacles were changed as consistent, strong sucking, suckers intact (day 0); soft, could not suck when touched, suckers start to fall out (day 10); and flaccid, not sucking, suckers falling out (day 15).

QIM scheme for sensory evaluation of Loliginid squids. In this study, we established a QIM scheme for Loliginid squids. It included 10 parameters and recorded the sensory changes during storage time with a score from 0 to 28 (Table 1).

Changes in the Loliginid squid stored on ice. Table 2 features the changes in the sensory parameters of Loliginid squids that occurred during storage.

Loliginid squids demonstrated obvious The changes in the sensory quality during ice storage. It could be divided into four levels: days 0-5, days 5-10, days 10-15, and days 15-25. The levels were numbered from I to IV, respectively. At level I (days 0-5), the squids were raw and fresh, and there was no change in the texture, color, or smell. Most of the sensory parameters scored 0, and the quality index did not exceed 10. At level II (days 5-10), the sensory parameters started to change: the body color and the smell of body and mouth area scored from 0 to 1 with the quality index at about 10-15. At level III (days 10-15), there were apparent changes in the sensory parameters: the skin lost its shine, pinkish pigment spots appeared, the flesh became opalescent, and the smell increased. Most of the sensory parameters got 1-2 scores, and the quality index fluctuated from 15-20, showing that the squids started to deteriorate. At level IV (> 15 days), the sensory parameters changed: the whole body reddened, patchy dark pigment

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Parameters		Description	Score
Color	Skin	Translucent color	0
	1		
		Mildly opalescent white, pink spots, slightly reddish	2
		Pink, red, with blurry purple-red pigment spots	3
	Flesh	Translucent white	0
		Mildly opalescent white	1
		Pearly white	2
		Opalescent white, milky white	3
	Corneal/eye tissues	Translucent	0
		Mildly opalescent	1
		Opalescent	2
		Milky white with black fluid	3
Smell	Body	Seaweedy, fresh, or seafood smell	0
		Mildly fishy	1
		Strongly fishy, mildly sour	2
		Heavily fishy, foul, spoiled	3
	Mouth area	Seaweedy, fresh, seafood smell	0
		Mildly seaweed fishy	1
		Fishy, mildly foul	2
		Heavily fishy, foul, spoiled	3
Status	Flesh structure	Consistent, highly elastic	0
		Consistent, elastic	1
		Less elastic, soft	2
		Flaccid, viscous	3
	Eye shape	Convex, round pupils	0
		Less convex, less round pupils	1
		Flat eyes, pupils not rounded	2
		Slightly concaved, pupil ruptured	3
	Body mucus	Transparent	0
		Transparent, sticky, thin layer	1
		Less sticky, watery	2
		Not detected	3
	Mouth mucus	Transparent	0
		Transparent, sticky	1
		Poor and yellowish	2
	Tentacles	Firm, strong sucking, suckers intact	0
		Soft, could not suck when touched, suckers start to fall out	1
		Flaccid, not sucking, suckers falling out	2
	Range of QIM score		0–28

Table 1 QIM scheme for sensory evaluation of Uroteuthis (Photololigo) chinensis L.

appeared, the flesh became floppy, and the foul smell became strong. Most parameters scored 2–3, showing clear signs of spoilage. The deterioration became especially evident on days 20-25: the whole body became purple-red and started to emanate a strong foul smell, while the flesh was flaccid and viscous. Therefore, the shelf life for squids preserved on ice equaled 10-12 days.

Figure 4 shows changes in the sensory parameters of Loliginid squids stored on ice. The quality index demonstrated a meaningful linearly increase over time with P < 0.05.

The recession equation was: $Y = 1.083 X_{tg} + 2.866 (R^2 = 0.9908).$

Verifying the QIM scheme for Loliginid squids. Table 3 sums up the results of determining storage time and remaining shelf life of squids using the equation (*) and real remaining time.

In this study, we established a QIM scheme that fully described the sensory changes that occurred in *Uroteuthis* (*Photololigo*) chinensis according to their biochemical transformation during ice storage. The scheme involved such parameters as skin and pigment color, flesh and mucus texture, and body and mouth smell. Pink color often appears on the cephalopod bodies. Skin pigments deter Loliginid iorate easily as a result of oxidation and enzyme tyroprotease during the storage period, and the skin goes black, pink, or red [15–17]. The flesh texture softens mostly due to the endogenous protease [18, 19].

Tabl	e 2	Changes i	n sensory	parameters	of	Uroteuth	is (I	Photol	loligo)	chinensis	L.	during	ice	storage
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Storage time, days	Sensory parameters	Quality index	Level
0	Translucent body, seaweedy, fresh, or seafood smell; firm, consistent, well elastic flesh; convex eyes, round translucent pupils; translucent mucus; firm, well sucking tentacles with intact suckers	0.39 ± 0.50	Ι
5	Translucent white body, with black pigment spots; seaweedy smell; mildly opalescent white and firm flesh; less convex eyes, less round black pupils; translucent mucus; rather soft tentacles with intact suckers but not sucking.	8.83 ± 0.71	II
10	White body with clear pigment spots, mildly fishy smell, white and firm flesh; mildly convex eyes with opalescent not rounded pupils; some mucus remains; rather soft tentacles with suckers starting to fall out, no sucking	14,44 ± 0,51	II
15	Mildly opalescent white body with some pink pigment spots, reddish skin; strong fishy and mildly foul smell; pearly white flesh, soft and less elastic; flat eyes with mildly opalescent corneal, not rounded pupils; less mucus remains; soft tentacles with suckers fallen out.	19.89 ± 0.47	III
20	Pinkish body with reddish pigment spots all around the body; strong fishy, foul smell; opalescent white soft flesh; mildly concaved eyes with opalescent corneal, broken pupils; no mucus; flaccid tentacles with suckers completely fallen out.	24.56 ± 0.51	IV
25	Purple-pink body with pigment spots all over; foul smell; milky white, soft and viscous flesh; mildly concaved eyes with opalescent corneal, broken pupils; no mucus; flaccid tentacles with suckers completely fallen out.	28.00 ± 0.00	IV



Figure 4 Quality index changes in the Loliginid squids during ice storage

Table 3 Determining storage time and estimating the remaining shelf life of squids during ice storage using QIM scheme

Sample	Quality	Storage	Estimating	Real
	index	time based	the remaining	remaining
		on the	shelf life	time, days
		equation,	based on the	
		days	equation, days	
Mo1	4.13	1.17	10.83	10.30 ± 0.26
Mo2	6.54	3.40	8.60	8.17 ± 0.36
Mo3	11.21	7.72	4.28	4.77 ± 0.25
Mo4	16.43	12.56	-0.56	-
Mo5	9.15	5.81	6.19	5.80 ± 0.26
Mo6	12.46	8.88	3.12	3.33 ± 0.58
Mo7	13.57	9.91	2.09	2.630 ± 0.32
Mo8	18.74	14.69	-2.69	_
Mo9	5.36	2.31	9.69	9.93 ± 0.12
Mo10	14.89	11.13	0.87	0.83 ± 0.29

We concluded that the shelf life of Loliginid squids was 10–12 days, which is equivalent to and slightly longer than that of other squid species in the previous QIM studies. For instance, raw cuttlefish and shortfin squids

proved to have 9–10 days of shelf life, while that of common octopus was only 8–10 days [13–15, 20].

The quality index equation introduced in this article correlated with the reports from previous authors that the quality index value increased linearly during ice storage in various kinds of seafood, e.g. fish, shrimps, and octopus [12, 15, 21, 22]. The verifying result also showed that the real remaining shelf life was similar to the calculated one. Therefore, the developed equation proved to be a scientifically based tool that could be used to evaluate the freshness and quality of raw Loliginid squids.

The obtained QIM scheme and quality index equation set a new quality control standard for the Loliginid squid in Vietnam. As they are simple enough to be used on the international seafood market, they also contribute to the QIM schemes that can be used worldwide.

CONCLUSION

In this study, we successfully established a quality index method (QIM) scheme for sensory evaluation of Uroteuthis (Photololigo) chinensis L. stored on ice. The scheme included ten parameters, and the quality index value was 0-28. We also developed a linear correlation equation of quality index and storage time with $R^2 = 0.99$. The best shelf life for Loliginid squids stored on ice proved to be from 10 to 12 days. The QIM and quality index equation can be combined with other chemical quality parameters of freshness to form a full-quality assessment program for Loliginid squids. The program could provide a user-friendly, quick, and efficient scientific-based tool that could help customers, fishers, seafood traders, seafood industry businesses, and controlling entities to specify the storage time and estimate the remaining shelf life for Loliginid squids.

CONTRIBUTION

The idea behind the analysis belongs to B.T.T. Hien. B.T.T. Hien, P.T. Diem, P.V. Tuyen, N.V. Thanh, D.V. An, L.A. Tung, N.K. Bat, N.V. Nghia, and N.T.M. Tu collected the data. B.T.T. Hien, P.T. Diem, P.V. Tuyen, and H.-D. Tran contributed data and performed the analysis. B.T.T. Hien, P.T. Diem, N.K. Bat, and H.-D. Tran wrote the paper. All the authors proof-read the manuscript and agreed to its published version.

CONFLICT OF INTEREST

The authors declare no conflict of interests related to

the publication of this article.

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Amaranth as a bread enriching ingredient

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Abstract:

Amaranth is a promising raw material for enriching foods with protein, minerals, vitamins, dietary fibre, squalene, and other nutrients. However, its varieties differ significantly in composition and properties. The research included two stages. At first, we studied the composition of eight amaranth varieties grown in a collection nursery of Voronezh State Agrarian University. Their composition was a factor that determined their functional use as an enriching ingredient. We found that amaranth grain of the Universal variety could be best used to increase the biological value of foods, whereas the Universal and Valentina varieties could be recommended as multifunctional ingredients. The addition of enriching ingredients into foods, including breads, often leads to changes in their traditional consumer properties. Therefore, our next step was to study changes in the composition of Universal amaranth during extrusion using IR spectroscopy. Also, we assessed the effect of amaranth extrudate on the baking properties of model wheat flour and extrudate mixtures as the main factor of the product's consumer properties. The results showed a redistribution of moisture between flour gluten proteins and extrudate dietary fibre. We also established amounts of amaranth extrudate needed to ensure the preservation of crumb appearance and structure close to the traditional ones.

Keywords: Grain, foods, gluten, extrudate, flour, IR-spectra, leaves

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INTRODUCTION

Improving people's diet involves enriching foods with a variety of essential nutrients. First of all, these include vitamins, some macro- and microelements, dietary fibre, polyunsaturated fatty acids, and other substances [1–7]. Protein is another important component of a balanced diet [1, 4]. Amaranth is a promising raw material and a rich source of nutrients [8-10]. Numerous studies, both in Russia and abroad, have focused on enriching breads and bakery confectionery products with amaranth [11-24]. However, these developments have not yet been commercialized on a large scale. This confirms the need to continue the search for applications of amaranth and its products in the food industry, especially in the production of staple foods such as breads. Thus, we need to develop a technology based on amaranth - not only its composition

depending on variety, but also its functional and technological properties.

Our aim was to assess amaranth as a raw ingredient for breads, based on the study of its composition, as well as its functional and technological properties.

STUDY OBJECTS AND METHODS

Market research was conducted to identify people's attitudes to enriching ingredients, taking into account permissible changes in traditional consumer properties of a product determined by changes in its formulation. The sample of 405 respondents was representative of the general population in the region. The statistical error did not exceed 5% at a confidence level of 95%.

The object of study was amaranth grown in a collection nursery of Voronezh State Agrarian University. Geographically, the nursery is located in the

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Amaranth variety	Amaranth variety Content, %					Ash on dry basis, %
	moisture	protein	fat	cellulose	mono- and disaccharides	
Voronezh-36	10.1	15.78	6.4	6.9	2.5	2.98
Voronezh	10.5	16.21	6.3	5.7	2.4	3.41
Imperator	10.2	19.57	11.7	7.5	2.4	3.99
Rubin	11.4	20.66	9.8	7.1	2.7	4.75
Universal	9.4	26.47	13.8	8.9	4.1	8.08
Gigant	10.1	19.29	10.3	7.1	2.9	3.03
Dobrynya	10.9	16.79	7.2	16.3	2.4	4.33
Valentina	11.6	16.10	6.9	19.4	2.6	6.04

Table 1 Amaranth grain composition

forest-steppe zone of the Central Black Earth Region. The experiments were carried out on leached, medium loamy chernozem with medium and high availability of nitrogen, phosphorus, and potassium mobile forms. The humus content was 4.5%, pH 5.4–5.8. Precipitation during a period of temperatures above +10°C reached 250–260 mm. Total active temperatures amounted to 2581°C. The seeding took place in the second half of May.

The study covered the following amaranth varieties bred by Voronezh State Agrarian University and the Federal Research Centre of Vegeculture: Voronezh-36, Voronezh, Imperator, Rubin, Universal, Gigant, Dobrynya, and Valentina.

The composition of amaranth grain and leaf mass was determined at the All-Russian Research Veterinary Institute of Pathology, Pharmacology and Therapy according to the following state standards:

- protein: State Standard 10846-91^{II};
- fat: State Standard 29033-91^{III};
- cellulose: State Standard 31675-2012^{IV};

- total sugar (mono- and disaccharides): State Standard 15113.6-77^v;

- carotene: State Standard 13496.17-95^{IX};

- copper and zinc: State Standard 30692-2000^x; and

- iron and manganese: State Standard 32343-2013^{XI}.

The content of digestible protein was determined by the PDCAAS method, with the degree of digestibility being 75% for amaranth grain protein and 65% for amaranth leaf protein [25, 26].

The quality of flour was determined according to State Standard 9404-88^{XII} (moisture) and State Standard 27839-2013^{XIII} (gluten quantity and quality).

We used top-grade baking wheat flour with a moisture content of 14.43%, a gluten content of 32%, and gluten quality rated as "satisfactory weak" (gluten deformation index, IDK).

Amaranth grain was subjected to extrusion. The extrudate was obtained from whole low-fat grains of Universal amaranth through an EUM-1 compact laboratory extruder at 110–120°C. Then it was ground to a particle size of max. 125 µm in a laboratory mill.

The infrared absorption spectra were determined in the range of $400-4000 \text{ cm}^{-1}$ on a Bruker VERTEX 70 FTIR spectrometer in the reflection mode.

Model mixtures were made of top-grade baking wheat flour and amaranth extrudate. The studies were performed at the Dokuchaev Scientific Research Institute of Agriculture of the Central Chernozem Zone. The data were processed in accordance with State Standard R 51414-99^{XIV}. The quantity and quality of gluten were determined according to State Standard 27839-2013¹³.

[–] moisture: State Standard 13586.5-2015^I;

⁻ ash: State Standard 27494-2016^{VI};

[–] phosphorus: State Standard 26657-97^{VII};

⁻ calcium: State Standard 26570-95^{VIII};

¹State Standard 13586.5-2015. Grain. Method of moisture content determination. Moscow: Standartinform; 2019. 13 p.

¹¹ State Standard 10846-91. Grain and products of its processing. Method for determination of protein. Moscow: Standards Publishing; 2001. 8 p.

^{III} State Standard 29033-91. Grain and derived products. Determination of fat content. Moscow: Standards Publishing; 1992. 5 p.

^{1V} State Standard 31675-2012. Feeds. Methods for determination of crude fibre content with intermediate filtration. Moscow: Standartinform; 2014. 10 p.

^v State Standard 15113.6-77. Food concentrates. Methods for determination of sucrose. Moscow: Standartinform; 2011. 11 p.

^{VI} State Standard 27494-2016. Flour and bran. Methods for determination of ash content. Moscow: Standartinform: 2019. 11 p.

^{VII} State Standard 26657-97. Fodders, mixed fodders, mixed fodder raw materials. Methods for determination of phosphorus content. Moscow: Standards Publishing; 2002. 10 p.

^{VIII} State Standard 26570-95. Fodder, mixed fodder and mixed fodder raw material. Methods for determination of calcium. Moscow: Standards Publishing; 2002. 14 p.

^{1X} State Standard 13496.17-95. Forage. Methods for determining carotin. Moscow: Standartinform; 2011. 5 p.

^x State Standard 30692-2000. Fodders, mixed fodders and animal raw foodstuff. Atomic absorption method for determination of copper, lead, zinc and cadmium. Moscow: Standards Publishing; 2002. 10 p.

^{XI} State Standard 32343-2013. Feeds, compound feeds. Determination of the contents of calcium, copper, iron, magnesium, manganese, potassium, sodium and zinc by atomic absorption spectrometry method. Moscow: Standartinform; 2014. 17 p.

^{XII} State Standard 9404-88. Flour and bran. Method of moisture content determination. Moscow: Standards Publishing; 2001. 4 p.

XIII State Standard 27839-2013. Wheat flour. Methods for determination of gluten quantity and quality. Moscow: Standartinform; 2014. 19 p.

^{XIV} State Standard R 51414-99. Wheat flour. Physical characteristics of doughs. Determination of water absorption and rheological properties using a valorigraph. Moscow: Standartinform; 2007. 8 p.

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Figure 1 Digestible protein content in amaranth grain varieties: 1 – Voronezh-36, 2 – Voronezh, 3 – Imperator, 4 – Rubin, 5 – Universal, 6 – Gigant, 7 – Dobrynya, 8 – Valentina

RESULTS AND DISCUSSION

The comparative characteristics of the amaranth varieties studied are shown in Tables 1, 2.

We found that the composition of amaranth grain differed widely, depending on its variety. The highest content of protein was observed in Universal and Rubin, fat – in Universal and Imperator, mineral substances – in Universal and Valentina. Universal also contained the maximum amount of mono- and disaccharides and digestible protein (Fig. 1). Thus, this variety seemed to have the best composition for being used as an enriching ingredient to increase the biological value of foods, including breads. In addition, this grain is white with a slight yellowish tinge, which makes it suitable for any product, regardless of its intended color.

Another important aim of food fortification is an increase in dietary fibre and minerals.

We found that the varieties of Valentina and Dobrynya had the highest cellulose content. Universal and Valentina were also rich in minerals. The results of additional studies of amaranth mineral composition are presented in Table 2.

Calcium and iron are two key minerals that Russian diet has a deficiency of [12]. We found that the varieties of Valentina, Universal, and Dobrynya had the highest calcium content, whereas Voronezh-36 and Universal were rich in iron.

As for other minerals, we found a high content of phosphorus in Universal, copper in Gigant and Universal, zinc in Voronezh, and manganese in Valentina. The content of copper and manganese should be considered as a limiting factor when developing food formulations. It is also noteworthy that Valentina has a dark colour, which may affect the sensory perception of the finished product.

Another form of amaranth use is its leaf mass. After harvesting, amaranth leaves were dried by convection at 30–35°C. This method was used to preserve the maximum amount of biologically active substances.

The comparative characteristics of amaranth leaf mass by variety are given in Tables 3, 4.

Amaranth leaf mass showed significant differences in composition. The highest content of protein and digestible protein (Fig. 2) was found in Rubin and Dobrynya, cellulose – in Universal and Valentina, total sugar – in Universal and Gigant. All the varieties had a low fat content and a high ash content. On the whole, the

Amaranth variety		Content						
	phosphorus, %	calcium, %	copper, mg/kg	iron, mg/kg	zinc, mg/kg	manganese, mg/kg		
Voronezh-36	0.46	0.17	8.6	110	32.5	52		
Voronezh	0.54	0.24	7.2	77	35.8	55		
Imperator	0.45	0.25	7.2	82	31.0	51		
Rubin	0.55	0.17	6.1	73	30.8	51		
Universal	0.63	0.36	13.0	90	30.9	45		
Gigant	0.54	0.20	14.9	77	32.7	29		
Dobrynya	0.50	0.36	8.1	72	28.2	47		
Valentina	0.46	0.48	5.4	76	31.3	82		

Table 2 Mineral substances in amaranth grain

Table 3 Amaranth leaf mass composition

Amaranth variety		Ash on dry basis, %				
	moisture	protein	fat	cellulose	mono- and disaccharides	
Voronezh-36	8.1	12.02	1.6	10.1	3.2	24.02
Imperator	8.7	10.48	2.0	10.3	3.9	18.46
Rubin	7.5	20.13	1.6	11.0	1.0	20.36
Universal	7.8	17.73	2.2	17.5	4.3	19.02
Gigant	8.1	11.77	2.1	9.9	4.4	20.55
Dobrynya	8.6	19.56	1.8	12.1	0.8	21.32
Valentina	8.3	14.08	1.5	14.3	3.7	17.55

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Figure 2 Digestible protein content in amaranth leaves by variety: 1 – Voronezh-36, 2 – Imperator, 3 – Rubin, 4 – Universal, 5 – Gigant, 6 – Dobrynya, 7 – Valentina



Figure 3 Carotene content in amaranth leaves by variety: 1 – Voronezh-36, 2 – Imperator, 3 – Rubin, 4 – Universal, 5 – Gigant, 6 – Dobrynya, 7 – Valentina

Amaranth variety	Content								
	phosphorus, %	calcium, %	copper, mg/kg	iron, mg/kg	zinc, mg/kg	manganese, mg/kg			
Voronezh-36	0.42	6.00	1.4	0.2	0.4	1.5			
Imperator	0.33	5.01	1.3	0.2	0.5	1.7			
Rubin	0.35	4.32	1.8	0.7	0.4	1.4			
Universal	0.31	4.80	1.6	0.4	0.4	1.2			
Gigant	0.39	4.86	1.5	0.7	0.5	2.6			
Dobrynya	0.42	4.60	1.6	3.0	1.1	1.9			
Valentina	0.36	2.88	1.6	0.2	4.7	3.0			

Table 4 Mineral substances in amaranth leaves

leaf mass of these varieties can be regarded as a protein ingredient. In addition, it can be used as a source of dietary fibre and mineral substances.

The data presented in Table 4 were used to find effective ways of correcting diets with macro- and microelements by introducing supplements from amaranth leaf mass.

We found that amaranth leaf mass was rich in calcium, especially Voronezh-36 and Imperator. Therefore, amaranth can be classified as a functional food ingredient. Compared to grain, amaranth leaves had a much lower content of iron, copper, zinc, and manganese. Of all the varieties, Valentina showed the highest content of zinc and manganese.

A high carotene content was found in the leaves of Dobrynya and Valentina (Fig. 3). Due to the antioxidant properties of carotenes, the leaves of these varieties can be recommended as enriching supplements.

Thus, the comparative analysis of amaranth composition showed the following results:

- amaranth grain and leaf composition differed significantly, depending on the variety; therefore, when developing formulations for functional or specialised foods, it is important to specify the recommended variety;

 Universal amaranth grain is best suited for improving the biological value of foods with a minimal effect on their color range; – Valentina and Dobrynya grain, as well as Universal and Valentina leaf mass, can be recommended as a source of dietary fibre; however, the color of amaranth grain and its products should be taken into account;

- Valentina grain, as well as Voronezh-36 and Imperator leaf mass, can be used in foods to increase their calcium content, with an allowable level of manganese being max -5 mg/day^{xv} ;

– Dobrynya amaranth leaves can be recommended for foods with antioxidant properties; and

– Universal and Valentina varieties can be used as polyfunctional ingredients.

Further studies were conducted with Universal amaranth variety, taking into account its color range and potential uses as an enriching ingredient in breads. To increase digestibility and adjust taste and smell, amaranth grain mass was subjected to extrusion. The extrudate was ground to a particle size of max. 125 µm.

The Universal extrudate is a loose powdery semifinished product with a light cream color, a nutty aroma, and a faint bitterish taste, characteristic of amaranth. It contains 4.76% moisture, 27.51% protein, 4.53% fat, and 3.20% cellulose.

^{XV} MR 2.3.1.2432-08 Normy fiziologicheskikh potrebnostey v ehnergii i pishchevykh veshchestvakh dlya razlichnykh grupp naseleniya Rossiyskoy Federatsii [Methodological guidelines MG 2.3.1.2432-08 "The norms of physiological needs for energy and nutrients for various population groups in the Russian Federation"].



Figure 4 IR absorption spectra of Universal amaranth (1) and Universal amaranth extrudate (2)



Amount of amaranth extrudate in the model mixture, %

Changes in the fractional composition of amaranth during extrusion were studied by IR spectroscopy (Fig. 4).

Absorption bands were identified in the short-wave $(3600-2600 \text{ cm}^{-1})$ and long-wave $(1800-900 \text{ cm}^{-1})$ spectral regions. The first band was characteristic of C–H stretching vibrations of methyl and methylene fragments at 2923–2933 cm⁻¹ and 2855 cm⁻¹, as well as O–H and N–H stretching vibrations at 3280–3300 cm⁻¹ [22]. In the long-wave region, intense absorption was observed at 1000–1050 cm⁻¹, with a well pronounced band at 1150 cm⁻¹, which corresponded to vibrations of the C–O–C ester group in the structure of cellulose [27]. The spectral behaviour of amaranth and amaranth extrudate samples was identical. Comparing the relative heights of the absorption bands for amaranth and amaranth extrudate, we found less intensive



Figure 5 Attitudes to consumer properties of enriched breads: 1 – respondents preferring traditional characteristics,

2 – respondents accepting minor differences,

3 – respondents accepting significant differences

Figure 6 Gluten content in the model mixtures of wheat flour and amaranth extrudate

vibrations of the carboxyl group (1740 and 1650 cm⁻¹) and amide bonds (1650 and 1540 cm⁻¹), as well as a lower height of the "ester band" (1050 cm⁻¹). This was due to the fact that extrusion causes destructive changes mostly in protein components, rather than in cellulose substances. On the whole, the IR absorption spectra analysis confirmed a partial destruction of amaranth proteins and polysaccharides during extrusion. On the one hand, this suggested a better digestibility of the extrudate compared to grain. On the other hand, this indicated the extrudate's sorption activity with respect to heavy metal cations due to the preserved native structure in some part of the carbohydrate-based biopolymers.

Thus, the study proved the efficacy of extrusion as a way of preparing amaranth for being used as a



Figure 7 Gluten quality in the model mixtures of wheat flour and amaranth extrudate

Indicator	Of model m of wheat flou	ixtures at different ratios r and amaranth extrudate	Wheat flour : amaranth extrudate ratio		
	90:10	85:15	80:20	70:30	
Water absorption capacity, %	57.76	58.42	62.14	62.82	
Duration of dough development, min	4 min 30 s	6 min 10 s	5 min 30 s	5 min 30 s	
Dough stability, min	4 min 05 s	3 min 55 s	3 min 30 s	2 min 50 s	
Dilution, units	90	80	85	85	
Valorimetric rating, units	70	72	72	71	

Table 5 Flour strength indicators





Figure 8 Valorigrams of flour mixtures with a ratio of wheat flour and amaranth extrudate of: a) 90:10; b) 85:15; c) 80:20; d) 70:30. Left – replication No. 1, right – replication No. 2

food ingredient. The extrudate of Universal amaranth variety was used in further research as a bread enriching ingredient.

One of the problematic issues in food fortification is changes in traditional sensory properties, primarily in product appearance, shape, condition, and texture (structure of porosity). Our market research showed that most consumers (80%) were not ready for drastic changes in these characteristics, even when the product had an increased nutritional value and an improved nutrient composition (Fig. 5).

Bread's shape, crumb, porosity, and other characteristics mainly depend on the baking properties of flour. New grain ingredients usually bring about quantitative and qualitative changes in the proteinproteinase and carbohydrate-amylase flour complex, altering the product's sensory properties. Therefore, the next stage of our research was to study the baking properties of model mixtures of top-grade wheat flour and amaranth extrudate. The choice of top-grade flour was motivated by a lower standardized amount of gluten and, therefore, a more pronounced effect of raw ingredients on the baking properties of the flour mix. The model mixtures were made of wheat flour and amaranth extrudate in the ratios of 95:5, 90:10, 85:15, 80:20, 75:25, and 70:30.

We studied the effect of amaranth extrudate on the content and elasticity of gluten proteins and, therefore, on their ability to form dough with certain rheological properties (Figs. 6, 7).

We found that an increased amount of amaranth extrudate lowered a gluten content in the model mixture. In addition, it raised the IDK index (gluten quality index). Gluten washed out from the model mixture was less elastic. However, it is noteworthy that the changes in gluten quality did not exceed 7% of the initial IDK value.

More detailed information on the effect of amaranth extrudate on the baking properties of the model mixture was obtained using a valorigraph (Fig. 8).

Table 5 presents flour strength indicators obtained from the analysis of the valorigrams.

We found that a higher content of amaranth extrudate in the model mixture raised its water absorption capacity. This can be explained by an increased waterbinding ability of amaranth dietary fibre. Water absorption properties of amaranth extrudate were established in [28].

The increased duration of dough development might be due to the redistribution of water towards amaranth extrudate and the consequent slowing down of gluten swelling. This happened when the amount of amaranth extrudate was up to 15%. With a higher extrudate content, the duration of dough development decreased and almost stabilized. This was probably due to a lower content of gluten proteins in the model mixture (Fig. 6).

The decrease in dough stability appeared to be caused by a lower amount of gluten, since it is gluten that gives dough a three-dimensional structure. Dilution is defined as a difference between the maximum dough consistency achieved during kneading and its consistency at the end of kneading. Excessive mechanical impact weakens dough consistency. A lesser dilution of the dough with a greater amount of wheat flour could probably be explained by the temperature parameters characteristic of water binding by protein substances. Another factor might be a better retention of the hydration shell on the protein globules.

All the model mixtures had similar valorimetric values (Table 5). Depending on the ratio of wheat flour and amaranth extrudate, the deterioration of some characteristics was compensated by the improvement of others. This confirmed a possibility of producing breads from a mix of wheat flour and up to 30% of amaranth extrudate with a crumb structure close to traditional. An extrudate content of up to 15% brought about smaller changes in the crumb shape, appearance, and structure, whereas an amount of up to 30% was more effective in increasing the product's nutritional value. To establish the optimal amount of amaranth extrudate, we need to study the changes in rheological properties during dough maturation and evaluate the product's taste, color, and nutritional value.

CONCLUSION

On the whole, the study confirmed the potential of extruded amaranth of the Universal variety as an ingredient that can enrich wheat flour breads with protein and dietary fibre and ensure consumer properties close to traditional.

CONTRIBUTION

Authors are equally related to the writing of the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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Hepatoprotective effect of breads with extracts of plants growing in the Far East

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Abstract:

Breads with proven hepatoprotective properties can make a significant contribution to preventing liver disease. This work aimed to study hepatoprotective and antioxidant effects of breads enriched with water and ethanol extracts of polyphenol-containing viburnum (*Viburnum sargentii Koehne* L.), magnolia-vine (*Schisandra chinensis* L.), and grapes (*Vitis amurénsis* L.). It was based on an experimental model of toxic hepatitis in mice intoxicated with carbon tetrachloride. Experimental groups of animals were fed on bread with extracts for 7 days and control groups had a bread-free diet. We analysed their body weight, liver lipid metabolism, "lipid peroxidation – antioxidant protection" system, and antiradical activity. The level of reduced glutathione and malonic dialdehyde was determined by micro-thin-layer chromatography. Superoxide dismutase, glutathione reductase, and glutathione peroxidase activity was measured to analyse the antioxidant system. The total content of common polyphenols in breads was determined by the colorimetric method with the Folin-Chocalteu reagent. The animals on a bread-free diet showed an impaired lipid metabolism and higher activity of liver enzymes. They had a 22% increase in liver weight and a 1.9 times depletion of antiradical protection (6.65 ± 0.15 Trolox units/mg protein vs. 13.15 ± 0.21 Trolox units/mg protein in the control; P < 0.001). We also registered a 2.5 times decrease in superoxide dismutase, a key enzyme of the antioxidant defence system. The animals fed on breads with the above extracts showed a statistically significant normalization of the parameters, compared to the bread-free group. We found that those breads had hepatoprotective and antioxidant effects on the animals, stabilizing their general condition and normalizing their biochemical parameters and antioxidant system.

Keywords: Bread, plant extracts, viburnum, magnolia-vine, grapes, polyphenols, antioxidant, hepatoprotector

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INTRODUCTION

Current environmental problems are increasingly exposing the world population to various stress factors such as foreign substances (xenobiotics). The liver is commonly known as the main barrier in the human body that neutralizes xenobiotics and toxic substances. Thus, preventing liver disease is highly important.

According to modern studies, free radical reactions play a significant role in the development of pathology during toxic liver damage. Reactive oxygen species activate the process of lipid peroxidation in hepatocyte cell membranes, causing liver dysfunction. Inhibiting such processes with various biologically active substances (BASs), including natural antioxidants, can have a preventative or therapeutic effect.

Foods with proven hepatoprotective and antioxidant properties can make a significant contribution to solving

this problem. Today, bread is a basic food product, and breadmaking is a socially important industry [1]. Bread is part of a daily diet for the vast majority of consumers in many countries. Thus, it can serve as a basis for preventative and dietetic specialized products that can contribute to the prevention of various liver diseases [2].

Introducing plant polyphenols into the product formulation is one of the ways to develop breads with antioxidant and hepatoprotective properties. Characterized by high antioxidant activity, plant polyphenols are a source of effective hepatoprotectors. They are able to eliminate increased lipid peroxidation during toxic hepatitis and improve the antitoxic function of liver cells [3].

In contrast to synthesized polyphenols, natural polyphenols have an extremely low toxicity and do not cause adverse reactions (allergies, addiction, or

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accumulation). According to studies, plant phenolic compounds display a wide range of biological (including hepatoprotective) effects. This is due to their antioxidant properties and an ability to act as traps for free radicals of various types [4, 5].

Being plant secondary metabolites, phenolic compounds are contained in traditional plant products used by humans (fruits, vegetables, berries, etc.). Thus, they are evolutionarily adapted for the human body, form complexes with transition metal ions and block lipid peroxidation processes [6–8].

However, of all the plant materials studied (plants, fruits, vegetables, cereals, seeds, bark, etc.), only 10% are sufficiently rich in polyphenolic compounds and can be used as effective natural antioxidants [9, 10]. Both foreign and Russian studies have revealed a need for enriching breads with plant polyphenols to ensure their antioxidant and hepatoprotective properties. For this, we can use such innovative ingredients as berry and fruit extracts, pectin-rich fruits, blueberry paste, and bioflavonoids from larch, vegetables (onions, beets), or ground coffee [17–19].

We evaluated sensory and physicochemical properties of breads containing various concentrations of polyphenols. The bread showed an increased antioxidant activity. Preclinical studies in animals with various diseases are vital in justifying the choice of new ingredients, their efficacy and biological effects in foods with preventative properties. However, scientific literature lacks data on experimental studies in animals that confirm the preventive efficacy of hepatoprotective ingredients in breads. This determined the aim of our study.

The Far East, particularly the Ussuri taiga with its wild plants, is a valuable source of plant raw materials rich in various BASs and used in medicine, pharmacology, animal husbandry, and food industry. BASs are often used as physiologically functional ingredients for dietetic (medicinal and preventative) foods [20].

People have to consume enormous quantities of plants to obtain the required amount of macro- and microelements, as well as other BASs, which is often impossible for many reasons. In this regard, food products are enriched with individual components of plant materials and their extracts that have high biological activity.

Water and ethanol extracts of plant materials are concentrates of biologically active substances. They are effective in small amounts and therefore can be added to products in amounts similar to dried berries [21]. Low contents of biologically active physiologically functional ingredients ensure the preservation of traditional sensory characteristics in the products, which is an important factor for the consumer. Baking technology (with temperatures up to 200–220°C) can neutralize the alcohol component of water and ethanol extracts and their specific sensory properties. This makes such extracts a promising ingredient for breads with dietetic and preventative properties.

For this study, we developed breads with three biologically active dietary supplements (BADSs): Caliphene, Eklikit, and Diprim. They are water and ethanol extracts developed at V.I. Ilyichov Pacific Oceanological Institute, Russia.

Caliphene was obtained from viburnum (*Viburnum* sargentii Koehne L.) processing waste with proven membrane- and hepatoprotective, antioxidant, antiradical, and other properties. The content of common polyphenols in it was 32.8 ± 2.4 g/L.

Eklikit was extracted from magnolia-vine (*Schisandra chinensis* L.) processing waste. Its polyphenol complex included proanthocyanidins, leucoanthocyanins, catechins, flavonols, organic acids, free amino acids, etc. The content of common polyphenols was 14.4 ± 1.7 g/L.

Diprim was made of grape (*Vitis amurénsis* L.) stalks. Its main component was such polyphenolic compounds as catechins and their polymer forms, leucoanthocyanins, flavonoids, procyanidins, oligomeric tannins, and lignin. The content of common polyphenols was 35.4 ± 2 g/L [20].

Biomedical studies have found a multifactorial positive effect of Caliphene, Eklikit, and Diprim on the human body, both when used as a dietary supplement and as part of foods, including breads [3, 22–25].

The quality and safety evaluation of breads enriched with water and ethanol extracts showed their compliance with the regulatory documents of the Russian Federation and the Eurasian Economic Union [26, 27].

The preclinical tests of extract-containing breads in animals using standard pharmaceutical models demonstrated their stress-protective (or adaptogenic) and actoprotective effects [28].

We believe that hepatoprotective and antioxidant properties of breads containing water and ethanol extracts are determined by the extract chemical composition as reported in [29, 30], a significant content of polyphenols, and its stability in the products.

Our experimental study in animals with toxic hepatitis aimed to evaluate the hepatoprotective and antioxidant effects of breads enriched with polyphenolcontaining functional ingredients, namely viburnum, magnolia-vine, and grapes water and ethanol extracts (hereinafter referred to as "extracts").

STUDY OBJECTS AND METHODS

The objects of our experimental studies were breads produced by the traditional method from wheat flour with the addition of water and ethanol extracts of viburnum, magnolia-vine, and grapes [31].

To determine the potential efficacy of introducing Caliphene, Eklikit, and Diprim, the total content of common polyphenols was measured by the colorimetric method with the Folin-Chocalteu reagent using gallic acid as a standard [32].

We applied a standard experimental model of toxic hepatitis in mice through their intoxication with carbon tetrachloride, one of the strongest stimulants of lipid peroxidation. Carbon tetrachloride is an organ-specific toxin with a hepatotropic effect. Its toxicity is primarily associated with the prooxidant effect of free radicals that form during its metabolism, trichloromethyl and trichloromethyl peroxyl. These radicals initiate lipid peroxidation, leading to profound disruption of the functional properties of liver cell membranes, their lysis, and death¹.

The experiments were performed on white mice bred in the Pacific Institute of Bioorganic Chemistry, Russia.

For the experimental model of toxic liver damage by carbon tetrachloride, we used adult male mice with an average weight of 25.00 ± 1.56 g. The animals were kept in standard vivarium conditions in compliance with all the rules and recommendations of the European Convention for the Protection of Vertebrate Animals (Strasbourg, 1986).

The experimental model of carbon tetrachloride intoxication (toxic hepatitis) in animals followed the methodological guidelines for studying hepatoprotective activity of pharmacological substances [33, 34].

1.25 mL/kg of carbon tetrachloride (50% solution in olive oil) was injected into the dorsal nuchal fold of mice for 4 days. After that, the mice were given portions of breads containing Caliphene, Eklikit, and Diprim with a total complex of common polyphenols for 7 days. A grid with individual cells was placed in the cage to feed the animals and removed after feeding.

The study used three control groups and three experimental groups, 18 mice in each.

The control groups included:

- group 1: intact mice fed on the normal diet;

- group 2: mice with toxic hepatitis fed on the normal diet; and

– group 3: mice with toxic hepatitis 7 days after the toxicant withdrawal.

The experimental groups included:

- group 4: mice with toxic hepatitis fed on Diprimcontaining bread for 7 days after the toxicant withdrawal;

- group 5: mice with toxic hepatitis fed on Caliphenecontaining bread for 7 days after the toxicant withdrawal; and

- group 6: mice with toxic hepatitis fed on Eklikitcontaining bread for 7 days after the toxicant withdrawal.

The biomaterial was obtained as follows. Blood samples were taken from the cervical vein of mice in all the groups and collected in test tubes with heparin to measure antioxidant protection. Mice blood serum was used to study lipid metabolism and some biochemical parameters. Mice livers were extracted, washed in a physiological solution, and used to evaluate weight and biochemical parameters of mice.

The antioxidant system was assessed by measuring the activity of superoxide dismutase, glutathione reductase, and glutathione peroxidase, the antiradical activity of blood, as well as the level of reduced glutathione and malondialdehyde [35–37].

The "lipid peroxidation – antioxidant protection" system was studied with biochemical methods. Common lipids were extracted from liver tissue and prepared according to Folch *et al.* [38]. Micro-thin-layer chromatography on silica gel was used to measure the quantity of phospholipid and neutral lipid fractions. The Russian KSK silica gel was used as a sorbent.

The chromatographic distribution of neutral lipids was performed by one-dimensional thin-layer chromatography on silica gel in a solvent system of "hexane:sulfuric ether:acetic acid" in a ratio of 90:10:1 (v/v/v). Lipid stains were identified using purified preparations of Russian origin. Neutral lipid fractions were quantified according to Amenta [40]. After chromatography, standards and samples were detected with iodine vapours.

The fractions (triacylglycerols, cholesterol, free fatty acids, cholesterol esters, and fatty acid esters) were transferred from the plates to a tube with a special spatula. The tubes were filled with 2 mL of bichromate reagent and heated on a boiling water bath for 15 min. After the samples cooled, 4 mL of distilled water was added to them. They were then stirred and centrifuged at 3000 rpm for 10 min. Optical density was measured on a spectrophotometer at a wavelength of 440 nm. The results were expressed as a percentage of the sum of all fractions.

The results were processed with Instat 3.0 (GraphPad Software Inc. USA, 2005). The parametric Student's t-test or the non-parametric Mann-Whitney U-test were used to determine the statistical significance of the differences depending on the distribution parameters. The differences were considered statistically significant at P < 0.05.

RESULTS AND DISCUSSION

As shown in Table 1, the content of polyphenols in, and the antiradical activity of, the breads containing Diprim, Caliphene, and Eklikit extracts are statistically significant and higher than in the control sample (bread without additives). The antiradical activity of breads with plant extracts is determined by polyphenolic structures.

The data confirmed the efficacy of adding Diprim, Caliphene, and Eklikit as functional ingredients to breads with preventative properties in preclinical studies.

¹ MUK 2.3.2.721-98 Opredelenie bezopasnosti i ehffektivnosti biologicheski aktivnykh dobavok k pishche [Guidelines for establishing safety and efficacy of dietary supplements]. Moscow: Ministry of Health of the Russian Federation; 1999. 89 p.

Table 1 Antiradical activity and common polyphenols in the breads $(M \pm m)$

Name of product	Antiradical activity, µmol trolox/g product	Common polyphenols, mg/g product
Bread (control)	0.78 ± 0.013	120 ± 1.8
Bread + Diprim	0.97 ± 0.012^3	$388\pm2.4^{\scriptscriptstyle 3}$
Bread + Caliphene	$0.95 \pm 0.018^{\scriptscriptstyle 3}$	385 ± 2.6^3
Bread + Eklikit	$0.94 \pm 0.015^{\scriptscriptstyle 3}$	$384\pm 2.5^{\scriptscriptstyle 3}$

Table 2 Weight changes in carbon tetrachloride intoxicated mice and their correction with extract-containing breads $(M \pm m)$

Mice groups	Mice	Mice liver
	weight, g	weight, g
Group 1 control	25.40 ± 0.88	1.99 ± 0.06
Group 2 carbon tetrachloride	$19.43 \pm 0.57^{\scriptscriptstyle 3}$	$2.55\pm0.12^{\scriptscriptstyle 3}$
Group 3 deprivation (toxicant withdrawal)	$21.52 \pm 0.95^{\scriptscriptstyle 1}$	$2.39\pm0.08^{\scriptscriptstyle 3}$
Group 4 deprivation + bread with Caliphene	$25.87\pm0.72^{\circ}$	$2.09\pm0.06^{\rm b}$
Group 5 deprivation + bread with Diprim	$25.29\pm0.89^{\circ}$	$2.06\pm0.08^{\text{b}}$
Group 6 deprivation + bread with Eklikit	$25.18\pm0.98^{\circ}$	$2.07\pm0.09^{\text{b}}$

The differences are statistically significant at: 1 - P < 0.05; 2 - P < 0.01; 3 - P < 0.001 vs. control; a - P < 0.05; b - P < 0.01; c - P < 0.001 vs. group 3

Four days of carbon tetrachloride injections resulted in the mice developing toxic hepatitis with its characteristic symptoms. Their hair became dull and stuck together. They were apathetic and had a poor appetite. Their liver metabolic reactions were impaired, manifesting in:

- weakened antioxidant defences;
- activated lipid peroxidation;
- poor membrane permeability;
- imbalanced lipid metabolism;
- intensified lipolytic processes; and

- poor cholesterol metabolism (esterifying liver function).

Tables 2–4 present the indicators of metabolic processes in the control groups of mice (intact and mice with toxic hepatitis), compared with those in the experimental groups of mice (fed on breads with Diprim, Caliphene, and Eklikit extracts after carbon tetrachloride withdrawal).

After four days of carbon tetrachloride injections, the weight of the mice decreased to 19.43 ± 0.57 g, which was 24% (P < 0.001) lower than that of the intact control mice (25.40 \pm 0.88 g). This confirmed the development of toxic hepatitis (Table 2).

We found a statistically significant normalization of the parameters, starting from day 4, in the mice fed on breads with plant extracts after carbon tetrachloride intoxication (groups 4–6, compared with the control). Their weight returned to the control values; their hair became smooth and fluffy; they began to eat well and move actively. Their liver weight was higher than that in the control group, but slightly lower than in the animals with toxic hepatitis. This decrease in liver weight of the animals fed on breads with Diprim, Caliphene, and Eklikit indicates the stability of the extracts and their hepatoprotective role in the product.

The development of toxic hepatitis in the control mice intoxicated with carbon tetrachloride was manifested in both the impaired biochemical parameters of their blood, indicative of free-radical processes in the body, and lipid metabolism in their livers. The activity of alanine aminotransferase (ALT), an enzyme marker of liver damage, in the blood serum of mice in this group, was almost 20 times as high ($34.2 \pm 1.80 \mu mol/mL/h$) as in the control group ($1.72 \pm 0.09 \mu mol/mL/h$; P < 0.001). This was due to the release of the enzyme from the liver cells (hepatocytes) into the blood caused by impaired membrane permeability (Table 3).

Hepatocyte membranes became more permeable as a result of lipid peroxidation. This was indicated by an increase in malondialdehyde (MDA) to $6.83 \pm 0.09 \ \mu mol/mL$ of blood plasma in the mice with toxic hepatitis, compared to $3.70 \pm 0.10 \ \mu mol/mL$ (P < 0.001) in the control group of intact mice (Table 3).

Membrane permeability was impaired by trichloromethine and chlorine (CC1₃ and Cl^{*}), the

Table 3 Biochemical blood parameters of carbon tetrachloride intoxicated mice and their correction with extract-containing breads ($M \pm m$)

Mice groups	ALT, μmol/mL/h	ARA, trolox units/mg protein	SOD, activity units/mL blood	MDA, μmol/mL
Group 1 control	1.72 ± 0.09	13.15 ± 0.21	678.49 ± 6.47	3.70 ± 0.10
Group 2 carbon tetrachloride	34.24 ± 1.80^{3}	6.65 ± 0.15^{3}	276.56 ± 8.64^3	6.83 ± 0.09^{3}
Group 3 deprivation (toxicant withdrawal)	13.38 ± 1.20^3	8.85 ± 0.65^{3}	$435.55 \pm 2.75^{\scriptscriptstyle 3}$	5.62 ± 0.05^{3}
Group 4 deprivation + bread with Caliphene	$1.90\pm0.08^{\circ}$	$12.80 \pm 1.01^{\circ}$	$683.43\pm4.48^{\circ}$	$3.72\pm0.13^{\circ}$
Group 5 deprivation + bread with Diprim	$1.83\pm0.07^{\circ}$	$12.54 \pm 0.89^{\circ}$	$685.74\pm4.32^{\circ}$	$4.03\pm0.16^{\rm c}$
Group 6 deprivation + bread with Eklikit	$1.87\pm0.07^{\circ}$	$12.02 \pm 0.75^{\circ}$	$665.43 \pm 4.78^{\circ}$	$3.61\pm0.14^{\circ}$

The differences are statistically significant at: 1 - P < 0.05; 2 - P < 0.01; 3 - P < 0.001 vs. control; a - P < 0.05; b - P < 0.01; c - P < 0.001 vs. group 3. Abbreviations: ARA – antiradical activity, SOD – superoxide dismutase, MDA – malondialdehyde

Mice groups	Reduced glutathione (µmol/g liver)	Glutathione reductase (nmol/min/mL plasma)	Glutathione peroxidase (nmol/min/mL plasma)
Group 1 control (intact)	4.70 ± 0.15	88.21 ± 4.26	139.0 ± 4.83
Group 2 carbon tetrachloride	3.13 ± 0.14^{3}	39.68 ± 3.25^3	110.1 ± 3.43
Group 3 deprivation (toxicant withdrawal)	3.09 ± 0.11^{3}	50.32 ± 1.54^2	105.3 ± 6.04^2
Group 4 deprivation + bread with Caliphene	$4.87\pm0.33^{\circ}$	$92.48\pm2.46^{\circ}$	$138.3\pm2.19^{\text{b}}$
Group 5 deprivation + bread with Diprim	$4.90\pm0.33^{\circ}$	$91.75 \pm 3.62^{\circ}$	$137.3 \pm 2.32^{\circ}$
Group 6 deprivation + bread with Eklikit	$4.87\pm0.20^{\rm c}$	90.61 ± 2.59°	$137.4 \pm 1.53^{\circ}$

Table 4 Indicators of antioxidant liver and blood system of carbon tetrachloride intoxicated mice and their correction with extract-containing breads $(M \pm m)$

The differences are statistically significant at: 1 - P < 0.05; 2 - P < 0.01; 3 - P < 0.001 vs. control; a - P < 0.05; b - P < 0.01; c - P < 0.001 vs. group 3

radicals forming during carbon tetrachloride metabolism in the cytochrome P-450 system after intoxication [40].

The control group of mice with toxic hepatitis that did not receive bread displayed an imbalance and depletion of enzymatic antioxidant processes and antiradical protection. This was indicated by a decrease in antiradical activity to 6.65 ± 0.15 trolox units/mg protein, compared to the control group of intact mice (13.15 ± 0.21 trolox units/mg protein; P < 0.001). Moreover, the activity of superoxide dismutase, a key enzyme of the antioxidant defence system, was 2.5 times as low as in the control group (276.56 ± 8.64 units/mL of blood compared to 678.49 ± 6.47 units/mL of blood, respectively; P < 0.001), as shown in Table 3.

A study of the liver and blood antioxidant system revealed the following simultaneous changes in antioxidant indicators: a 21% decrease in glutathione peroxidase activity (GPO; P < 0.001), a 55% decrease in glutathione reductase (GR; P < 0.001), and a 33% decrease in reduced glutathione (P < 0.001), as shown in Table 4.

Such changes in the antioxidant protection system of carbon tetrachloride intoxicated mice can be regarded as evidence of its depletion.

A study of lipid metabolism in the liver of carbon tetrachloride intoxicated mice revealed a pronounced

metabolic disorder. This confirmed the efficacy of the experimental model and proved the development of toxic hepatitis in the experimental animals after the toxicant administration (Table 5).

Table 5 shows an increase in triacylglycerols (TAGs) by 17% (P < 0.001), cholesterol (CS) by 9% (P < 0.001), and free fatty acids (FFAs) by 14% (P < 0.001). These disorders were caused by a breakdown of triacylglycerols in adipose tissue (chemical stress), a release of fatty acids and glycerol into the blood, and their resynthesis as triacylglycerols in the liver. Due to impaired synthesis of phospholipids from triacylglycerols, fatty acids and TAGs accumulated in hepatocytes, leading to fatty infiltration of the liver. The increase in cholesterol was due to the inhibition of mitochondrial oxidation of acetyl CoA in the Krebs cycle, whose condensation lead to cholesterol production. A lower level of fatty acid esters (FAEs) (by 9%; P < 0.05) and cholesterol esters (CSEs) (by 22%; P < 0.001) indicated an impairment of cholesterol metabolism and esterifying function of the liver. This was due to the inhibition of the ACAT enzyme (acyl-CoA: cholesterol-acyltransferase) during carbon tetrachloride intoxication. This ratio of lipid fractions confirmed the fatty degeneration of the liver caused by the impairment of its esterifying function.

Table 5 Neutral lipid content in carbon tetrachloride intoxicated mice liver and their correction with extract-containing breads $(M \pm m)$

Mice groups	TAGs	FFAs	FAEs	CS	CSEs	Residual
						fraction
Group 1 control	21.91 ± 0.60	16.24 ± 0.24	15.49 ± 0.50	14.95 ± 0.19	16.97 ± 0.11	13.65 ± 0.15
Group 2 carbon tetrachloride	25.54 ± 0.42^{3}	$18.47 \pm 0.32^{\scriptscriptstyle 3}$	14.15 ± 0.27^{1}	16.15 ± 0.17^3	$13.31 \pm 0.17^{\scriptscriptstyle 3}$	12.37 ± 0.44
Group 3 deprivation	$26.30\pm0.35^{\scriptscriptstyle 3}$	$17.27 \pm 0.23^{\scriptscriptstyle 1}$	$14.19\pm0.24^{\scriptscriptstyle 1}$	$15.69 \pm 0.15^{\scriptscriptstyle 1}$	$13.53 \pm 0.23^{\scriptscriptstyle 3}$	13.51 ± 0.35
(toxicant withdrawal)						
Group 4 deprivation + bread with	$21.89\pm0.31^{\circ}$	$15.85\pm0.28^{\circ}$	$16.72\pm0.23^{\circ}$	$14.44\pm0.26^{\circ}$	$17.05\pm0.19^{\circ}$	14.05 ± 0.25
Caliphene						
Group 5 deprivation + bread with	$21.70\pm0.33^{\circ}$	$15.63\pm0.26^{\circ}$	$16.75\pm0.27^{\circ}$	$14.44\pm0.23^{\circ}$	$17.25\pm0.20^{\circ}$	14.23 ± 0.21
Diprim						
Group 6 deprivation + bread with	$21.80\pm0.31^{\circ}$	$16.30\pm0.34^{\rm a}$	$16.74\pm0.28^{\circ}$	$14.80\pm0.33^{\rm a}$	$15.34\pm0.21^{\circ}$	15.02 ± 0.42
Eklikit						

The differences are statistically significant at: 1 - P < 0.05; 2 - P < 0.01; 3 - P < 0.001 vs. control; a - P < 0.05; b - P < 0.01; c - P < 0.001 vs. group 3. Abbreviations: TAGs – triacylglycerols, FFAs – free fatty acids, FAEs – fatty acids esters, CS – cholesterol, CSEs – cholesterol esters

The impaired metabolic reactions in the mice liver proved the efficacy of the experimental toxic hepatitis model through carbon tetrachloride intoxication.

Seven days after the toxicant withdrawal (deprivation period) did not normalize biochemical parameters in the liver of the mice with a bread-free diet. This indicated that their bodies' own defences were too weak to withstand pathologic development caused by intoxication with hepatotropic poison. The animals were lethargic; they had a poor appetite and dull hair.

The malondialdehyde content in the bread-free group remained 52% (P < 0.001) higher than in the control group, indicating a still high level of lipid peroxidation.

Further decrease in reduced glutathione to $3.09 \pm 0.11 \ \mu mol/g$ in group 3 confirmed the on-going destabilisation of liver cell membranes, causing their imbalance and depleting their antioxidant protection. The activity of superoxide dismutase was 57% (P < 0.001) higher than in group 2 (carbon tetrachloride), due to the toxicant withdrawal, but it was 36% (P < 0.001) lower than in the control group. Likewise, the antiradical activity in group 3 was 35% (P < 0.001) higher than in group 2 (carbon tetrachloride), but 32% (P < 0.001) lower than in the control. The results indicated the continuing free-radical processes.

Those indicators were associated with a high activity of alanine aminotransferase, a marker of liver damage, which was eight times as high as in the control group $(13.38 \pm 1.2 \,\mu\text{mol/mL/h}; P < 0.001)$.

Among neutral lipids, the content of triacylglycerol, free fatty acids, and cholesterol remained significantly high, compared to the control groups. The blood serum of the mice with a bread-free diet showed an impaired cholesterol metabolism – a significantly low content of fatty acid and cholesterol esters – indicating a suppressed esterifying function of the liver. This was consistent with high ALT activity – 8 times as high as in the control group $(13.38 \pm 1.2 \,\mu\text{mol/mL/h}; P < 0.001)$.

Thus, the lipid spectrum of the liver of mice that did not receive bread with extracts indicated a continuing deterioration of metabolic reactions even in the absence of the toxic agent (Tables 2–5).

Feeding the carbon tetrachloride intoxicated mice with bread containing plant extracts (groups 4–6) resulted in the correction and normalization of the parameters studied. The activity of ALT, an enzyme marker of toxic hepatitis, was not significantly different from the control values. This suggested that bread treated with plant extracts had membrane-stabilizing properties.

We found a complete normalization of the membrane lipid peroxidation parameters (in particular,

malonic dialdehyde) in groups 4–6 vs. the control. The antiradical and antioxidant protection systems also showed a full recovery.

The analysis of lipid metabolism in the liver of mice in groups 4–6 showed a marked decrease in triacylglycerols, free fatty acids, fatty acid esters, and cholesterol. At the same time, there was an increase in fatty acid esters and cholesterol, compared to group 3 (deprivation). This indicated the efficacy of Caliphene, Diprim, and Eklikit extracts in restoring the esterifying function of the liver and reversing fatty infiltration.

We believe that the biochemical mechanism of restoring the liver function with functional foods after carbon tetrachloride intoxication is based on the localization of polyphenols in the lipid bilayer of the hepatocyte plasma membrane, stabilizing its permeability [41, 42].

CONCLUSION

Thus, the experimental model of toxic hepatitis showed that breads enriched with the plant extracts of Caliphene, Diprim, and Eklikit had pronounced hepatoprotective and antioxidant properties.

Those properties were due to the effect of polyphenols contained in the extracts on the metabolism and function of the liver. In particular, polyphenols inhibited free radical reactions, increased the liver's antiradical and antioxidant activity, and reduced the amount of toxic lipid peroxidation products. They also stabilized hepatocyte membranes, normalized the liver's esterifying function, and restored the weight of the animals and their liver, as well as ALT activity [43-45]. Thus, breads enriched with water and ethanol extracts obtained from viburnum (Viburnum sargentii Koehne L.), magnolia-vine (Schisandra chinensis L.), and grapes (Vitis amurénsis L.) waste can be regarded as products with preventative properties and recommended as part of a hepatoprotective diet.

CONTRIBUTION

Authors are equally related to the writing of the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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perechnya otdel'nykh vidov sotsial'no znachimykh prodovol'stvennykh tovarov pervoy neobkhodimosti, v otnoshenii kotorykh mogut ustanavlivat'sya predel'no dopustimye roznichnye tseny, i perechnya otdel'nykh vidov sotsial'no znachimykh prodovol'stvennykh tovarov, za priobretenie opredelennogo kolichestva kotorykh khozyaystvuyushchemu sub"ektu, osushchestvlyayushchemu torgovuyu deyatel'nost', ne dopuskaetsya vyplata voznagrazhdeniya" [The RF Government Decree No. 530 of July 15, 2010 "Approving the Rules for establishing maximum allowable retail prices for certain types of basic foodstuffs, the List of certain types of basic foodstuffs for which maximum allowable retail prices can be established, and the List of certain types of basic foodstuffs for the acquisition of a certain amount of which the retailer is not entitled to remuneration"].

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Effects of dust phenomenon on heavy metals in raw milk in western Iran

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Abstract:

Introduction. After the Iraq war, the dust phenomenon has increased in western Iran. Our study aimed to evaluate the effect of the dust phenomenon on the content of heavy metals in raw milk in Ilam province.

Study objects and methods. The dust samples were collected during one year. The concentrations of dust particles were determined with the Enviro Check Laser System, using the Dust Monitor Check. The concentration of heavy metals in dust was determined by using the high volume air samplers and glass fiber filters.

Results and discussion. Heavy metals (lead, arsenic, zinc, copper, and iron) were measured at four sampling sites in raw milk by the atomic absorption method. The mean and standard deviations of dust particulate matter (PM_{10} and $PM_{2.5}$) were 105.6 ± 90.5 and 25.9 ± 15.4 µg/m³, respectively. The amounts of arsenic, zinc, lead, and copper were higher in the spring and summer. Lead levels in western and southern regions were higher than those in the east, center, and north.

Conclusion. We found similar trends for arsenic, zinc, copper, and iron in raw milk. Our results showed the potential effect of the dust phenomenon on the presence of heavy metals in raw milk.

Keywords: Heavy metal, dust phenomena, raw milk, atomic absorption

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INTRODUCTION

A dust storm is one of many air pollutants known to humans. About 800 trillion grams of dust particles are spread in Asia. Particulate matter (PM) is usually suspended in arid, semiarid, and desert areas [1]. Today, dust storms are one of environmental problems that threaten the world [2]. The phenomenon of dust in the atmosphere causes the spread of PM around the globe [3]. Dust phenomena can be triggered by such factors as environmental change, global drought, and land cover [4]. The dry currents of the Saudi air and lack of attention to the environment and desertification in Iraq have dried up many of the marshes there and created dusty areas. In the past, Iran, Iraq, and Saudi Arabia jointly funded the mulching of these lands during a particular season. The Iraq war obliterated this work, resulting in a spike in western Iran, and eventually almost throughout the whole country. According to

Harrington *et al.*, the United States soldiers involved in the Iraq war had respiratory problems due to the dust phenomenon in that country [5].

Biological particles, salt sprays and, in particular, dust phenomena have been reported to contain numerous heavy metals [6]. Furthermore, these metals lead to climate change in temperature and other seasonal changes, such as the wind speed and patterns [7]. Although trace heavy metals are fundamental to living organisms for a normal and healthy life, excessive levels of heavy metal contamination in the environment could cause harm [8, 9]. To reduce environmental pollution and mitigate the resulting degradation of soil and water resources, it is important to precisely determine heavy metal concentrations [10, 11].

Some heavy metals, for example, chrome (Cr), lead (Pb), cadmium (Cd), and mercury (Hg) in the form of suspended particles in the air produce significant toxicological effects on people and other

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organisms by contaminating food and drinking water in the environment [12]. Particulate matter, which is contaminated with heavy metals, can pollute groundwater. It has been indicated that contamination is transmitted from the soil to the plant. As a result, contaminated plants ingested by humans or animals can cause a toxic effect. Consequently, the concentration of heavy metals in animal products, such as raw milk, also causes toxicity. The extent of toxicity depends on different factors, such as plant processes and the amount of raw materials used [13]. Moreover, heavy metals such as lead, cadmium, chrome, nickel, and cobalt can contaminate cows and their surroundings. Heavy metals are absorbed by plant roots from the soil. As a result, this pollution causes serious problems, changing the amount and structure of milk [14].

The International Agency for Research on Cancer (IARC, 2016) classifies arsenic (As) and chrome (Cr) as carcinogenic metals and lead as a possible carcinogen. Such metals can cause different types of cancer through dermal contact, inhalation, and ingestion [15].

The western and southwestern climates of Iran are influenced by environmental conditions and markers such as geological, climatic, hydrological, and geomorphological characteristics [3]. Among the factors that cause dust storms are the development of deserts in Iraq, a decrease in volume and flow of rivers, and the Turkish dam on the rivers [16]. With a population of 172 000 people, the city of Ilam is located on the western border of Iran (33°38'N, 46°25'E). Due to a small population and a mountainous area, the city has low traffic and also no air pollution industry.

With the onset of spring, the phenomenon of dust comes to Iran from Iraq. This situation was aggravated by the Iraq war. In this study, we investigated the concentration of suspended particles in the environment and evaluated the effect of heavy metals on cow raw milk in the west of the country, Ilam province.

STUDY OBJECTS AND METHODS

PM concentration in dust. The dust samples were collected from four districts of Ilam province: northern and central, southern, western, and eastern (28 samples in each). The sampling was performed on the roofs of buildings seven meters above the ground and two meters from the roof surface. During the sampling, we complied with all the standards of the US Environmental Protection Agency. In particular, we kept the required distance from natural and artificial obstacles, pathways, and sources of contamination. Suspended particles PM₁₀ and PM_{2.5} were measured with the Enviro Check Laser System using the Dust Monitor Check (Grimm). This apparatus can directly and simultaneously measure the particle count, PM₁₀ and PM_{2.5}. The system automatically saves the values in its memory and calculates the average on an hourly and a daily basis [17].

On normal days, the sampling was carried out every six days and on days with dust (a concentration

above 150 μ g/cm³), according to the Meteorological Organization and satellite sites, on a daily basis. The peak concentrations of PM₁₀ and PM_{2.5} were recorded and measured on average every hour. The data obtained during a year were analyzed with the SPSS software [18].

Heavy metals in dust. Teflon and fiberglass filters were used to investigate heavy metals in the dust phenomenon. A 100-cm section of a filter was cut and transferred to a 100 mL beaker. Then, we added 50 mL of Aqua Regia (HNO₃+3 HCl) and heated it to 140°C until the filter section was dry. Then, we removed it and washed the beaker with 10% nitric acid. This work was repeated three times. In the end, the prepared sample was kept at room temperature until it was cooled. Then, we transferred it into a 100-mL volumetric flask and diluted to volume with 10% HNO3. The concentrations of heavy metals (lead, arsenic, zinc, copper, and iron) were determined by a Perkin-Elmer Analyst 800 atomic absorption spectrometer, including an AS-800 Autosampler equipped with Zeeman-effect background correction. Each result was an average of three readings. Blank filters were prepared by digesting clean glass fiber filters with the same digestion method used for the dust samples. Also, the dust samples were prepared in different seasons [19].

Heavy metals in raw milk. A total of 112 samples of cow raw milk were collected from four districts of Ilam province: northern and central, southern, western, and eastern (28 in each), at the same places as dust samples. All the samples were collected in nitric acidwashed polyethylene containers. They were immediately transported to the laboratory in a cooler with ice packs and stored at -20°C until analysis. The raw milk samples were analyzed based on AOAC official methods. The amounts of heavy metals (lead, arsenic, zinc, copper, and iron) were measured by a Perkin-Elmer Analyst 800 atomic absorption spectrometer, including an AS-800 Autosampler equipped with Zeeman-effect background correction. Each sample was studied three times [20]. The limit of detection and the limit of quantitation of the atomic absorption device were 0.08 ppm and 0.15 ppm, respectively.

Statistical analysis. The SPSS 21 software was used to extract the data (P < 0.05). The results of three repetitions were analyzed by ANOVA, using an SPSS statistics package.

RESULTS AND DISCUSSION

Ilam is bordered by Iraq and close to the countries of Saudi Arabia and Kuwait, which are the main sources of dust events in the Middle East (Fig. 1).

According to Table 1, the sampling took 87 days. The average PM_{10} and $PM_{2.5}$ were about 105.6 ± 82.9 and 25.9 ± 15.4 µg/m³, respectively. The maximum particle size of PM_{10} and $PM_{2.5}$ was about 806.3 and 213.2 µg/m³, respectively.

Kirkuk ليمانية كركوك Sanandaj Hawija Eslamshahr Varamin es سنندج 190 Saveh ورامير لاوه Hamedan Kamyaran همدان كاميارات Qom Tikrit Kalar كلار قم نكرر Kermanshah Nat Malave (hanagin كرمانشاه Samarra ملاير Arak سامراء Kashan اراک slamabad-e-Gharb Mahalat بهاوند Borujerd کاشان سلام آباد غرب Baqubah بروجرد حلات بعقوبة llam ايلام Kuhdasht Khorramabad Dorud Ramad كوهدت Aligudarz Baghdad خرم آباد دورود اليگودرز الرمادي 510 م Al-Fallujah الفلوحة 0 Iraq Isfahan Karbala Kut اصفهان كريلاء Dezful الكور Hillah Shahrekord دزفول شهركرد الحلة Shooshtar Najaf Shahreza Shush لنخف Amarah ليهرضا Izeh العمارة يده Ahvaz اهواز Nasirivah Samawah الناصرية لسماوة Yasuj Basrah Behbahan باسوح Bandar البصرة يهبهان •Mahshahr Abadan

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Figure 1 Geographical location of Ilam province and Iraq (Google map)

Months	Number of	Aver	rage	Max	imum	Minii	num	Mec	lian	Standard	deviation
	sampling days	PM ₁₀	PM _{2.5}	PM_{10}	PM _{2.5}	PM ₁₀	PM _{2.5}	PM ₁₀	PM _{2.5}	PM ₁₀	PM _{2.5}
March-April	12	178.3	42.7	725.1	171.1	31.7	8.3	179.1	31.2	185.2	41.7
April-May	9	142.4	31.3	562.3	210.4	62.7	12.5	142.3	37.1	127.5	32.4
May-June	16	210.3	56.6	806.3	213.2	91.8	17.7	181.3	35.8	197.3	30.1
June-July	7	112.7	27.2	351.7	98.2	51.2	10.3	125.2	29.6	110.2	12.5
July-August	9	139.9	37.5	393.4	99.1	57.4	12.1	139.1	28.8	123.1	13.1
August-September	6	79.8	25.1	185.2	55.3	26.6	8.7	76.6	12.5	31.5	9.2
September-October	4	56.7	12.7	100.1	21.1	18.7	6.2	43.9	8.7	26.7	6.7
October-November	5	58.7	13.3	110.7	20.7	19.2	6.0	45.2	8.4	27.1	7.3
November-December	4	62.7	14.1	211.2	18.7	30.1	11.8	52.2	9.7	30.7	10.9
December-January	4	48.4	12.2	89.6	12.9	15.2	4.4	38.3	3.5	20.3	5.2
January-February	4	39.6	11.5	75.3	12.1	13.3	4.1	35.2	4.1	18.7	4.9
February-March	7	137.5	26.9	300.8	121.6	41.3	9.6	127.5	11.2	97.3	10.4
Study period	87	105.6	25.9	325.9	87.8	38.3	9.3	98.8	18.4	82.9	15.4

Table 1 Concentrations of PM_{10} and PM_{25} in different months of sampling in Ilam province, $\mu g/m^3$

Five heavy metals (lead, arsenic, zinc, copper, and iron) were measured in all the samples. Figure 2 shows the range and mean concentrations (ng/m^3) of the selected heavy metals analyzed at the sampling stations. In the spring and summer, the amounts of heavy metals were higher than in the other seasons, especially iron. Lead and copper levels were lower in all the seasons compared to other metals.

Average values of iead content in the raw milk samples are shown in Table 2. The highest average lead

level was determined in the western region (57.1 μ g/kg). The statistical analysis revealed a significant difference in lead concentrations between the western and southern regions compared to the east and north of Ilam province (*P*-value < 0.05).

Average concentrations of arsenic in the milk samples are shown in Table 3. Although arsenic was higher in the south and west compared to the northern and central region or the east of the province, we found no significant difference. The highest average





Figure 2 Seasonal average concentrations of heavy metals (lead, arsenic, zinc, copper, iron) in dust phenomena. Dust phenomenon in spring (D Ph sp), non-dust phenomenon in spring (N Dph sp), dust phenomenon in summer (D Ph su), non-dust phenomenon in fall (D Ph f), non-dust phenomenon in fall (N Dph f), dust phenomenon in winter (D Ph w), non-dust phenomenon in winter (N Dph w), * *P*-value < 0.05, ** *P*-value < 0.001 vs. the other group

Northern a	and Central region	Eas	stern region	Southern region		We	stern region
Sample	Content, µg/kg	Sample	Content, µg/kg	Sample	Content, µg/kg	Sample	Content, µg/kg
1	29	1	31	1	49	1	48
2	29	2	33	2	48	2	55
3	23	3	35	3	53	3	49
4	28	4	29	4	59	4	63
5	27	5	37	5	57	5	61
6	31	6	39	6	61	6	65
7	25	7	38	7	55	7	59
Average	27.4	Average	34.6	Average	54.6	Average	57.1
Max	31	Max	39	Max	61	Max	65
Min	23	Min	29	Min	48	Min	48

Table 2 Lead contents in raw milk samples

Table 3 Arsenic contents in raw milk sampl	es
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Northern	Northern and Central region Eastern region		Sou	thern region	Western region		
Sample	Content, µg/kg	Sample	Content, µg/kg	Sample	Content, µg/kg	Sample	Content, µg/kg
1	11	1	10	1	13	1	12
2	11	2	10	2	12	2	13
3	12	3	12	3	12	3	12
4	10	4	12	4	14	4	14
5	10	5	13	5	10	5	12
6	12	6	10	6	13	6	11
7	11	7	9	7	12	7	13
Average	11	Average	10.9	Average	12.3	Average	12.4
Max	12	Max	13	Max	14	Max	14
Min	10	Min	9	Min	10	Min	11

arsenic level was observed in the west, amounting to 12.4 mg/kg.

Table 4 shows average zinc values in the raw milk samples. The highest average amount of zinc was determined in the western region (4582.8 μ g/kg). According to the statistical analysis, there was a

significant difference in zinc concentrations between the western and southern regions compared to the east and north of Ilam province (*P*-value < 0.05).

The concentrations of copper in different regions are shown in Table 5. As we can see, the average level of copper in the western and southern regions was

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Northern and Central region		Ea	Eastern region		uthern region	We	Western region	
Sample	Content, µg/kg	Sample	Content, µg/kg	Sample	Content, µg/kg	Sample	Content, µg/kg	
1	3120	1	3210	1	4650	1	4740	
2	3190	2	3460	2	4770	2	4400	
3	2870	3	3110	3	4390	3	4810	
4	3150	4	3060	4	3880	4	5030	
5	3210	5	2930	5	4810	5	4560	
6	3010	6	3420	6	4230	6	4330	
7	2460	7	3250	7	4560	7	4210	
Average	3001.4	Average	3205.7	Average	4470	Average	4582.8	
Max	3210	Max	3460	Max	4810	Max	5030	
Min	2460	Min	2930	Min	3880	Min	4210	

Table 4 Zinc contents in raw milk samples

Table 5 Copper contents in raw milk samples

Northern a	Northern and Central region		Eastern region		hern region	Western region	
Sampla	Contont ug/kg	Sampla	Contont ug/kg	Sampla	Content ug/kg	Sampla	Contont ug/kg
Sample	Content, µg/kg	Sample	Content, µg/kg	Sample	Content, µg/kg	Sample	Content, µg/kg
1	210	1	200	1	230	1	170
2	300	2	320	2	690	2	480
3	190	3	170	3	410	3	550
4	180	4	350	4	520	4	400
5	250	5	420	5	450	5	670
6	310	6	300	6	360	6	510
7	200	7	380	7	590	7	430
Average	234.3	Average	305.7	Average	464.3	Average	458
Max	310	Max	420	Max	690	Max	670
Min	180	Min	170	Min	230	Min	170

Table 6 Iron contents in raw milk samples

Northern and Central region		Eastern region		Southern region		Western region	
Sample	Content, µg/kg	Sample	Content, µg/kg	Sample	Content, µg/kg	Sample	Content, µg/kg
1	2554	1	2654	1	3865	1	3845
2	2129	2	2863	2	3978	2	3693
3	2763	3	2341	3	4762	3	4236
4	3327	4	3496	4	3687	4	4122
5	2645	5	2029	5	4831	5	3541
6	2431	6	2585	6	3573	6	3655
7	3504	7	3051	7	2985	7	4032
Average	2764	Average	2717	Average	3954	Average	3874
Max	3504	Max	3496	Max	4831	Max	4236
Min	2129	Min	2029	Min	2985	Min	3541

higher than in the eastern and northern regions. The lowest average copper concentration (234.3 μ g/kg) was observed in the northern and central region.

According to Table 6, there was a significant difference between the amounts of iron in the west and south compared to the east and north of the province. The highest average concentration of iron (3954 μ g/kg) was found in the southern region.

Typically, dust particles with a diameter of $616-660 \ \mu m$ remain in their places of origin. Particles sized $31-62 \ \mu m$ are dispersed over approximately $320 \ km$ from their origin, while those sized $16-30 \ \mu m$, up to

1600 kilometers. Particles below 16 μ m travel longer, and particles ranging from 2–50 μ m have been reported to mostly originate in deserts like Iraq, Saudi Arabia, and Africa [21]. The main source of dust phenomenon in the southwest of Iran is the deserts of Iraq [22].

In our study, the mean and standard deviations of PM_{10} and $PM_{2.5}$ were 105.69 ± 0.5 and $25.9 \pm 15.4 \ \mu g/m^3$ at the time of sampling, and the maximum PM_{10} and $PM_{2.5}$ concentrations were 806.3 and 213.2 $\ \mu g/m^3$ in June, respectively. According to Draxler *et al.*, the main sources of dust phenomenon in the southwest and west of Iran are Kuwait, Iraq, and Saudi Arabia.

The authors showed that PM_{10} concentrations exceeded 3000 µg/m³ [23]. In the study by Shahsavani et al., which agrees with our results, the mean and standard deviations of PM₁₀ and PM_{2.5} in Ahwaz, southwest of Iran, were 407.07 ± 319.1 and $83.2 \pm 69.5 \,\mu\text{g/m}^3$, while the maximum PM₁₀ and PM₂₅ concentrations were 5337.6 and 910.9 μ g/m³ in June, respectively [24]. In another study, in the southwest of Iran, the mean and standard PM₁₀ and PM₂₅ deviations in the entire study period were 775.3 \pm 598.9 and 129.5 \pm 114.9 μ g/m³, whereas their maximum concentrations reached 4730.1 and 774.4 µg/m³ in February, respectively [17]. The difference between the maximum concentrations found by the authors and our results may be due to differences in geographical and atmospheric conditions and the distance from the dust source in the period of particle measurement. Also, in another study of 2007, the mean total concentration of suspended particles was 282 μ g/m³ and the PM₁₀ and PM₂₅ concentrations were 165 and 67 µg/m³, respectively [25]. Finally, a 2010 study conducted in China reported the mean concentrations of PM_{10} and $PM_{2.5}$ to reach $322 \pm 237.4 \ \mu g/m^3$ and $141.5 \pm 108.8 \ \mu g/m^3$, respectively [26].

According to Jacobs et al., of 16.4 million homes in the United States with more than one child below six, 25% still had significant amounts of lead-contaminated deteriorated paint, dust, or adjacent bare soil [27]. Lu et al. detected the presence of heavy metals in soil by spectroscopic methods [28]. In another study, the voltammetric method found cadmium (0.06 µg/L) and lead (0.65 μ g/L) in soil [29]. However, the atomic absorption technique is also very important for analyzing heavy metals in air samples. Factors such as high sensitivity, high performance, low cost, and accuracy make this method a good choice. The amount of heavy metals in the atmosphere depends on the origin and the distance from the source of pollution. Seasonal changes also affect concentrations of heavy metals in the atmosphere [30]. Our results showed that the amounts of arsenic, zinc, lead, and copper were higher during the spring and summer. Zinc and iron had a higher level compared to the others. Due to high temperatures during the spring and summer, dust is denser than in the fall or winter, and it is easier for animals and humans to inhale them. In a study by Al-Dabbas et al., the X-ray powder diffraction method was used to detect the presence of heavy metals (Fe, Co, Ni, Cu, Zn, and Pb) and dust particles in the streets of southern Iraq [31]. Also, in Ahwaz, southwest Iran, heavy metals (Cd, Cr, Co, Ni, Pb, Zn, and Al) were identified in particles with PM₁₀[32].

It has been well demonstrated that heavy metals such as cadmium, chrome, nickel, and cobalt, which contaminate the environment around animals, e.g. cows, penetrate into cow milk and cause tissue problems. Heavy metals can penetrate into plants through their roots. Through contaminated drinking water and water used in agriculture and food production, they enter animal and human bodies [33]. As reported by Razafsha *et al.*, plants contaminated with particles containing heavy metals increase the risk of raw milk contamination [34].

Dairy products are a vital part of a healthy diet, and milk is widely used in feeding infants and children. Therefore, studying the presence of metals in milk is especially important to ensure the safety of milk production that is greatly reduced in a contaminated and toxic environment [35, 36].

According to the FAO/WHO guidelines and the Codex standard, the levels of lead, arsenic, zinc, and iron in milk and dairy products are 2.0, 0.050, 0.9, and $0.6 \mu g/kg$, respectively [37].

We examined the amounts of heavy metals in cow milk in Ilam, west of Iran, and found that lead levels in the western and southern regions were higher than in the east, center, and north. This situation was also consistent with arsenic, zinc, copper, and iron. The average concentrations of arsenic and copper were generally lower than those of zinc, lead, and iron. Farm animals, which are used for milk and meat, tend to get polluted with heavy metals through the environment. According to recent observations, the concentrations of remaining heavy metals in milk are significantly higher than those approved by international authorities [38].

Consistent with our study, Konuspayeva et al. reported that seasonal changes influenced the level of lead in camel milk: it was lower in the spring compared to other seasons [39]. They also found that the presence of arsenic in camel milk and its contamination level depended on the distance from the source of pollution, wind and farm topography (soil type, vegetation type), etc. [40]. Another study reported that the amount of iron and copper in the milk collected in industrial areas was higher than that of lead in traffic-intensive and industrial regions [20]. This shows the effect of the environment on the content of heavy metals in milk. Awasthi et al. and Malhat et al. reported that the levels of cadmium, iron, and zinc in cow milk were higher in industrial areas compared to others [41, 42]. A study in Egypt found that the amount of cadmium in cow milk produced in contaminated air was significantly higher [43].

It has been shown that all the milk samples collected from different governorates contained lead and iron in higher concentrations than those recommended for milk by the IDF standard (1979). Lead is an environmental pollutant that is toxic to humans and animals [44].

Similar to our study, Kabir *et al.* presented the average concentrations of metals in 50 samples of cow milk from contaminated environments in the following order: Fe > Cr > Mn > Zn > Ni > PB > Hg > Sc > Cd > As [45].

CONCLUSION

We analyzed the particle matter and the amounts

of heavy metals in the dust phenomenon in western Iran (Ilam province) over one year and also studied the presence of heavy metals in the raw milk samples collected in its four regions. As a result, we can conclude that the dust phenomenon that comes from Iraq to Iran is probably one of the sources of milk contamination in western Iran.

CONTRIBUTION

Naser Abbasi designed the work and took the lead in writing the manuscript together with Elahe Karimi; Ali Aidy performed the experiments; Monireh Yari and Hori Ghaneialvar derived the models and analyzed the data; Hamid Reza Kazemi and Reza Asadzadeh contributed to the interpretation of the results.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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ETHICAL STATEMENT

The authors confirm that they have adhered to the journal's ethical policies specified on its author guidelines page, and received the approval of the appropriate ethical review committee. The authors also confirm that they have followed the EU standards for the protection of animals used for scientific purposes and feed legislation.

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Effects of lingonberry extract on the antioxidant capacity of meat paste

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Abstract:

Introduction. Modern meat scientists are currently interested in combining meat raw materials with plant ingredients to enrich products with biologically active substances of natural origin, including antioxidants. In this regard, we studied the antioxidant capacity of a dry lingonberry marc extract introduced into meat paste and analyzed its effects on the product's color and stability during storage. *Study objects and methods.* Our objects of study were a dry lingonberry marc extract originating in the Republic of Buryatia, forcemeat, and a ready-made paste in a casing. The extract was obtained by water-alcohol extraction using microwave irradiation. We investigated the physicochemical characteristics of the dry extract, including its contents of phenolic compounds, benzoic acid, and antioxidants.

Results and discussion. During the experiment, we analyzed the extract's effect on the paste's total antioxidant capacity, coloring, and shelf life. The results showed that increasing the extract's amount from 0.1% to 0.4% changed the color of the paste from gray-brown to purple-brown, respectively, due to anthocyanins. In further tests, we used a 0.2% concentration of lingonberry extract – the optimal amount that retained the usual brown color of the paste while increasing the content of antioxidant substances. Then, we analyzed the degree of fat oxidation in the paste samples made with and without sodium lactate during storage. According to the results, the lingonberry marc extract used without the acidity regulator and with it inhibited lipid oxidation by 12.7% and 20%, respectively, by neutralizing free radicals. Finally, we tested the presence of pathogenic microorganisms in the end products. We detected no *E. coli* bacteria in the samples and found an inhibited growth of mesophilic anaerobic and facultative anaerobic microorganisms due to the extract's bactericidal effect established in our earlier studies.

Conclusion. Thus, our results indicated that the dry lingonberry marc extract introduced into meat paste increased the product's total antioxidant capacity and improved its stability during storage.

Keywords: Meat products, berry extract, lingonberry, paste, phenolic compounds, antioxidants, oxidation, peroxide value

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INTRODUCTION

Introducing slaughter by-products into food formulations and technology is a promising direction in the meat industry that ensures a rational use of protein raw materials. Modern meat scientists are developing new products using heat-treated offal of farm animals [1-4].

Such products include liver sausage, paste, head cheese, and jellied meat. Meat pastes are especially popular. They have a spreading consistency and can be packaged in a casing or container. According to the standards, pastes are classified into "meat pastes" (category A) with at least 20% of muscle tissue and "meat-containing pastes" (category B) with 0 to 20% of muscle tissue. Pastes are affordable meat products due to a lower cost of liver, skirt, lungs, kidneys, and meat trimmings compared to meat. Our meat market traditionally offers pastes in a casing that are popular among students, schoolchildren, tourists, and passengers on trains and planes. These products are made from inexpensive protein-containing ingredients with a high nutritional value and are packaged in small portions.

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According to literature, modern meat scientists are interested in combining meat products (including pastes) with plant ingredients to enrich the product with biologically active substances of natural origin (microand macroelements, vitamins, amino acids, antioxidants, etc.) and increase its functional, technological, and other properties.

For example, Gurinovich *et al.* formulated a meat paste by combining animal protein with that of pine nut oilcake. This way, the authors improved the functional properties of meat systems and enriched the end product with a plant-origin ingredient [5].

Bazhenova *et al.* mixed forcemeat with wheat flour containing selenium, an essential trace mineral. The authors described how they selected their method of introducing selenium-enriched flour into the forcemeat. They concluded that a 10-15% protein-fat emulsion with selenized flour increased the functional and technological parameters of forcemeat and provided 50-70% of our daily requirement of selenium [6].

Giro and Chirkova proposed enriching paste with iron [7]. They aimed to develop functional meat-based products for people predisposed to, or suffering from, iron-deficiency anemia. Their study showed that offalbased pastes enriched with chickpea could be used to prevent disturbed hematopoiesis caused by iron deficiency. These products contain highly bioavailable microelements that help the body to quickly mobilize its compensatory reactions.

Another study by Okuskhanova *et al.* looked into the composition and properties of maral deer pastes fortified with beans and protein. The authors developed three formulations with varying amounts of the protein fortifier and beans: no protein fortifier or beans; 15% protein fortifier and 20% beans; and 25% protein fortifier and 10% beans. The study showed that the third formulation had a higher content of essential and non-essential amino acids compared to the first two variants [8].

Pastes from hypoallergenic horse meat and lamb were formulated by Lyakh *et al.* with the addition of dried dill and Polisorbovit-95, a biologically active dietary supplement. According to their results, this combination of ingredients improved the product's sensory and physicochemical properties [9].

As we can see, meat scientists have created various formulations of pastes with plant ingredients rich in biologically active substances.

Further, modern scientific literature shows increased interest in studying antioxidant capacities of natural plant ingredients in order to introduce them into food products to improve their functional properties and inhibit fat oxidation processes [10–21]. Antioxidants can neutralize the destructive effects of free radicals on a human body. Our antioxidant system is one of the main mechanisms for stabilizing our adaptive potential. This is especially important for people who live in adverse environmental conditions and have an unbalanced diet containing synthetic ingredients.

For example, Lisitsyn et al. studied the antioxidant activity of aromatic plant extracts (black pepper, rosemary, sage, and thyme) at the GORO Research Center for Ecological Resources (Rostov-on-Don, Russia). The scientists commercialized a new technology for processing aromatic raw materials - supercritical CO₂ extraction. This technology produces extracts with a significantly different composition from those obtained in traditional ways. Supercritical extracts contain a variety of terpene compounds, as well as waxes, pigments, high molecular weight saturated and unsaturated fatty acids, alkaloids, vitamins, and phytosterols. These substances have high biological, antimicrobial, and antioxidant activities. According to the results, the highest and the lowest contents of antioxidants were found in sage and black pepper extracts (3.1% and 0.07%, respectively). It is generally accepted that natural extracts with an antioxidant content of at least 0.1% can be considered as a dietary supplement with antioxidant properties. Therefore, the authors recommended using sage, rosemary, and thyme extracts as antioxidant ingredients for meat products [10].

Another group of researchers, Zabalueva *et al.*, looked at antioxidant contents in water-alcohol infusions of medicinal plants, depending on the method of their preparation. They found that the concentration of watersoluble antioxidants in infusions obtained by maceration did not differ significantly from those prepared by ultrasound and an ultra-high frequency electromagnetic field. The study showed the potential of using wateralcohol infusions from rose hips and barberry fruits as antioxidant supplements in the production of meat products [15].

A wide range of plant materials (vegetables, fruits, berries, and herbs), including wild plants, are introduced into meat products in the natural form or as extracts, infusions, and decoctions treated in various ways. Edible and medicinal plants are collected, processed, and utilized almost without waste. However, waste from processing wild plants is not always used rationally, being an environmentally friendly, renewable raw material that could be used as a source of biologically active natural substances. These wild plants include lingonberries growing in Transbaikalia (east of Lake Baikal) that are rich in biologically active compounds with medicinal properties. Lingonberry leaves contain phenolic glycosides (arbutin and methylarbutin), vaccinine, lycopene, hydroquinone derivatives, acids (ursulic, tartaric, gallic, quinic, and ellagic), tannin, hyperoside, and other flavonoids. Lingonberries are rich in sugars, ascorbic acid, carotene, and organic acids [22].

The chemical composition of lingonberry leaves and fruits indicates a high antioxidant capacity of respective products. In fact, lingonberries are processed in large quantities for juice production. However, their byproducts – such as husks, pulp or marc – could also be used as a source of biologically active substances. Some authors propose enriching meat products (e.g. liver paste) with fresh or dried lingonberry and cranberry pulp [18, 19].

For example, Bitueva and Ayusheeva introduced dried cranberry or lingonberry pulp, pre-crushed and reconstituted, into ground meat products. The powdered pulp was added at the stage of forcemeat preparation, replacing 13–15% of bread. This method enriched the meat products with biologically active substances [18].

In another study, Ivanova and Izosimova proposed a formulation of meat paste with 19% polyfunctional additives – lingonberry or cranberry marc. The marc contains citric and malic acids that shift the medium's pH away from the isoelectric point, enhancing the dissociation of the main and acid groups of protein, as well as increasing bound moisture and the yield of the end product. A high content of low-ester pectins in the berry marc also contributed to the system's stabilization. Biologically active substances and antioxidants increased the microbiological resistance of the meat and plant paste. The product also had an improved vitamin and mineral composition [19].

In our previous work, we prepared a water-alcohol extract of lingonberry marc which was then dried [23]. The dry extract is a powder rich in biologically active nutrients that is easy to store and transport. Due to a high concentration of dry substances, the extract can be introduced in small amounts into the formulation of meat products, providing them with functional properties and eliminating negative effects on the product's sensory and physicochemical characteristics.

Thus, it is extremely relevant to create products based on a combination of meat and plant materials to enrich them with micro- and macroelements, vitamins, amino acids, and antioxidants. Many types of plant materials contain a variety of compounds with an antioxidant effect. Even low concentrations of antioxidants in the human body can slow down or prevent oxidation processes which are known to cause premature aging and disease. Thus, we can inhibit fat oxidation by introducing antioxidants into food products.

In view of the above, we aimed to develop a meat paste formulation with an extract from lingonberry marc (originating in Transbaikalia), as well as evaluate the total content of antioxidants and effect on the color and stability of the paste in the casing during storage.

STUDY OBJECTS AND METHODS

The objects of the study included a dry lingonberry marc extract (DLME), forcemeat, and a ready-made paste in the casing.

A dry extract of lingonberry marc was obtained in accordance with Patent No. 2626565. Lingonberries were pressed for juice and the remaining marc was placed on a baking sheet in a 5-8 mm layer and then dried in an oven with infrared radiation at $35-40^{\circ}$ C for 40-50 min to 10-15% moisture. The dried marc was crushed to a powder state and subjected to extraction with a wateralcohol solution using a microwave electromagnetic field (700 W, 2450 MHz).

The water-alcohol extracts were filtered and concentrated on a rotary evaporator under vacuum in a water bath at temperature below 45°C until a syrup consistency was reached (40–50% dry matter). The syrup was then vacuum-dried at a temperature below 50°C for 3.5-4.5 h to obtain a powder with a residual moisture of < 5%. Such process parameters do not cause any qualitative changes in thermolabile substances. They preserve maximum biological activity of active substances and ensure high quality of the extract.

The main ingredients in the formulation of paste samples were cheek meat, beef liver, meat trimmings, soy isolate, and semolina. To evaluate the DLME effects on forcemeat characteristics, samples were made with 0.1, 0.2, 0.3, and 0.4% DLME previously dissolved in water in a ratio of 1:5. The paste was made in a casing according to the traditional technology.

A DLME-free paste sample was used as a control. To evaluate the shelf life of the paste, we conducted two experiments. For the first experiment, we prepared control and DLME samples and stored them for 14 days. For the second experiment, we used a 0.2% acidity regulator – sodium lactate (E325) and stored the samples for 18 days.

To evaluate the samples' antioxidant activity, we performed amperometric measurement of the total content of antioxidants in terms of quercetin. The test samples were subjected to extraction with bidistilled water to isolate water-soluble compounds with an antioxidant effect. The total content of antioxidants was measured on a Tsvet Yauza-01-AA analyzer. Quercetin solutions were used to construct calibration graphs [24]. The extraction efficiency was determined by the amount of phenolic compounds isolated spectrophotometrically using the Folin-Ciocalteu reagent. The content of benzoic acid was measured by the HPLC method.

The optical density of the colored aqueous extracts of the test and control samples was determined by the photocolorimetric method on a KFK-3-01 ZOMZ photometer. This method is based on measuring the polychromatic radiation of the visible part of the spectrum. The dependence between light absorption and the radiation wavelength is expressed by a curve (spectrum) of light absorbed by this solution. In the graph, wavelengths are plotted along the abscissa, while optical densities are plotted along the ordinate.

The sensory evaluation of the paste samples was carried out on a nine-point scale according to State Standard 9959. The peroxide value was determined by a method based on the interaction between fat oxidation products (peroxides and hydroperoxides) and potassium

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Indicator	Meaning
Appearance	loose mass
Taste and smell	sweet and sour, tangy, with lingonberry flavor
Color	burgundy
Acidity, pH units	3.24 ± 0.08
Moisture, %	4.52 ± 0.08
Phenolic compounds, %	6.63 ± 0.04
including anthocyanins	3.58 ± 0.04
Benzoic acid, %	1.34 ± 0.02
Total antioxidants, mg/g	382.60 ± 8.70

iodide in a solution of acetic acid and chloroform, followed by the quantification of iodine released in a sodium thiosulfate solution by the titrimetric method (State Standard R 51487-99¹).

The microbiological parameters of the paste samples were assessed according to State Standards R $50454-92^{II}$ and $9958-81^{III}$.

The experiments were performed in triplicate. Statistical processing of the data was carried out in Microsoft Excel.

RESULTS AND DISCUSSION

First, we analyzed the chemical composition of the lingonberry marc extract originating in Transbaikalia (Table 1).

As we can see, the main component of the lingonberry marc extract is a group of phenolic compounds (6.63%), including water-soluble pigments, anthocyanins, and benzoic acid (1.34%). In a preliminary study [25], we used the disk diffusion method and found that the extract had antimicrobial activity, partly due to the presence of benzoic acid with bactericidal properties. Thus, introducing the lingonberry marc extract into food products, namely meat, can inhibit the growth of microorganisms.

Table 1 also shows a high total content of antioxidants, including polyphenols, anthocyanins, vitamin C, and other compounds (382.6 mg/g). Anthocyanins (3.58%), accounting for half of all phenolic compounds, give lingonberries bright red or burgundy coloring. They include malvidins and peonidins (polyphenolic compounds from the flavonoid group) which contain mono- and diglycosides decomposing into sugar and aglycon (anthocyanidins) upon hydrolysis.

Anthocyanins are widely used in the food, medical, pharmacological, and cosmetic industries. A daily intake of brightly colored berries (160–2000 mg) leads to the absorption of anthocyanins (0.005–0.1%), which can have an antioxidant effect. Solutions of anthocyanins neutralize almost all radical forms of oxygen and nitrogen four times as efficiently as ascorbate or α -tocopherol. Even low concentrations of antioxidant substances can slow down or prevent oxidative processes. For example, adding only 0.001–0.01% of antioxidants to oil can slow down its oxidation for a long time [26].

When dissolved, the lingonberry marc extract retains its dark red color. When it is added to gray non-nitrite forcemeat, the latter acquires a purple hue. Anthocyanins are known to act as pigments and the color of plants depends on their concentration, as well as the medium pH. They are red in acidic media, purple in neutral and blue in alkaline media. In this regard, we studied how the concentration of the dry lingonberry extract affected the forcemeat pH (Fig. 1).

The forcemeat pH decreased with the introduction of the dry extract, while remaining closer to the neutral region. The extract's acidity was quite high (3.24, see Table 1) due to the use of marc, whose biologically active substances are better extracted into the solution than those of the fruit juice. Therefore, the marc extract is rich in acids (about 2.5%) – citric, malic, benzoic, oxalic, acetic, glyoxylic, pyruvic, hydroxypyruvic, ketoglutaric, ascorbic, and others, with the highest content of benzoic acid (1.34%). This high concentration of acids provides the extract with a low pH, so even very small amounts of the dry extract can significantly decrease the forcemeat pH.

Before the extract was introduced into the forcemeat, it was pre-hydrated for uniform distribution. The DLME concentrations of 0.1, 0.2, 0.3, and 0.4% reduced the forcemeat pH by 1.49, 2.98, 3.7, and 4.47%, respectively. However, the absolute value of the forcemeat pH remained close to neutral, which did not affect the functional and technological properties of the forcemeat system.

Adding the DLME in concentrations from 0.1 to 0.4% affected the forcemeat color. To select the optimal



Figure 1 Amounts of dry lingonberry marc extract vs. forcemeat acidity

¹ State Standard R 51487-99. Vegetable oils and animal fats. Method for determination of peroxide value. Moscow: Standartinform; 2008. 6 p.

¹¹ State Standards R 50454-92. Meat and meat products. Detection and enumeration of presumptive coliform bacteria and presumptive Escherichia coli (Reference method). Moscow: Standartinform; 2010. 7 p.

^{III} State Standards 9958-81. Sausage products and meat products. Methods of bacteriologikal analysis. Moscow: Izdatel'stvo standartov; 2001. 14 p.

 Table 2 Paste color with different concentrations of dry lingonberry marc extract

Paste samples	Color in section
Control (without DLME)	Gray-brown
Test (with DLME):	
0.1%	Gray-brown
0.2%	Dark gray-brown
0.3%	Brown with a light purple hue
0.4%	Brown with a light violet hue

concentration, we analyzed the color characteristics of ready-made pastes in the casing after heat treatment (Table 2).

As we can see, 0.1% and 0.2% DLME concentrations did not change the habitual sectional color of paste, graybrown or slightly darker. However, larger amounts of the extract (even 0.3%) gave the paste a purple hue. This change is associated with the presence of anthocyanins, water-soluble plant pigments, in the extract.

Lingonberries may contain such anthocyanins as cyanidin-3-galactoside, peonidin-3-galactoside, cyanidin-3-arabinoside, peonidin-3-arabinoside, cyanidin-3-glucoside, and others. However, their qualitative composition depends on the growth conditions. Of great importance for the color of plant pigments is the pH of vacuoles where pigments accumulate. The same pigment in different media can exhibit varying colors: yellowgreen in an alkaline medium, purple in a neutral, and red in an acidic medium [27].

In the DLME, just like in lingonberries, the medium is strongly acidic (pH 3.24), so the color is bright red. When the extract is introduced into the forcemeat, whose medium is close to neutral, it acquires a purple tint from water-soluble anthocyanins. The color, however, depends on the concentration of pigments in the forcemeat.

For the quantitative and qualitative analysis of watersoluble compounds, we determined the optical density of the paste samples with different DLME concentrations, using the spectrophotometric method (Fig. 2). Optical density is known to be directly proportional to the concentration of compounds in a solution. The scale of the abscissa did not show a significant difference in the samples. However, a thorough analysis indicated that the highest peaks for the control sample (curve 1), 0.1% DLME sample (curve 2), 0.2% DLME sample (curve 3), and 0.3% DLME sample (curve 4) were at wavelengths of 554 nm, 557 nm, 557 nm, and 559 nm, respectively (Fig. 2). As we know, the spectral range from 500 to 560 nm corresponds to purple, while that from 560 to 575 nm to purple. Our study showed the same results: the sample with 0.3% DLME had a violet hue (Table 2).

Optical density along the ordinate axis characterizes the color intensity. It means that the height of the peaks corresponds to the concentration of dissolved substances (polyphenols) in the samples. As we can see, the optical density values for the control sample (curve 1), 0.1% DLME sample (curve 2), 0.2% DLME sample (curve 3), and 0.3% DLME sample (curve 4) were 0.86, 0.92, 0.93, and 0.93, respectively. The results show that larger amounts of the extract led to higher concentrations of water-soluble compounds, having reached a maximum on curve 4 (0.3% DLME sample).

Thus, we found that increasing the DLME concentration to 0.3% provided the paste with a high content of antioxidants, but added a purple hue to its color due to the presence of anthocyanins, which might spoil the product's appearance.

Antioxidants, including phenolic compounds, neutralize lipid peroxidation, all radical forms of oxygen and nitrogen. Therefore, we analyzed the samples for the total content of antioxidants (Fig. 3).

Figure 3 shows a correlation between increased amounts of lingonberry marc extract and higher total antioxidant capacity of the paste. According to the results, the total antioxidants in the test samples with 0.1, 0.2, 0.3%, and 0.4% DLME was higher than that of the control by 1.3, 1.5, 1.8, and 2.05 mg/g, respectively. The extract antioxidant complexes were rich in polyphenols



1 - control, 2 - 0.1% DLME sample, 3 - 0.2% DLME sample, 4 - 0.3% DLME sample

Figure 2 Optical density of paste samples with different concentrations of dry lingonberry marc extract

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Figure 3 Total content of antioxidants in paste samples with different concentrations of dry lingonberry marc extract

(6.63%; Table 1). They also contained organic acids, vitamins, lycopene, and other antioxidant compounds.

Further, we performed a sensory evaluation of the paste on a nine-point scale to establish how the extract affected the product's consumer appeal (Table 3).

According to the results, small amounts of the dry lingonberry marc extract did not significantly affect the texture, smell, or taste of the end product. However, its concentrations above 0.2% had a negative effect on the paste color in section. As we could see in Table 2, the sample with 0.3% DLME acquired a light purple hue and that with 0.4% DLME, a light purple tint. The sensory evaluation showed a concentration of 0.2% as optimal since it did not spoil the characteristics of the end product while enriching it with antioxidant compounds (Fig. 3). Therefore, we used this concentration in further studies. For a physicochemical analysis, we prepared a control and a test sample with 0.2% DLME (Table 4).

The results showed that the dry lingonberry marc extract did not affect the quality of the paste, but it doubled the total content of antioxidants. The content of phenolic compounds in the extract is the most important indicator of its biological value, which determines its antioxidant activity.

To assess the extract's antioxidant capacity, we studied the process of fat oxidation. For this, we prepared a control and a test paste samples and determined the peroxide value which characterizes the accumulation of primary lipid decomposition products during storage.

 Table 3 Sensory characteristics of paste samples with dry lingonberry marc extract

Characteristic	Control	Test samples with DLME					
		0.1%	0.2%	0.3%	0.4%		
Appearance	8.5 ± 0.2	8.5 ± 0.1	8.5 ± 0.2	8.3 ± 0.1	8.2 ± 0.1		
Texture	8.4 ± 0.2	8.5 ± 0.2	8.4 ± 0.1	8.4 ± 0.2	8.3 ± 0.2		
Color and	8.6 ± 0.2	8.6 ± 0.1	8.6 ± 0.1	8.0 ± 0.1	7.4 ± 0.1		
appearance							
in section							
Smell and taste	8.7 ± 0.2	8.7 ± 0.1	8.7 ± 0.2	8.3 ± 0.2	8.1 ± 0.2		

Table 4 Physicochemical characteristics of paste in casing (0.2% DLME)

Characteristics	Paste in casing			
	Test	Control		
Content, %				
moisture	71.5 ± 0.1	71.8 ± 0.03		
protein	13.6 ± 0.1	13.3 ± 0.1		
fat	11.9 ± 0.05	12.1 ± 0.2		
minerals	1.4 ± 0.02	1.2 ± 0.1		
salt	1.6 ± 0.02	1.6 ± 0.02		
Total antioxidants, mg/100 g	67.84	31.23		

Storage periods were selected in accordance with State Standard R $55334-2012^{IV}$ (10 days for pastes in polyamide casings and 15 days for pastes with acidity regulators). Our experiment consisted of two tests. In the first test, the control and the test samples (without DLME and with 0.2% DLME, respectively) were made without acidity regulators and stored for 14 days (Fig. 4). In the second test, the control and the test samples (without DLME and with 0.2% DLME, respectively) contained 0.2% sodium lactate as an acidity regulator and were stored for 18 days (Fig. 5).

The reason for this experiment was that acidity regulators are necessarily used in production, especially in summer, to increase the shelf life of perishable meat products. Thus, the experiment could show the DLME role in the inhibition of peroxidation of animal lipids. The samples were stored under identical conditions, in the dark at $2 \pm 2^{\circ}$ C.

Figure 4 shows the effect of DLME on the process of fat oxidation in the paste.

In Fig. 4, we can see an irreversible process of fat oxidation with the accumulation of primary fat decomposition products. Animal fats contained in the paste undergo auto-oxidation or peroxidation. The polyamide casing cannot completely prevent oxidation, since it is caused by a complex of factors: oxygen, light, positive temperature, unsaturated fatty acids, etc.

According to regulatory documents, the peroxide value for a high-quality fat product, low-oxidized raw materials, and fat raw materials should not exceed 0.5, 3.5, and 10 mmol of active oxygen per 1 kg of fat.

As we can see in Fig. 4, the peroxide value in the control and test samples immediately after preparation was 0.71–0.75 mmol O/kg. After six days of storage, it increased 3.17 times in the control and 2.98 times in the test sample, reaching 2.38 and 2.12 mmol O/kg, respectively. We found that on day 6, the peroxidation process in the test sample slowed down by 10.9% compared to the control. After 10 days (the shelf life for this type of product), the process of lipid oxidation continued to intensify and the peroxide value increased to 3.1 mmol O/kg in the control sample (6.2 as high as

^{1V} State Standard R 55334-2012. Meat and meat containing pate. Specifications. Moscow: Standartinform; 2014. 34 p.

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Figure 4 Effect of dry lingonberry marc extract on the peroxide value of paste without acidity regulators during storage



Figure 5 Effect of dry lingonberry marc extract on the peroxide value of paste with sodium lactate during storage

the initial value) and to 2.7 mmol O/kg in the test sample (3.8 times as high as the initial value). On day 10, the difference between the control and the test samples reached 12.9%.

Further analysis of the oxidation process showed that after 12 days, the peroxide values in the control and the test samples were 3.55 and 3.1 mmol O/kg, respectively. After 14 days, the process accelerated and the values reached 4.1 and 3.7 mmol O/kg, respectively. The difference between the control and the test samples was 12.7% on day 12 and 9.7% on day 14. Thus, the steadily lower peroxide value in the test sample, compared to the control, indicated a decreased rate of lipid peroxidation reactions throughout the whole storage period. This result can be explained by the presence of DLME rich in antioxidant compounds that can neutralize the effects of free radicals playing a significant role in chain reactions of lipid oxidation.

The first test showed that introducing DLME into the paste made without acidity regulators helped to slow down fat oxidation and increase the shelf life by two days (total of 12 days without signs of oxidative damage).

In the second test, the control (without DLME) and test (0.2% DLME) samples contained 0.2% sodium lactate as an acidity regulator (Fig. 5).

The growth of peroxide values indicated the accumulation of fat oxidation products in the paste samples with sodium lactate throughout storage. However, we found a certain inhibition of the oxidation process compared to the first test, in which the samples were made without an acidity regulator. For example, on day 10, the peroxide values of the DLME samples without and with sodium lactate were 2.7 and 2.5 mmol O/kg, respectively. Thus, we can see a synergistic effect of sodium lactate and DLME antioxidant compounds during fat oxidation in the paste.

Further, we compared the peroxide values in the control and test samples with sodium lactate. We found that after 15 and 18 days of storage, the peroxide value of the test samples was 20% lower compared to the control, which was significantly higher than in the samples without sodium lactate (12.9%).

At this stage, we concluded that a combination of lingonberry extract with sodium lactate produced a more pronounced antioxidant effect. At the end of the storage period (18 days), the peroxide values of the control and test samples were 3.5 and 2.7 mmol of active oxygen per 1 kg of fat. This means that the paste's shelf life could be extended by three days.

Thus, our experiment showed that although DLME contributed to the inhibition of lipid oxidation, its synergism with sodium lactate could significantly slow down these reactions.

The shelf life of paste containing a large amount of water (71–72%) is affected by not only oxidative

Table 5 Microbiological indicators of control and test samples during storage

Indicator	Storage of paste without sodium lactate, days							
		DLME	test samples			Со	ntrol	
	0	6	10	12	0	6	10	12
QMAFAnM, CFU/g	1.4×10^{2}	4.1×10 ²	6.2×10 ²	7.3×10 ²	1.4×10^{2}	5.8×10 ²	7.5×10 ²	8.5×10 ²
Coliforms in 1 g				not	detected			
Indicator			Stora	ge of paste wi	th sodium lact	tate, days		
		DLME	test samples		Control			
	0	7	15	18	0	7	15	18
QMAFAnM, CFU/g	1.4×10^{2}	4.5×10 ²	8.4×10 ²	1.6×10 ³	1.4×10^{2}	6.1×10 ²	1.0×10 ³	2.1×10 ³
Coliforms in 1 g	1 g not detected							

processes, but also by the growth of microorganisms. According to Table 1, the dry lingonberry extract is rich in benzoic acid (1.34%) that has strong antimicrobial, antiseptic, and bactericidal effects inhibiting decay and fermentation processes. As a result, lingonberries last quite a long time without canning. Also, previous studies have proven the antimicrobial activity of DLME added to bakery products [25].

In our study, we investigated a possibility of inhibiting microorganisms in the DLME paste samples with and without sodium lactate (Table 5).

As we can see, all the samples showed a growth of microorganisms. However, it was less intensive in the DLME test samples with and without sodium lactate, compared to the controls. Thus, the presence of benzoic acid with strong bactericidal action slowed down the growth of microorganisms and had a positive effect on the test samples' shelf life.

As for pathogens, no *E. coli* bacteria were detected in any of the test samples, which might be due to the preliminary heat treatment of the raw materials and the use of a polyamide casing that excludes the product's contact with air, containers or equipment.

CONCLUSION

Thus, our study showed that 0.2% of dry lingonberry marc extract was the optimal amount to be introduced into paste forcemeat. This amount increased the

nutritional and biological value of the paste and maintained high consumer appeal. We found that the extract provided the product with a high content of polyphenols with antioxidant properties, including anthocyanins. Rich in antioxidant compounds, the extract inhibited fat oxidation in the paste and, in combination with sodium lactate, produced a synergistic effect on lipid peroxidation processes. In addition, the dry lingonberry marc extract slowed down the growth of microorganisms due to a high content of benzoic acid with antimicrobial and bactericidal properties. The integrated effect of the extract's components extended the shelf life of the paste in a casing by two or three days.

CONTRIBUTION

Authors are equally related to the writing of the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Effects of non-meat proteins on the quality of fermented sausages

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Abstract:

Introduction. Non-meat proteins are widely used in meat processing. In our study, we analyzed the effects of whey and soy protein isolates on the physicochemical and sensory properties of domestic fermented sausage.

Study objects and methods. Five groups of sausages were traditionally fermented under industrial conditions. The sausage group without the additives was labelled the control, while other sausages were manufactured with the addition of 0.5% and 1.5% protein isolates of whey and soybean. Using a quantitative descriptive test, we assessed the sensory characteristics of the sausages and instrumentally determined their color, hardness, water activity (a_w) , and pH.

Results and discussion. The proteins added to fermented sausages improved emulsification, texture, as well as water and fat binding capacity, which was confirmed by the results for hardness. Using a 0.5% soy protein isolate resulted in a firmer product. The additives had a minor effect on the color: the samples with the additives had a slightly lower L^* value, and those with a soy protein had higher yellowness (b^*).

Conclusion. Using the additives did not have a significant effect on the chemical composition and overall sensory quality of all tested samples (P > 0.05).

Keywords: Meat products, sausages, whey proteins, soy proteins, sensory quality, color, hardness

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INTRODUCTION

Today, there are many different meat products on the market. Domestic fermented sausages belong to a group of dry fermented sausages, which are produced in a traditional way and have desirable sensory properties. Their sensory characteristics depend on various factors including the selection and quality of raw materials and basic ingredients, the formulation of sausage emulsion, the metabolic activity of epiphytic microflora, the physicochemical changes during smoking and drying, the enzymatic decomposition of proteins and fats, the conditions and length of ripening, as well as external factors (temperature, relative humidity, and air circulation) [1].

The quality of fermented sausages, as well as the changes that occur during fermentation, drying, and ripening, depends not only on the basic components of the sausage emulsions, but also on the additives that affect the transition of the emulsion into the fermented product. Spices, additives, flavoring agents, enzymes, sugars, carbohydrates, fibers, and protein products are commonly used in manufacturing meat products. When using additives, we should preserve the characteristic properties of meat products [1]. Introducing non-meat ingredients into meat products improves their quality and reduces the cost of production. The most commonly used ingredients are dairy products, eggs, plants, and probiotics, which contribute to increased nutritional value, consumer acceptance, and benefits for human health [2, 3].

The first impression about the quality of fermented sausage is based on the visual experience, or the size, shape, color, and gloss of products, either coated or packed. The outer surface of the sausage depends, above all, on the type and quality of coating and the intensity of smoking (type of wood) and drying. Also, the sensory evaluation of sausages includes the cross-section color. According to the generally accepted criteria for sensory properties of fermented sausages, the filling on the cross-section should have the appearance of a mosaic composed of approximately equal pieces of meat (stable and uniform red color) and fat tissue (whitish color). The filling ingredients must be evenly arranged and firmly interconnected, with no visible cavities or cracks in the cross-section [4].

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The formation of odor and flavor of fermented sausages depends on the fermentation of carbohydrates, lipolysis and lipid oxidation, on proteolytic processes, as well as the type and quantity of used spices, salt, and additives [5, 6].

Non-meat proteins, such as soybean and whey proteins, are often used to improve the texture of meat products. These ingredients play an important role in changing the functional properties such as emulsifying, water and fat binding capacity, and texture. They are used as additives that can improve yield and potentially reduce the cost of products [7].

The previous works have studied the use of non-meat proteins in cooked and semi-dry sausages, but there are few studies on their effect on fermented sausages.

The main goal of this research was to study the effect of soy and whey protein isolates on the quality of domestic sausages traditionally fermented under industrial conditions. The proteins were added to improve the quality of sausage, rather than as a substitute for meat. Adding soy and whey protein to domestic fermented sausages and modelling their quantitative ratio during product development can improve the quality of the new product and reduce the manufacturing time.

STUDY OBJECTS AND METHODS

Domestic fermented sausages were produced in a traditional way under industrial conditions. The emulsion consisted of mature pork (59.3%) and beef meat (7.6%) of first and second category, pork back fat (28.7%), nitric salt for curing (2.5%), spices (0.3% garlic in granules, 0.4% hot red pepper, 0.4% sweet red pepper, 0.3% ground black pepper), and additives (0.3% glucono-delta-lactone GDL/TARI S 77 and 0.3% MIOCOLOR VS (a homogeneous mixture of antioxidants based on the salt of ascorbic acid, edible organic acids, and dextrose)).

For this study, we made five samples of domestic fermented sausages: the control (without isolates); with 0.5% of whey isolate (Impact Whey isolate, Myprotein, Norwich, UK); with 1.5% of whey isolates; with 0.5% of soy isolate (IZOPROT S, Ireks Aroma, Zagreb, Croatia), and with 1.5% of soy isolate. Duplicate batches were prepared. The weight of each batch was 40 kg.

After grinding and mixing in the cutter, the sausage emulsion was poured into natural coatings (pork intestine) with a diameter ~ 30 mm. The sausages were first tempered (22°C), then smoked (beech wood) for three days (18°C to 20°C), and finally left for fermentation (ripening) at 16°C. The relative humidity gradually decreased from 85% at the beginning to 65% at the end of ripening. Following the ripening stage, the final sausages were vacuum packed and stored in a cooling chamber at 4°C until sampling. Seven randomly selected sausages were taken after the ripening stage and during storage periods (1, 2, 3, and 6 months). CIE L^* , a^* , b^* color values (L^* – lightness, a^* – redness, b^* – yellowness) were determined with a Konica Minolta CM 2600d camera (Osaka, Japan). The measurements were carried out on a fresh cut of sausage samples. Five measurements were taken on three crosssections of two sausages from each treatment. The mean of 30 measurements was recorded for each color parameter.

The hardness/softness was determined by a universal texture meter, a TA.XT plus Texture Analyzer (Stable Micro Systems, Godalming, UK). The cutting force was measured by a Warner-Bratzler contact pin (parameters: 25 kg force, 4 mm/s rate, 20 mm distance). The test samples were prepared by using a mold with eight rectangular shapes (1×1 cm, approximately 5 cm) in which the measurements were performed. The mean of 20 measurements was recorded.

The water activity (a_w) was determined by a LabMaster-aw hygrometer (Novasina, Switzerland) at a constant temperature of 25°C. The mean of 5 measurements was recorded.

pH was measured by a digital pH meter (HANNA HI 99161, Cluj-Napoca, Romania) equipped with a combined penetration tip, which had been calibrated with buffer solutions at pH 4 and 7. The mean of 5 measurements was recorded.

Using quantitative descriptive analysis (ISO 6564:1985¹), we evaluated the sensory properties of sausages (external appearance, cross-section appearance and color, odor, flavor and taste, texture and overall acceptability). Ten panelists (6 females, 4 males, average age of 35) took part in the evaluation. Based on the average value of ratings for individual characteristics, we calculated the overall quality score of the sausages.

Standard methods were used to analyze the chemical quality parameters: water content – ISO 1442:1997^{II}; total fat content – ISO 1443:1973^{III}; total protein content – ISO 937:1978^{IV}; total ash content – ISO 936:1998^V; total phosphorus content – ISO 13730:1996^{VI}; sodium chloride content – ISO 1841-1:1996^{VII}; and nitrite content

¹ ISO 6564:1985. Sensory analysis. Methodology. Flavour profile methods. Geneve: International Organization for Standardization; 1985. 8 p.

^{II} ISO 1442:1997. Meat and meat products. Determination of moisture content (Reference method). Geneve: International Organization for Standardization; 1997. 8 p.

^{III} ISO 1443:1973. Meat and meat products. Determination of total fat content. Geneve: International Organization for Standardization; 1973. 4 p.

^{IV} ISO 937:1978. Meat and meat products. Determination of nitrogen content (Reference method). Geneve: International Organization for Standardization; 1978. 3 p.

^v ISO 936:1998. Meat and meat products. Determination of total ash. Geneve: International Organization for Standardization; 1998. 10 p.

^{VI} ISO 13730:1996. Meat and meat products. Determination of total phosphorus content. Spectrometric method. Geneve: International Organization for Standardization; 1996. 12 p.

^{VII} ISO 1841-1:1996. Meat and meat products. Determination of chloride content. Part 1: Volhard method. Geneve: International Organization for Standardization; 1996. 6 p.

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Parameter	Control	0.5% whey isolate	1.5% whey isolate	0.5% soy isolate	1.5% soy isolate
Moisture, %	21.95 ± 1.44	21.20 ± 1.44	21.80 ± 1.44	21.97 ± 1.28	22.10 ± 1.80
Ash, %	4.92 ± 0.35	5.01 ± 0.41	4.91 ± 0.28	4.99 ± 0.29	5.06 ± 0.36
Fat, %	49.38 ± 3.44	49.55 ± 2.10	49.58 ± 2.67	50.14 ± 1.54	48.55 ± 1.63
Proteins, %	20.17 ± 1.89	20.75 ± 1.44	20.12 ± 1.15	19.83 ± 1.52	20.92 ± 1.21
Fat-proteins ratio	2.44	2.40	2.47	2.47	2.30
Moisture-proteins ratio	1.19	1.10	1.17	1.18	1.13
NaCl, %	4.50 ± 0.17	4.60 ± 0.36	4.57 ± 0.02	4.52 ± 0.27	4.56 ± 0.32
Total phosphates, %	0.47 ± 0.06	0.47 ± 0.04	0.49 ± 0.07	0.49 ± 0.06	0.51 ± 0.06
Nitrites, mg/kg	3.04 ± 1.80	3.42 ± 1.37	2.84 ± 1.03	3.78 ± 1.41	4.22 ± 1.94

Table 1	Chemical	composition of	of sausages	with	protein	isolates	(average	value \pm S	D)

Table 2 Water activity and pH values of sausages with protein isolates (average value \pm SD)

Storage	e period,	Control	0.5% whey isolate	1.5% whey isolate	0.5% soy isolate	1.5% soy isolate
a	0	$0.832^{a,A}{\pm}\ 0.0005$	$0.807^{\text{b,A}} {\pm 0.0005}$	$0.819^{c,A} \pm 0.0005$	$0.819^{\rm c,A}{\pm}\ 0.0005$	$0.823^{\text{d,A}}{\pm}~0.0005$
	1	$0.816^{a,B}\!\pm 0.0005$	$0.820^{\rm b,B}\!\pm 0.0005$	$0.826^{\rm b,B}\!\pm 0.0005$	$0.818^{a,c,A}{\pm}\ 0.0005$	$0.821^{\rm b,c,A}{\pm}~0.0005$
	2	$0.808^{a,C}\!\pm 0.0005$	$0.822^{\rm b,B}\!\pm 0.0005$	$0.836^{c,C} \pm 0.0005$	$0.842^{\rm d,B} {\pm 0.0005}$	$0.834^{e,B} \pm 0.0005$
	3	$0.824^{\mathrm{a},\mathrm{D}} \!\pm 0.0005$	$0.822^{\text{b},\text{B}} \!\pm 0.0005$	$0.830^{\rm c,D} {\pm 0.0005}$	$0.807^{\text{d},\text{C}} {\pm 0.0005}$	$0.817^{\text{e},\text{C}} \pm 0.0005$
	6	$0.823^{a,D}\!\pm 0.0005$	$0.822^{a,B}\!\pm 0.0004$	$0.831^{\text{b,D}} {\pm 0.0004}$	$0.807^{c,C} {\pm 0.0008}$	$0.815^{\text{d,C}} {\pm 0.0004}$
pН	0	$5.34^{\text{a,A}}\pm0.063$	$5.54^{\text{b,c,A}} \pm 0.001$	$5.37^{\text{a,A}}\pm0.020$	$5.51^{\text{b,A}} \pm 0.014$	$5.64^{c,A} \pm 0.016$
	1	$5.57^{\text{a,c,B,C}} \pm 0.020$	$5.45^{\rm b,B}\pm 0.050$	$5.53^{\text{a,b,B}}\pm0.005$	$5.62^{\rm c,B}\pm 0.020$	$5.63^{\text{c,A}}\pm0.030$
	2	$5.70^{a,D}\pm 0.005$	$5.64^{\rm b,C}\pm 0.010$	$5.60^{c,C} \pm 0.012$	$5.59^{\text{c},\text{B}}\pm0.010$	$5.68^{a,A} \pm 0.007$
	3	$5.68^{\rm a,C,D}\pm 0.037$	$5.70^{a,b,C}\pm 0.008$	$5.71^{a,b,D} {\pm 0.025}$	$5.68^{\text{a,C}}\pm0.010$	$5.77^{\text{b,B}} \pm 0.030$
	6	$5.48^{\text{a,b,B}}\pm0.041$	$5.48^{a,b,A,B}\pm 0.010$	$5.50^{\rm a.B}\pm 0.023$	$5.44^{\text{b},\text{D}}\pm0.012$	$5.55^{c,C} \pm 0.012$

a–d values in the same column with different superscripts are significantly different (P < 0.05)

A–D values in the same row with different superscripts are significantly different (P < 0.05)

- ISO 2918:1975^{VIII}. All measurements were carried out in 5 repetitions. All analyses were performed immediately after production (0) and during storage (1, 2, 3, and 6 months).

Statistical analysis. Our results were presented as mean values accompanied with standard deviations. A factorial analysis of variance (ANOVA) was performed using the Statgraphic Plus 5.1 Professional Edition (1994–2001, Statistical Graphics Corporation, USA). The Multiple Range test was used to identify significant (P < 0.05) differences between treatments. Repeated measures ANOVA was used to test the differences during storage periods.

RESULTS AND DISCUSSION

The chemical composition of all the samples of domestic fermented sausages is shown as mean values of parameters measured after 0, 1, 2, 3, and 6 months of storage (Table 1).

The moisture content of the sausage samples during the period monitored ranged from 21.2% to 22.1%. However, numerous studies report higher moisture contents for similar products [8–10]. Using coatings with a narrow diameter (about 30 mm), longer ripening or a higher fat content could result in a lower moisture content [11]. The fat content of the final product varied between 48.55% and 50.14%, which depended primarily on the recipe, with similar fat contents reported by numerous other studies [8, 12]. According to our results, soy and whey proteins in concentrations of 0.5 and 1.5% did not have significant effects on the total protein content (P > 0.05), which agreed with earlier studies [13, 14]. The difference between the contents of fat and protein was large, due to a high content of fat in the formulation, while the moisture and protein values were almost identical, compared to other data for traditional products [5].

The salt content ranged from 4.5 to 4.6%, and other authors obtained similar or higher values for traditionally fermented sausages [15, 16]. The ash contents ranged from 4.9 to 5.06%, while the use of additives, whey and soy protein isolates, did not have a significant effect on the values studied [17, 18].

The average values of total phosphorus during storage ranged from 0.47 to 0.51%, with no major differences between the samples. This result was quite expectable as the meat protein content, the main source of phosphorus, did not change significantly (P > 0.05).

The values of residual nitrite content after production and during storage ranged from 2.84 to 4.22 mg/kg. These data confirm the fact that the nitrites were decomposed during ripening and fermentation, which

^{VIII} ISO 2918:1975. Meat and meat products. Determination of nitrite content (Reference method). Geneve: International Organization for Standardization; 1975. 3 p.

Samples	Storage period, months						
	0	1	2	3	6		
Control	$1.43^{a,A} \pm 1.30$	$2.12^{\scriptscriptstyle a,B}\pm1.01$	$2.02^{\rm a,B}\!\pm 0.67$	$1.98^{\rm a,b,B} {\pm 0.61}$	$2.05^{\mathtt{a},\mathtt{B}} \!\pm 0.62$		
0.5% whey isolate	$1.12^{\mathrm{a,A}}\pm0.63$	$1.66^{a,B} \pm 1.14$	$1.60^{a,A,B}\!\pm 0.92$	$1.59^{a,A,B}\!\pm 0.78$	$1.62^{b,B} \pm 0.62$		
1.5% whey isolate	$1.14^{a,A} \pm 0.62$	$1.62^{\scriptscriptstyle a,B} \!\pm 1.04$	$1.77^{a,B}\!\pm 0.85$	$1.81^{a,b,B} \pm 0.71$	$1.84^{a,b,B} \pm 0.59$		
0.5% soy isolate	$1.00^{a,A} \pm 0.29$	$3.70^{b,B} \pm 1.64$	$3.41^{b,B} \pm 1.35$	$3.16^{c,B} \pm 1.08$	$3.04^{c,B} \pm 0.79$		
1.5% soy isolate	$1.06^{a,A} \pm 0.41$	$2.06^{\text{a,B}}\pm1.16$	$2.02^{a,B} \pm 0.77$	$2.11^{b,B} \pm 0.80$	$2.07^{a,B}\!\pm 0.59$		

Table 3 Hardness of sausages with protein isolates (average value \pm SD)

a, b values in the same column with different superscripts are significantly different (P < 0.05)

A, B values in the same row with different superscripts are significantly different ($P \le 0.05$)

was reported by many authors [8, 19]. In the Slavonian sausage of Kulen, the content of nitrite after ripening was 2.93–14.3 mg/kg [20]. As we can see, there were no significant differences (P > 0.05) in the chemical composition between the samples.

The degree of reducing the a_w value depends on the composition of sausages, temperature, relative humidity, and the ripening time. During drying and ripening, the concentration of water in the product decreases, followed by dehydration and reduction of a_w [11].

The results of water activity can be seen in Table 2. After production, the a_w values of the analyzed samples ranged from 0.807 to 0.832, which was confirmed by Suvajdžić [16]. Mastanjević received higher values in the study of Slavonian kulen [21]. Operta *et al.* reported that the activity of water in traditional fermented sausages ranged from 0.83 to 0.89 at the end of drying, which was also the case for dry fermented chicken sausages with the addition of corn oil and soybean isolates [22, 23]. During storage, there were noticeable significant differences (P < 0.05), with values of a_w ranging from 0.807 to 0.842 (Table 2). Operta *et al.* reported similar results indicating that the products with a soybean protein isolate showed a slight decrease in a_w values during storage [22].

After the production, the pH values of the samples were from 5.34 to 5.64 (Table 2). During storage, they ranged from 5.44 to 5.77, with noticeable significant differences (P < 0.05). Many authors cited similar or lower values as a characteristic of fermented sausages [24, 25].

Table 3 shows changes in the samples texture after production and during a six-month storage period.

After production, there were no significant differences between the sausages (P > 0.05), with hardness ranging from 1.00 to 1.43. Similar values were recorded in other studies as well [1, 26]. Some authors cite higher values [9, 16]. Lee found that the products with the addition of a soybean protein isolate show slightly higher hardness values compared to the control sample [27].

After the first month of storage, there was an increase in hardness, especially in the sample containing 0.5% soy protein isolate compared to other samples (P < 0.05). The texture of fermented sausages is related to the fat and salt content, as well as pH [16].

The hardness test showed a noticeable effect of the additives. The samples with a whey protein isolate had a lower cutting force than the control, while the sample with 0.5% soy protein isolate had significantly higher hardness values during the entire storage period (P < 0.05).

Priyadarshi pointed out that added soy and whey proteins increased the hardness of cooked pork sausage, while many authors stated the opposite for cooked sausages and burgers [17, 28, 29]. Akesowan found that an amount greater than 2% of soy protein isolate affected the strength of cooked pork sausages [13].

The lightness (L^*) values of the sausage samples are shown in Table 4. As we can see, they were consistent after production, ranging from 49.07 to 50.20. Many studies featured similar values [31, 32]. Kim *et al.* reported higher L^* values, while most authors found significantly lower values, ranging from 30 to 45 [5, 18, 23, 32].

During storage, the L^* values changed significantly (P < 0.05) from 40.72 to 50.92, although there was generally a slight decrease. Some studies showed similar results [21, 33]. The decrease in the L^* values was related

Table 4 L^* values of sausages with protein isolates during storage (average \pm SD)

Samples			Storage period, months			
	0	1	2	3	6	
Control	$49.61^{\mathtt{a},\mathtt{A}}\pm4.26$	$49.79^{\text{a},\text{A}} \pm 5.91$	$42.52^{\text{a,b,B}}\pm6.21$	$50.41^{\mathtt{a},\mathtt{A}}\pm5.47$	$46.52^{\scriptscriptstyle a,C}\pm3.09$	
0.5% whey isolate	$50.16^{\rm a,A,C}\pm 7.22$	$50.92^{\text{a,A}}\pm6.31$	$42.93^{\text{a,b,B}}\pm6.09$	$44.29^{\text{a,A,C}}\pm5.12$	$46.94^{\mathrm{a},\mathrm{C}}\pm4.89$	
1.5% whey isolate	$49.41^{\mathtt{a},\mathtt{A}}\pm4.64$	$48.15^{\text{a,b,A}}{\pm}4.73$	$44.57^{\text{a},\text{B}} \pm 5.05$	$44.36^{\text{b,B}}\pm5.17$	$45.15^{\mathtt{a},\mathtt{B}}\pm3.037$	
0.5% soy isolate	$50.20^{\mathrm{a,A}}\pm4.65$	$45.89^{\text{b},\text{B}}\pm4.04$	$42.94^{\text{a,b,C}}\pm3.145$	$43.09^{\text{b,C}}\pm3.91$	$45.65^{\text{a},\text{B}}\pm3.77$	
1.5% soy isolate	$49.07^{\text{a,A}}\pm5.18$	$45.54^{\text{b},\text{B}}\pm4.22$	$40.72^{\text{b,C}}\pm5.32$	$44.67^{\mathrm{b,B}}\pm4.32$	$45.83^{\text{a},\text{B}}\pm3.31$	

a, b values in the same column with different superscripts are significantly different ($P \le 0.05$)

A–C values in the same row with different superscripts are significantly different (P < 0.05)

Samples	Storage period, months					
	0	1	2	3	6	
Control	$14.50^{a,A} \pm 2.25$	$12.37^{\text{a},\text{B}}\pm2.88$	$16.56^{\mathrm{a,c,C}}\pm2.45$	$12.32^{\text{a},\text{B}}\pm2.77$	$14.85^{\text{a,A}}\pm2.71$	
0.5% whey isolate	$12.72^{\rm b,A,B}\pm 2.82$	$12.14^{\mathtt{a},\mathtt{B}}\pm2.87$	$14.53^{\text{b,C}} \pm 2.51$	$12.06^{\mathrm{a,B}}\pm1.81$	$14.01^{\mathtt{a},\mathtt{B},\mathtt{C}}\pm2.15$	
1.5% whey isolate	$14.15^{\text{a,b,A}}\pm2.29$	$12.12^{\mathtt{a},\mathtt{B}}\pm2.31$	$15.45^{\text{a,b,C}}\pm2.95$	$12.86^{\text{a,b,B}}\pm2.02$	$12.20^{\text{b},\text{B}}\pm1.91$	
0.5% soy isolate	$14.30^{\text{a,A}}\pm2.81$	$14.67^{\text{b,A}}\pm2.18$	$17.60^{c,B} \pm 3.00$	$14.58^{\text{c,A}}\pm2.26$	$16.48^{\mathrm{c,B}}\pm2.05$	
1.5% soy isolate	$14.24^{\mathtt{a},\mathtt{A}}\pm2.22$	$13.46^{\text{a,b,A}}\pm2.31$	$17.93^{\text{c,B}}\pm2.83$	$14.15^{\text{b,c,A}}\pm2.58$	$14.53^{\text{a,A}}\pm1.58$	

Table 5 a^* values of sausages with protein isolates during storage (average \pm SD)

a-c values in the same column with different superscripts are significantly different (P < 0.05)

A–C values in the same row with different superscripts are significantly different (P < 0.05)

to the loss of moisture and also to low fat and high water contents [4, 25].

Škaljac reported that the loss of water from the Petrovačka sausage during aging increased the concentration of myoglobin [11]. On the other hand, the dehydrated muscle tissue absorbed a higher amount of light which resulted in a darker color of the product and decreased L^* values.

In the soy protein samples, the L^* values remained approximately the same or decreased, although some authors had opposite results [13, 14, 23, 34]. Using whey protein also led to lower L^* values [18, 35]. Serdaroglu, however, claimed that milk additives slightly increased L^* values, with similar observations made by Hughes *et al.* [17, 36]. Barbut reported no significant changes caused by whey supplements [37].

The values of redness (a^*) are shown in Table 5. After production, these values ranged from 12.72 to 14.50. This color parameter was significantly different between the samples and during storage (P < 0.05). Many authors reported higher values and greater deviations [4, 31]. A reduction of the a^* value was due to a higher amount of lactic acid, which denatured myoglobin, nitrosylmyoglobin, and oxymyoglobin [4]. A lower protein content had the same effect, while a low fat level and a high water content led to increased a^* values.

Serdaroglu and Abdolghafour found no effect of dairy supplements on the a^* value, although some authors reported a decline in this value when using additives, which was confirmed by our study [17, 18, 37]. The use of soy protein resulted in lower a^* values [13, 34].

The values of yellowness (b^*) are shown in Table 6. Immediately after production, they ranged from 12.35 to 14.79 in the 0.5% whey protein and 0.5% soy protein samples, respectively. However, during further storage, this parameter reached 12.49 and 18.00 for the 0.5% whey protein and 0.5% soy protein samples, respectively. Thus, the differences between the samples and during storage were significant (P < 0.05). Similar data were reported by other authors [4]. Lower values were given by Skaljac et al. for sausages stored under controlled conditions in an industrial chamber [4]. Higher values for vacuum-packed Petrovska sausage were reported by Skaljac et al. [38]. The decrease in the b^* values was assumed to be caused by microorganisms that use oxygen during fermentation, thus reducing the amount of a muscle pigment that beneficially affected the b^* value [37]. Another study reported higher fermentation temperature and the addition of autochthonous starter culture as a cause of the decrease [21].

Most authors agree that adding whey and soy protein isolates decreases the b^* values or that they do not change significantly [34, 37]. Abdolghafour reported that the decrease of the b^* value was caused by soy protein, which was confirmed by our results [18]. Hughes *et al.* found that adding whey protein lead to an increase in lightness (L^*) and a decrease in redness (a^*) and yellowness (b^*) [36].

The external appearance of the sausages at the end of production, as well as during the storage period, was satisfactory without any statistically significant differences between the samples (P > 0.05). The coat was not separated from the emulsion, deformed or

Table 6 b^* values of sausages with protein isolates during storage (average \pm SD)

Samples	Storage period, months					
	0	1	2	3	6	
Control	$13.93^{a,b,A,B}\pm 3.13$	$14.20^{\mathtt{a},\mathtt{b},\mathtt{A},\mathtt{B}}\pm3.47$	$15.75^{\text{a,A}}\pm4.32$	$13.34^{\mathrm{a,b,B}}\pm3.83$	$15.86^{\text{a,c,A}}\pm4.53$	
0.5% whey isolate	$12.35^{\text{a},\text{A}}\pm2.78$	$12.55^{\scriptscriptstyle a,A}\pm3.70$	$12.53^{\text{b,A}}\pm3.44$	$12.49^{\mathtt{a},\mathtt{A}}\pm2.45$	$15.55^{\text{a},\text{B}}\pm4.09$	
1.5% whey isolate	$14.39^{\text{a,b,A,B}}\pm3.86$	$12.94^{\text{a},\text{A}}\pm3.11$	$16.25^{\text{a,B}}\pm4.83$	$13.58^{\text{a,b,A}}\pm2.35$	$12.58^{\text{b,A}}\pm4.29$	
0.5% soy isolate	$14.79^{b,A} \pm 3.24$	$15.88^{\text{b},\text{A},\text{B}}\pm3.65$	$17.85^{\text{a,B}}\pm5.43$	$13.99^{\mathtt{a},\mathtt{b},\mathtt{A}}\pm4.12$	$18.00^{c,B} \pm 3.54$	
1.5% soy isolate	$14.20^{\rm a,b,A,C}\pm 3.30$	$13.93^{\text{a,b,A}}\pm3.17$	$17.09^{\mathrm{a,B}}\pm3.06$	$14.83^{\text{b,A,C}} \pm 3.96$	$16.01^{\scriptscriptstyle a,c,B,C}\pm4.23$	

a–c values in the same column with different superscripts are significantly different (P < 0.05)

A–C values in the same row with different superscripts are significantly different (P < 0.05)





Figure 1 Sensory evaluation of cross-section color, odor, flavor, and taste of sausages with protein isolates

damaged; it was slightly wrinkled and highly graded for all the samples. Similar results were obtained by Vasilev *et al.* for a functional sausage with fatty tissue [39].

After production, the cross-section of the samples had a mosaic appearance, with slightly larger particles of fat tissue, which is characteristic of this product. Inside the sausage were no visible cracks, and the components were well connected. The appearance of the section was rated very highly in all the test samples during storage. The lowest values were recorded, as expected, after 6 months of storage, from 4.36 to 4.86. Most panelists pointed out the presence of a high content of fatty tissue at the intersection of the sausage, which was confirmed by an extremely high fat content in the samples. Bratulić *et al.* made the same conclusion, having examined sausages from the Istrian region [12].

The sensory evaluation of the cross-section color, as well as odor, flavor, and taste in the analyzed sausage samples during a six-month storage period are presented in Fig. 1. The cross-section color after production was adequate, with minimal deviations. The meat pieces were red and the particles of fat tissue were whitish. The grades ranged from 4.65 to 4.77, and later, between the 1st and the 3rd months, they varied from 4.38 to 5.00. At the end of the test period, after six months of storage, the ratings were lower, ranging from 3.84 to 4.48, but they were still acceptable. The color was noticeably lighter in the samples with soy protein, although the other samples were characterized as slightly brighter than expected (pieces of meat). After six months, a greater change in color was noticeable, especially at the edges, which was more expressed in the whey samples.

Abdolghafour and Zaki cited higher grades for the samples with added whey, which declined during storage [18, 35]. According to Akesowana, adding soybeans had a positive effect on the color, while Krasnowska *et al.* did not indicate a significant difference between the samples with soy and whey compared to the control sample [13, 34].

The most obvious changes in sensory characteristics were in odor, taste, and flavor during the storage period. We found that the use of additives hardly affected the characteristic pleasant smell of fermented products after ripening and the mild smell of smoke. The grades after production ranged from 3.77 to 4.92. Adding soy proteins during this period reduced the intensity of aroma and flavor, contributing to a bland taste. Many authors reported similar observations: adding up to 3% of soy protein masked the intensity of other flavors, reduced juiciness and salinity [40]. Serdaroglu concluded that whey caused the absence of meat flavor [17]. We found changes in sensory properties during storage. The samples with 1.5% of additives had less expressed characteristics, a mismatch of aroma and taste. The whey samples had a sour odor. Krasnowska et al. cited slightly lower grades for flavor and juiciness, and better grades for taste in the samples with soy and whey proteins [13, 30, 34].

The texture of the sausages after production was satisfactory, with minor deviations (Fig. 2). Observing a sausage cut, we found that the mass was compact and that the additives had a noticeable effect on chewiness. The ratings after production were from 4.00 to 4.92. During storage, there were changes in texture, with the grades ranging from 3.92 to 4.57. The whey samples became softer and less connected, while the soy samples were harder than expected. After six months, the grades were lower, ranging from 3.45 to 4.23. The products crumbled during the cutting and also demonstrated some toughness. The samples with a higher amount of added protein attained lower grades, while the 0.5% soy protein sample had the best texture. Many authors reported a positive effect of added soy and whey on the texture, and therefore on the grades, compared to the control sample [13, 18, 34].

The overall sensory quality of all the samples was quite high during the entire test period, with no major





Figure 2 Texture and overall sensory quality of sausages with protein isolates

deviations from the maximum quality (P > 0.05). After production, the grades ranged from 4.37 to 4.79. The whey samples received high grades (4.71), almost as the control (4.77), while the soy samples rated slightly lower (4.37 and 4.44, respectively). During the storage period, there were no major changes, with the mean scores ranging from 4.35 to 4.82, and the samples with smaller amounts of additives were rated slightly better. After six months of storage, the marks were somewhat lower (3.98 to 4.32). In this period, the soy samples were given better grades, just as the samples with smaller amounts of additives We found that all the samples showed good ratings and acceptability during the entire test period.

Krasnowska *et al.* found that the products with whey and soy proteins had better sensory parameters [34]. Many authors agree that whey and soy protein supplements have a positive influence on sensory characteristics [18, 30, 40].

CONCLUSION

The results of our study showed that protein supplements possessed excellent functional properties in fermented products, including the emulsifying and binding properties. We found a significantly noticeable reduction of water activity, which is very important in the production of fermented sausages in terms of the ripening rate. Slower moisture losses during storage were observed in the samples with additives. Another effect was that on hardness: a 0.5% soy protein isolate resulted in a tougher product. We also found a minor effect on the color: the samples with the additives showed a slightly lower L^* value, while those with soy protein had higher yellowness (b^*). The use of the additives did not have a significant effect on the chemical composition and sensory properties of the product (P > 0.05) because of their low concentrations. The main characteristics of meat products were preserved despite the addition of non-meat proteins.

However, the effect of non-fat proteins on the quality of fermented sausages needs further investigation to determine the optimal concentration for obtaining high quality products.

CONTRIBUTION

Authors are equally related to the writing of the manuscript and are equally responsible for plagiarism.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interests.

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Production of peptides and amino acids from microbial biomass in food and feed industries: biotechnological aspects

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Abstract:

Introduction. Microbial biomass is a popular source of food ingredients and feed additives. Its high use has made it focus of many relevant studies. Yeast and fungal biomasses proved to be useful substrates that improve the quality and biological value of functional products. They differ in the content and composition of proteins and polysaccharides. The present research dealt with the enzymatic decomposition of proteins found in a novel fungal and yeast biomass. The research objective was to describe the peptide and amino acid composition of their enzymatic hydrolysates.

Study objects and methods. The research featured a new fungal and yeast biomass mix. Aspergillus oryzae is a mycelial fungus and a popular industrial producer of hydrolytic enzymes in food industry. As for the yeast, it was the Saccharomyces cerevisiae strain, which is often used in baking.

Results and discussion. The total content of identified amino acids in the fungal and yeast biomass was 306.0 mg/g, which was 1.5 times higher than in the fungal biomass alone. The biomass mix demonstrated a higher biological value of proteins than the yeast biomass. A set of experiments made it possible to compile a scheme for the biocatalytic destruction of polymers in the fungal and yeast biomass under the effect of fungal intracellular and endogenous enzymes. The article also contains a thorough description of the obtained enzymatic hydrolysates with various fractional compositions of peptides and free amino acids. Peptides with the molecular weight in the range of up to 29.0 kDa decreased by 2.1 times after 5 h of hydrolysis and by 10.7 times after 18 h. The designed conditions doubled the release of amino acids and increased the content of low-molecular-weight peptides up to 75.3%.

Conclusion. The research provided a new algorithm for the biocatalytic conversion of microbial biomass. Regulating the conditions of enzymatic hydrolysis made it possible to obtain enzymatic hydrolysates with a desired degree of protein degradation. They could serve as peptides and amino acids in functional food and feed products.

Keywords: Microbial biomass, yeast, biocatalytic hydrolysis, enzymes, enzymatic hydrolysates, amino acids, molecular weight, peptide fractions

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INTRODUCTION

The modern concept of healthy diet means that the range of functional foods keeps expanding to satisfy various physiological needs of the human organism. There are many ways to balance the nutritional and biological value of functional products, e.g. new formulations, specific raw materials, optimal technological processes, functional and biologically active additives, etc. [1–3].

The biotechnological processing of microbial biomass proved to be a promising direction for the production of functional food and feed ingredients [4–6]. Microbial biomass is a source of protein substances, vitamins, polysaccharides, and trace elements. Bacterial cell walls contain many valuable polysaccharides, including β -glucans, mannans, aminopolysaccharides, etc. [7–9]. Fungal biomass is known to produce chitosanglucan biologics [10–12]. Certain components

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of cell walls possess sorption, antioxidant, and other valuable properties, which makes it possible to use them in food industry [13–16]. In addition, microbial cell protoplasm contains a biologically complete protein with the amino acid score approaching that of animal protein [4, 6]. However, commercial use of microorganisms in protein and amino acid production still requires further research.

The Saccharomyces cerevisiae strain of yeast has long been focus of scientific attention. Biotechnology employs it as a substrate for protein food and feed additives. Enzyme systems can increase the bioavailability of cellular contents. They catalyze the hydrolysis of subcellular structures and release biologically valuable components, e.g. proteins [6, 17, 18]. The functional and biomedical properties of enzymatic hydrolysates depend on the degree of biocatalytic decomposition of intracellular proteins. Proteolytic enzymes owe their regulatory role to their ability to catalyze the hydrolytic degradation of the protein by certain peptide bonds. This process results in physiologically active peptides, which, in turn, can be bioregulators of certain biological processes [18-21]. The primary structure of the peptides determines their functions. Biologically active peptides (BAP) have a low molecular weight, and their amount of amino acid residues can vary from 3 to 50 [21-24].

Aspergillus fungal mycelial biomass has also been a popular subject of scientific studies. Aspergillus oryzae produces industrially significant metabolites, e.g. enzymes, organic acids, etc. [4, 10-12, 14, 25-27]. Various studies of microbial biomass as a substrate for food and feed additives revealed differences in the amount of proteins and polysaccharides. Their structure and biochemical composition also vary, which can affect the functional properties of biological products. The biomass of A. oryzae fungus contains almost twice as little protein as the S. cerevisiae yeast. However, the fungal biomass proved a valuable source of polysaccharides [25]. A fungal and yeast biomass mix can improve the quality and biological value of functional products and is a promising direction in substrate production.

Protein substances, e.g. polypeptides, low-molecularweight peptides, and amino acids, are an important component of any balanced diet. Proteins and amino acids are responsible for the formation of all tissues in a living organism. They also play a regulatory role in metabolic processes. It is the composition and amount of key amino acids that matters. This fact proves the relevance of studies aimed at obtaining various functional ingredients of food and feed products from microbial biomass as a source of biologically complete protein.

The research objective was to study the processes of enzymatic decomposition of proteins in fungal and yeast biomass. The project also focused on the effect of peptide and amino acid composition of microbial biomass enzymatic hydrolysates on the functional properties of food and feed ingredients.

STUDY OBJECTS AND METHODS

The research was performed on the premises of the Russian Research Institute of Food Biotechnology – branch of Federal Research Center of Nutrition, Biotechnology, and Food Safety (Moscow). It featured the biomass of the *Aspergillus oryzae* mycelial fungus, an industrial producer of hydrolytic enzymes for the food industry, and the *Sacharomyces cerevisiae* strain of baker's yeast.

The *A. oryzae* fungal biomass was obtained by a 10-min centrifugation at 5000 rpm. The resulting mycelial biomass was mixed with yeast in a ratio of 1:2. It served as a substrate for the biocatalytic decomposition of intracellular polymers. After centrifugation, the filtrate of the culture fluid was used to obtain a complex enzyme preparation (CEP), which served as a source of proteinases and peptidases.

The biocatalytic decomposition of the fungal and yeast biomass happened because of the autolytic processes caused by intracellular fungal enzymes. The exogenous enzymatic systems of proteolytic (CEP) and β -glucanase (Brewzyme enzyme preparation) action were introduced to increase the polymer hydrolysis.

The enzymatic activity in the enzyme systems was measured using standard methods. The mannanase activity was determined by the degree of mannan hydrolysis under certain conditions with the formation of reducing carbohydrates. The chitinase hydrolysis was assessed according to the chitin hydrolysis. State Standard R 53974-2010^I was used to evaluate the general proteolytic activity, while State Standard R 53973-2010^{II} served to measure the β -glucanase activity.

We determined the hydrolysis of the fungal and yeast biomass mix according to the concentration of reducing substances, amine nitrogen, and amino acids during enzyme hydrolysis. The anthrone method made it possible to measure the concentration of reducing substances, while the copper method helped to define the concentration of amine nitrogen [28]^{III}.

We used high-pressure exclusion chromatography to assess the mass distribution of peptide molecules in the enzymatic hydrolysates. The superose 12 column $(1.0 \times 30 \text{ cm})$ was calibrated with standard globular water-soluble proteins provided by SERVA (Germa-

¹ State Standard 53974-2010. Enzyme preparations for food industry. Method for determination of proteolitic activity. Moscow: Standartinform; 2011. 16 p.

^{II} State Standard R 53973-2010. Enzyme preparations for food industry. Method for determination of β -glucanase activity. Moscow: Standartinform; 2011. 12 p.

^{III} OFS.1.2.3.0022.15 Opredelenie aminnogo azota metodami formol'nogo i yodometricheskogo titrovaniya [General Pharmacopoeia Article No. 1.2.3.0022.15 Determination of amine nitrogen by formol and iodometric titration].

ny) [29]. 0.2 M sodium chloride served as an eluent at elution rate = 0.4 cm³/min and a wavelength λ = 280 nm using a UV132 flow-through ultraviolet detector and a Multichrom 3.1 data processing software. The chromatograms were integrated by the gravimetric method. The range of molecular weights varied from free to full volume of the chromatographic column.

The research employed a KNAUER EUROCHROM 2000 chromatograph to measure the amino acid content in the microbial biomass and enzymatic hydrolysates. After that, the components were determined by a spectrophotometric Smartline UV Detector 2500 at a wavelength of $\lambda = 570$ nm (Germany). The aminograms were calculated by comparing the areas of the standard and the sample [30].

RESULTS AND DISCUSSION

The microbial biomass proved to vary in the amino acid composition (Table 1). The content of essential amino acids amounted to 53.13% of the total number in the fungal protein, while it was only 41.30% in the yeast protein. The level of tryptophan and methionine in the fungal protein was 2.2 times higher, leucine and tyrosine – by 1.8 times, and valine – by 1.5 times. As for the yeast protein, it appeared to contain proline; the amount of glutamic acid was by 1.4 times higer, lysine and threonine – by 1.2 times.

The yeast and fungal biomass mix had a total amino acid content of 306.0 mg/g, which was 1.5 times higher

Table 1 Amino acids in the microbial biomass mix(Aspergillus oryzae and Sacharomyces cerevisiae)

Amino acids	Amino acid content in microbial biomass						
	Yea	Yeast Funga		gal	Mi	Х	
	mg/g	%	mg/g	%	mg/g	%	
Aspartic acid	37.86	10.01	20.05	9.89	30.06	9.82	
Serine	22.15	5.86	11.04	5.44	19.25	6.29	
Threonine	18.57	4.91	8.40	4.14	15.63	5.11	
Glutamic acid	64.83	17.15	25.54	12.59	51.02	16.67	
Proline	36.05	9.54	_	_	14.06	4.60	
Glycine	17.21	4.55	10.03	4.95	14.46	4.73	
Alanine	25.71	6.80	11.00	5.42	18.37	6.00	
Valine	14.85	3.93	11.85	5.84	14.38	4.70	
Methionine	5.51	1.46	6.62	3.26	5.92	1.94	
Isoleucine	13.00	3.44	6.31	3.11	11.02	3.60	
Leucine	23.66	6.26	22.92	11.30	23.25	7.60	
Tyrosine	6.45	1.71	6.09	3.00	6.02	1.97	
Phenylalanine	14.87	3.93	8.46	4.17	12.75	4.17	
Histidine	11.66	3.08	5.47	2.70	8.21	2.68	
Lysine	27.96	7.40	12.74	6.28	23.95	7.83	
Tryptophan	25.57	6.76	30.45	15.02	28.62	9.35	
Arginine	12.16	3.22	5.83	2.87	9.03	2.95	
Total amount	378.07	100	202.80	100	306.00	100	
of amino							
acids							
Essential	156.15	41.30	107.75	53.13	135.42	44.26	
amino acids							

than that in the fungal biomass (202.8 mg/g). The yeast and fungal biomass had a slightly higher biological value of proteins, while the share of essential amino acids was 44.26% (Table 1).

We conducted a comparative analysis of the amino acid composition of the protein in the yeast and fungal biomass mix with that of the reference protein approved by the Food and Agricultural Organization (WHO). The reference protein shows to what degree a certain protein satisfies the physiological need of the body for essential amino acids [31].

The amino acid score (ACS) was calculated according to the formula:

$$ACS = A : S \times 100\%$$
(1)

where ACS - amino acid score;

A – essential amino acid content in a particular protein;

S – amino acid content in the reference protein.

The yeast and fungal biomass demonstrated a high biological value of the protein: the total content of essential amino acids was 1.2 times higher than in the reference protein. The biomass contained two limiting amino acids, namely phenylalanine and methionine. Their amino acid score was 70% and 55% of the reference protein, respectively (Fig. 1). Tryptophan, lysine, threonine, and leucine proved to have the highest amino acid score.

Therefore, the biomass fortified with essential amino acids obtained from proteins of the *S. cerevisiae* yeast strain and the *A. oryzae* fungus can be a promising substrate for the production of new biologically active peptide and amino acid additives with a wide range of functional properties.

The microbial biomass mix had a higher level of protein in the substrate, and its biological value also increased. In addition, it demonstrated a higher content of chitin-glucan and mannan polysaccharides, as well as intracellular enzymes.



Figure 1 Essential amino acids in the protein of the microbial biomass mix (*Aspergillus oryzae* and *Sacharomyces cerevisiae*) vs. reference protein

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Table 2	Enzymatic activity of en	nzyme preparations used f	or biocatalysis of th	e microbial biomass	mix (Aspergillus oryzae
and Sac	charomyces cerevisiae)				

Source of enzymes		Enzyme activity, unit/g (cm ³)						
	Protease	β-glucanase	Mannanase	Chitinase				
Fungal biomass	5.10	1.44	0.12	0.02				
Brewzyme BGX enzyme	0	600.00	78.00	0.76				
Complex enzyme preparation (CEP)	450.00	113.00	48.00	1.98				

The biocatalytic conversion made it possible to increase the bioavailability of polymers in the microbial biomass mix and to obtain easily digestible peptides and amino acids. The biocatalytic conversion included three stages (Fig. 1). Stage I featured the fungal biomass, which contained residual proteolytic and β -glucanase enzymes (Table 2). The autolytic decomposition of the microbial biomass polymers lasted 2 h at 50°C.

Enzymatic hydrolysate I of the biomass mix appeared after 2 h of autolysis under the effect of fungal intracellular enzymes.

The Brewzyme BGX enzyme preparation is known as a source of β -glucanases and other hydrolases (Table 2). The Brewzyme enzyme made it possible to increase the decomposition rate of cell walls during Stage II. Mannans and β -glucans, as well as protein-



Figure 2 Biocatalytic conversion of the microbial biomass mix (Aspergillus oryzae and Sacharomyces cerevisiae)

mannan and chitin-glucan complexes, were the main structural polymers [8, 9]. The proportion was 50 units of β -glucanase per 1 g of biomass dry matters. Stage II lasted 3 h at 40°C and produced enzymatic hydrolysate II after 5 h of hydrolysis (Fig. 2).

Complex enzyme preparation CEP was introduced during Stage III. It provided a deeper enzymatic hydrolysis of the main subcellular polymers of the microbial biomass, including protein substances. The hydrolysis resulted in the formation of easily digestible biologically active products. The CEP served as a source of a complex of proteinases and peptidases. The proportion was 20 units of protease per 1 g of biomass solids (Fig. 2). Fungal proteolytic enzymes are thermolabile, so the temperature was reduced to 30°C. Stage III lasted 13 h; the total biocatalysis time was 18 h. Stage III produced enzymatic hydrolysate III.

The enzyme system of the *A. oryzae* fungus and exogenous enzymes made it possible to obtain enzymatic hydrolysates from the yeast and fungal microbial biomass mix. The enzymatic hydrolysates varied in the degree of decomposition of intracellular polymers (Fig. 2).

The most intense formation of hydrolysis products of protein and carbohydrate polymers took place during the first 5 h. After 5 and 14 h, the concentration of soluble reducing carbohydrates increased by 9.3 and 12.1 times (from 2.1% to 25.5%), respectively. The concentration of amine nitrogen (NH₂⁺) increased by 6.4 times and 9.6 times (from 0.5% to 4.8%). The concentration of free amino acids increased by 8.0 times and 12.2 times, from 1.3% to 15.9% (Fig. 3).



Figure 3 Biochemical parameters of the enzymatic hydrolysates during hydrolysis of the microbial biomass mix (*Aspergillus oryzae* and *Sacharomyces cerevisiae*)

Table 3 illustrates the composition of the free amino acids in the obtained enzymatic hydrolysates and their amount. 26.4% of free amino acids were released during the hydrolysis of the microbial biomass mix by intracellular fungal enzymes (enzymatic hydrolysate I). After exogenous enzymes (β -glucanase and proteolytic effects) were introduced and the process time was prolonged, the release of amino acids increased by 1.5–2.0 times. It reached 38.1% in enzymatic hydrolysate II and 49.7% in enzymatic hydrolysate III. The content of free essential amino acids also increased (Table 3). The amount of essential free amino acids increased

Table 3 Composition of free amino acids in enzymatic hydrolysates of the microbial biomass mix (*Aspergillus oryzae* and *Sacharomyces cerevisiae*)

Amino acids		Amino acid content, mg/g	
	Enzymatic hydrolysate I	Enzymatic hydrolysate II	Enzymatic hydrolysate III
Aspartic acid	3.567	5.097	12.866
Serine	4.246	5.560	9.824
Threonine	6.960	10.476	10.968
Glutamic acid	11.233	14.442	14.487
Proline	2.611	3.439	7.810
Glycine	1.712	2.850	7.264
Alanine	6.304	7.517	9.130
Valine	5.125	6.540	8.509
Methionine	1.088	2.009	2.570
Isoleucine	4.189	5.744	6.558
Leucine	5.916	8.404	11.549
Tyrosine	3.040	4.627	5.497
Phenylalanine	3.575	5.346	6.648
Histidine	3.696	10.053	10.803
Lysine	6.111	9.240	9.527
Tryptophan	6.435	8.044	11.295
Arginine	4.829	7.249	7.714
Total amount of amino acids, where	80.637	116.637	152.019
essential amino acids	39.398	55.803	67.624
Content of free amino acids, % of total	26.4	38.1	49.7



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Figure 4 Molecular weight distribution of bioconversion products of protein polymers in the enzymatic hydrolysates of the microbial biomass mix (*Aspergillus oryzae* and *Sacharomyces cerevisiae*)

by 1.7 times in enzymatic hydrolysate III, rising from 39.398 mg/g to 67.624 mg/g, compared to enzymatic hydrolysate I.

Thus, controlling the enzymatic hydrolysis of the combined microbial biomass allowed us to obtain enzymatic hydrolysates with the desired degree of decomposition of microbial cell proteins.

We also measured the effect of process time on the molecular weight of the peptide fractions in the enzymatic hydrolysates of the biomass mix. Average yeast proteins consist of 466 amino acid residues and have a molecular weight of 53 kDa. Fungal proteases reduced the molecular weight of proteins after 2 h of autolysis (Fig. 4).

The molecular weight distribution of protein fractions during the hydrolysis of the biomass mix



Figure 5 Molecular weight distribution of peptides during the enzymatic hydrolysis of the microbial biomass mix (*Aspergillus oryzae* and *Sacharomyces cerevisiae*)

confirmed the effectiveness of the decomposition processes that produced lower-molecular-weight peptides (Figs. 4, 5). A longer enzymatic hydrolysis lowered the content of high-molecular-weight peptides and increased the number of low-molecular-weight peptides.

In enzymatic hydrolysate I, peptides in the range over 4.1 kDa accounted for 38.2% of the total amount of protein substances. Peptides in the range from 4.1 to 1.6 kDa constituted 26.7%, while those under 1.6 kDa made up 35.1%. The content of highmolecular-weight peptides decreased significantly during the hydrolysis of protein polymers. After 5 h of hydrolysis, the amount of peptides over 29.0 kDa fell by 2.1 times, after 18 h – by 10.7 times (Fig. 5). In enzymatic hydrolysate II, the fraction of low-molecular-weight peptides reached 45.4%. As for enzymatic hydrolysate III, the content of low-molecular-weight peptides in the range up to 4.1 kDa was 75.3%, while the share of those under 1.6 kDa accounted for 52.4%.

CONCLUSION

The present research revealed the composition of peptides and amino acids in the enzymatic hydrolysates of a new biomass mix that combined the *Saccharomyces cerevisiae* yeast strain and the *Aspergillus oryzae* fungus. A set of experiments confirmed that the enzymatic hydrolysates could be used to fortify food and feed products.

The new biomass mix demonstrated a higher content of proteins and essential amino acids, as well as other valuable components. The yeast increased the amount of proteins, while the fungus raised the content of essential amino acids. The fungus also increased the amount of intracellular enzymes, which are used during enzymolisis. As a result of the mutual fortification, the total amino acid content increased by 1.5 times due to the higher protein content in the yeast. In addition, the biological value of the proteins in the new biomass mix proved to be higher than that in the traditional yeast biomass. This fact means that the ingredients obtained from the biomass mix could contribute to a wider range of functional properties.

The comparative analysis showed the high biological value of the protein in the yeast and fungal biomass mix. The total content of essential amino acids was 1.2 times higher than in the reference protein. The biomass appeared to contain two limiting amino acids - phenylalanine and methionine. Their amino acid score accounted for 70% and 55% of their content in the reference protein, respectively. Tryptophan, lysine, threonine, and leucine demonstrated the highest score. A significant amount of tryptophan, typical for fungal biomass, might add extra functional properties to ingredients obtained from their peptides and amino acids. Tryptophan is known as an immunologically active amino acid. It is a dipeptide with a wide range of immunomodulatory effects [32, 33]. Tryptophancontaining drugs have an antidepressant effect and stimulate the production of vitamin B₃ (niacin). In addition, tryptophan hydroxylation produces serotonin, an important brain neurotransmitter [34].

The biomass mix fortified with essential amino acids of proteins obtained from the *S. cerevisiae* yeast strain and the *A. oryzae* fungus could be used as a commercial substrate. It was found capable of facilitating the production of new biologically active peptide and amino acid additives with a wide range of functional properties.

We developed a new algorithm for biocatalytic polymer conversion in the new microbial biomass mix. The algorithm made it possible to obtain easily digestible peptide and amino acid ingredients using fungal intracellular enzymes, as well as β-glucanase and proteolytic enzymatic preparations. The conditions of enzymatic hydrolysis proved to affect the fractional composition of the enzymatic hydrolysates. A fivehour hydrolysis lowered the amount of peptides in the range over 29.0 kDa by 2.1 times, and 18-h hydrolysis by 10.7 times. Intracellular proteinases and peptidases are known to catalyze the decomposition of proteins. As a result, the enzymatic system with proteinases and peptidases could provide food and feed ingredients that contained 75.3% of low-molecular-weight peptides and up to 50% of free amino acids that are responsible for biologically active factors with functional properties.

The low-molecular-weight peptides, free amino acids, and essential amino acids are involved into various biological processes. They improved the digestibility of the enzymatic hydrolysates obtained from the microbial biomass mix, which can be used as peptide and amino acid components of functional food and feed products.

CONTRIBUTION

Authors are equally related to the writing of the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests related to the publication of this article.

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Synergistic effects of *Lactobacillus plantarum* and *Staphylococcus carnosus* on animal food components

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Abstract:

Introduction. Various cultures of microorganisms have recently been used to accelerate technological processes. In this regard, it appears highly relevant to study the action of beneficial microorganisms on the components of food systems.

Study objects and methods. The study objects included a model mixture of beef muscle and pork fat tissue with 2% salt, as well as a model protein. *Lactobacillus plantarum* and *Staphylococcus carnosus* were used in an amount of 1×10^7 CFU/g of raw material. The compositions of free amino and fatty acids, carbohydrates, and other components were analyzed by liquid and gas chromatography with mass-selective detection.

Results and discussion. We studied the effect of *L. plantarum* and *S. carnosus* on protein, lipid, and carbohydrate components of food systems based on animal raw materials. We found that the combined effect of the cultures was by 25% as effective as their individual use at 4×10^9 CFU/kg of raw material. The three-week hydrolysis of proteins to free amino acids was almost a third more effective than when the cultures were used separately. The synergistic effect of *L. plantarum* and *S. carnosus* on fat components was not detected reliably. Free monosaccharides formed more intensively when the cultures were used together. In particular, the amount of free lactose almost doubled, compared to the cultures' individual action.

Conclusion. We described culture-caused quantitative changes in the main components of animal-based food systems: amino acids, fatty acids, carbohydrates, and basic organic compounds. Also, we identified substances that can affect the taste and aroma of final products when the cultures are used together or separately. These results make it possible to obtain products with a wide variety of sensory properties.

Keywords: Sensory properties, Lactobacillus plantarum, Staphylococcus carnosus, food systems, meat products, microorganisms

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INTRODUCTION

Microorganisms have long been widely used in the food industry: in winemaking and beverage production, dairy production, silage making and fermentation of plant materials, as well as in seafood fermentation [1–17].

Products of animal origin make up a significant part of total food consumption. Like any products, they undergo biochemical transformations during production and are exposed to microorganisms during long-term storage [18–19].

Literature describes various methods for producing foods under the influence of microorganisms contained in animal raw materials. One of such methods – aging - is mainly used to produce dry-cured products. This process involves curing raw materials and semi-products under certain conditions to expose them to a gradual, and sometimes fairly long, effect of the microorganisms' enzyme systems. As a result, the main food components transform and develop certain flavoring characteristics [20–22].

Today, formulators forcibly introduce starter cultures of microorganisms into raw materials to reduce process time. In particular, for food systems based on animal raw materials, they use *Staphylococcus* (*St. xylosus*, *St. carnosus*), *Lactobacillus* (*L. pentosus*, *L. plantarum*, *L. sakei*, *L. curvatus*), and *Pediococcus* (*P. pentosaceus*, *P. acidilactici*) [23–26]. Microorganisms can

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produce different effects and form a wide variety of flavors [27–28].

For convenience, microorganisms are commonly used as lyophilized solids, with culture cells deposited on the surface of a solid carrier, usually sugars. For products of animal origin, 1-2 g of a freeze-dried culture containing $(1-10)\times10^{12}$ CFU/100 g sucrose is usually added per every 5 or 10 kg of raw material [21, 29].

Although the action of microbiological cultures on various food materials is fully described, scientific literature lacks systematic data on the quantitative changes in the most important minor components of animal materials (individual amino acids, fatty acids, and monosugars) under the influence of *L. plantarum* and *S. carnosus*.

We aimed to identify the effects of microorganism cultures on protein, lipid, and carbohydrate components of animal-based food systems.

STUDY OBJECTS AND METHODS

Our objects of the study included a model food system – a mixture of beef muscle tissue *Longissimus dorsi* and pork fat tissue *Telae adipem* with 2% sodium chloride (75:25%) homogenized in a Buchi Mixer B400 blender (Switzerland), as well as a model protein. The model food system contained 18.5% protein, 23% fat, 2.5% carbohydrates, and 54% moisture.

The model protein was obtained by a 6 h extraction at 25°C of *Longissimus dorsi* of *Bubulae* beef with a 5% sodium chloride solution followed by desalting on G25 and freeze-drying [30]. The isolated protein was 93% pure and had a 6% moisture.

Lactobacillus plantarum ATCC 8014 (LP) and *Staphylococcus carnosus* ATCC 51365 (SC) were added in an amount of 1×10^7 CFU/g of raw material. We used preparations of culture on freeze-dried sucrose in an amount of 2×10^{10} CFU/g.

The model mixture was treated as follows. First, animal raw materials were kept in salt for 24 h at $2 \pm 2^{\circ}$ C. Then, we introduced starter cultures and packed the mixture in plastic bags to keep in the chamber for 5 days at 2–4°C, relative humidity (W) 85%, and an air flow speed of 0.1 m/s. Further treatment was carried out during 5 days (15°C, W 82%) and 10 days (12°C, W 75%). The control sample was kept in salt for 24 h at $2 \pm 2^{\circ}$ C.

The model protein was treated with starter cultures in a 2% sodium chloride solution (hydromodule 1:5) under similar conditions at pH 7.0.

To measure the proteolytic activity, we placed a 1% casein solution in 0.05 M Tris-PO4 buffer (pH 7.0) into two tubes (5 mL in each) and added 10 mL of distilled water to the first tube and 1 mL of a 1×10^{10} CFU/mL enzyme solution or 1 mL of the test solution to the second tube. After a 10-min exposure at 37°C, we added 5 mL of a 10% trichloroacetic acid solution to the test samples, filtered them through a 0.45 µm filter, and measured the optical densities of the transparent solutions against the control at 280 nm. The proteolytic activity (units/mg) was calculated as $A = (D_{280} \text{ sample} - D_{280} \text{ control})/10 \cdot \text{g}$, where g is the nominal enzyme concentration in the test sample. The standard unit of peptidase activity is the amount of enzyme required to release free amino acids during proteolytic decomposition. It is equivalent to a change in the absorption rate of the test solution (0.001D₂₈₀) per minute at 37°C and pH 7.0 [31].

The materials were treated with *L. plantarum* and *S. carnosus* in a 1:5 ratio: 1 g of the enzyme preparation per 5 kg of the formulation and 1 mg of the preparation per 5 g of animal protein.

The content of amino acids was determined on a Biotronic 6001 amino acid analyzer (Germany) by distribution chromatography after acid hydrolysis of proteins [31].

Free amino acids were determined after protein precipitation by adding 10% trichloroacetic acid, followed by neutralization with a 10 M sodium hydroxide solution to pH 2.0 and filtration through a Millipore membrane filter with a pore diameter of 0.22 μ m. Then, the filtrate was diluted in a buffer solution (pH 2.2). To quantify individual amino acids, we compared the peak areas in the aminogram obtained with the Winpeak Eppendorf-Biotronic integration system (Germany) by analyzing a standard mixture of amino acids that contains 2.5 μ mol of each amino acid in 1 mL of the solution [31].

Fatty acids and chemical components responsible for the product's taste and aroma were determined by chromatography-mass spectrometry [21, 31].

The components were analyzed on a 7890A gas chromatograph with a 5975C VLMSD mass selective detector (Agilent Technologies, USA) using a modified Folch method. In particular, a 1 g sample was subjected to a mixture of 10 mL chloroform and 10 mL methanol in the presence of a 1% KCl solution for 24 h to dissolve the lipid components. The extract was filtered through paper. After removing the excess solvents by evaporation to dryness, the residue was subjected to acid hydrolysis to obtain methyl esters of fatty acids, which were analyzed by gas chromatography.

A 0.01 g amount of lipids was treated with 3 mL of a 15% solution of acetyl chloride in methanol at 100°C for 2 h. Then, the mixture was neutralized by KOH (1.25 mL) in CH₃OH to pH 5.0–6.0. A few minutes after adding 3 mL of a saturated aqueous NaCl solution and 3 mL of hexane, we took for analysis 0.2 μ L from a clear hexane layer containing methyl esters of fatty acids. Chromatography was performed on a 30 m×0.32 mm×0.5 μ m HP-Innowax capillary column under the following conditions: the column temperature in the thermostat increasing from 100°C to 260°C at a rate of 10°C/min; injector temperature 250°C, detector temperature 300°C; hydrogen flow from the generator at 35 cm³/min; nitrogen flow at 20 cm³/min; flow division 1:100; analysis time 30 min; injection of 1 μ L of the sample. A NIST08 MS Library was used to measure the content of isomers, an automatic search and identification program for gas chromatography-mass spectrometry with a probability of peaks correlation above 65%.

The content of free fatty acids was determined by an acid-base titration of the samples according to the acid number. In 2 mg KOH/g of the product, it corresponded to 1% of the mass content of free fatty acids [31].

The composition of free carbohydrates was analyzed using a BioLC chromatographic system, including a GS50 gradient pump, an ED50 electrochemical detector, an EG50 eluent generator with 10 mN NaOH, and an LC25 chromatographic thermostat with a CarboPac PA20 column (Dionex, Germany). The content of free carbohydrates was determined in aqueous extracts of 0.01 g of the sample in 100 mL of demineralized HPLC-grade water filtered through a 0.45 μ m filter at 25°C.

The water retention capacity was determined by a standard method, recording bound moisture under load [31].

Our study used casein, tris (hydroxymethyl) aminomethane (tris), phosphoric acid, sodium chloride, sodium hydroxide, potassium hydroxide, and Sephadex G-25 (Sigma, USA). As standards of amino acids, we used a solution of mixed individual amino acids in a molar concentration of 2.5 μ mol/mL (Supelco, USA): glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tyrosine, methionine, cysteine, aspartic acid, glutamic acids, lysine, arginine, histidine, serine, and threonine.

As standards of fatty acids, we used a solution of mixed C6-C24 fatty acid methyl esters in methylene chloride with a mass concentration of 10 mg/mL (Supelco, USA): caproic (C6:0), octanoic (C8:0), decanoic (C10:0), decenoic (C10:1), undecanoic (C11:0), dodecanoic (C12:0), tridecanoic (C13:0), tetradecanoic (C14:0), cis-9-tetradecenoic (C14:1), pentadecanoic (C15:0), cis-10-pentadecenoic (C15:1), hexadecanoic cis-9-hexadecenoic (C16:0), (C16:1), heptadecanoic (C17:0), cis-10-heptadecenoic (C17:1), octadecanoic (C18:0), cis-9-octadecenoic (C18:1n9c), trans-9octadecenoic (C18:1n9t), cis-9,12-octadecadienoic (C18:2n6), *cis*-6,9,12-octadecatrienoic (C18:3n6), cis-(C18:3n3), 9,12,15-octadecatrienoic nonadecanoic (C19:0), eicosanoic (C20:0), cis-9-eicosenoic (C20:1n9), cis-11,14,17-eicosatrienoic (C20:3n3), cis-8,11,14eicosatrienoic (C20:3n6), cis-11,14,17-eicosatrienoic *cis*-5,8,11,14-eicosatetraenoic (C20:4n6), (C20:3n3),eicosapentaenoic (C20:5n3), heneicosanoic (C21:0), docosanoic (C22: 0), cis-13-docosenoic (C22:1n9), *cis*-13,16-docosadienoic (C22:2n6), *cis*-7,10,13,16,19docosapentaenoic (C22:5n3), cis-4,7,10,13,16,19docosahexaenoic (C22:6n3), tricosanoic (C23:0),tetracosanoic (C24:0), and cis-15-tetracosenoic (C24:1).

As carbohydrate standards, we used arabinose (Ara, $C_5H_{10}O_5$, D-(–)-arabinose $\geq 99\%$, A3131 Sigma), galactose (Gal, $C_6H_{12}O_6$, D-(+)-galactose $\geq 99\%$, G0750 Sigma-Aldrich), glucose (Glc, $C_6H_{12}O_6$, D-(+)-glucose $\geq 99.5\%$, G8270 Sigma), xylose (Xyl), mannose (Man, $C_6H_{12}O_6$, D-(+)-mannose from wood, $\geq 99\%$ M2069 Sigma), fructose (Fru, $C_6H_{12}O_6$, D-(–)-fructose $\geq 99\%$, F0127 Sigma), sucrose (Sug, $C_{12}H_{22}O_{11}$, α -D-glucose- $(1\rightarrow 2)$ - β -D-fructose, sucrose $\geq 99.5\%$ S9378 Sigma), ribose ($C_5H_{10}O_5$, D-(–)-ribose $\geq 99\%$ R7500 Sigma), lactose (Lac, $C_{12}H_{22}O_{11}$ ·H₂O, β -D-galactose- $(1\rightarrow 4)$ - α -D-glucose, α -Lactose monohydrate reagent grade L3625 Sigma-Aldrich), aqueous solutions in a concentration of 0.001 mg/mL.

RESULTS AND DISCUSSION

Animal-based products have a protein content of 10 to 25% [18, 29]. Fresh raw materials of animal origin usually contain from 0.001 to 0.01% of free amino acids, and their content increases with prolonged storage due to internal enzyme systems. We determined the amino acid composition of the model protein and the meat system protein before and after introducing Lactobacillus plantarum and Staphylococcus carnosus (Table 1). We found that the starter cultures significantly increased the total content of free amino acids both in the model protein and in the formulation. In the formulation, their content increased to $2.0 \pm 0.1\%$, $2.2 \pm 0.1\%$, and $2.8 \pm 0.1\%$ after using Lactobacillus plantarum, Staphylococcus carnosus, and an equimolar mixture of Lactobacillus plantarum and Staphylococcus carnosus, respectively.

With the same total concentration of introduced cultures at 4×10^9 CFU/kg, the mixture of *Lactobacillus plantarum* and *Staphylococcus carnosus* increased the rate of protein hydrolysis to free amino acids by 30–40% (P > 0.96), compared to their separate action.

To determine the treatment time, we observed changes in the release of free amino acids and the correlating values of water retention (Fig. 1). We found



Figure 1 Content of free amino acids (curves 1, 4) and water retention (curves 2, 3) vs. treatment time with *Lactobacillus plantarum* and *Staphylococcus carnosus* (1) and without them (4)

Table 1	Amino acid co	mposition of	f model and	food system	proteins and	the content	of free amino	acids after treat	ment with
Lactoba	icillus plantaru	m and Staph	ylococcus c	arnosus					

Name	Model	Food system proteins			Model pro	otein	Food system proteins		
of amino acid	protein	g/100g product	g/100g protein	LP	SC	LP+SC	LP	SC	LP+SC
Asparagine	7.98	1.43	7.24	0.14	0.04	0.17	0.19	0.17	0.16
Glutamine	16.4	2.95	15.9	0.81	0.31	0.54	0.87	0.73	0.56
Serine	1.59	0.28	1.52	0.01	0.01	0.10	0.03	0.13	0.13
Histidine	3.46	0.62	3.38	0.08	0.01	0.08	0.08	0.08	0.14
Glycine	2.24	0.40	2.19	0.08	0.01	0.12	0.11	0.13	0.11
Threonine	5.91	1.06	5.94	0.04	0.02	0.06	0.03	0.07	0.09
Arginine	7.80	1.40	7.77	0.10	0.05	0.13	0.12	0.13	0.12
Alanine	3.63	0.65	3.54	0.18	0.04	0.13	0.16	0.15	0.15
Tyrosine	3.98	0.71	3.76	0.04	0.01	0.09	0.05	0.07	0.12
Cysteine	1.18	0.21	1.15	0.02	0.01	0.04	0.01	0.02	0.04
Valine	5.56	1.00	5.48	0.07	0.01	0.08	0.07	0.06	0.09
Methionine	3.35	0.60	3.41	0.02	0.02	0.04	0.01	0.02	0.05
Phenylalanine	4.55	0.81	4.53	0.01	0.01	0.12	0.02	0.04	0.09
Isoleucine	4.93	0.88	4.91	0.01	0.02	0.14	0.02	0.08	0.13
Leucine	8.57	1.54	8.49	0.03	0.04	0.12	0.03	0.07	0.13
Lysine	10.6	1.91	10.5	0.22	0.17	0.58	0.16	0.17	0.54
Proline	3.10	0.55	3.06	0.03	0.03	0.08	0.03	0.06	0.12
Tryptophan	1.32	0.23	1.31	0.04	0.01	0.03	0.01	0.02	0.03
Σ	96.2	17.23	94.1	1.93	0.82	2.65	2.00	2.2	2.8

LP – Lactobacillus plantarum; SC – Staphylococcus carnosus

that the optimal treatment time was three weeks. This time ensured optimal quality parameters of the product, including water retention (Fig. 1).

According to Fig. 1, a joint use of the bacterial cultures ensured the maximum concentration of free amino acids in two weeks. In addition, the water retention capacity of the food system – an important technological indicator of product quality – was almost twice as high as when the process lasted a month without using bacterial cultures.

The proteolytic activity with respect to the model and food system proteins showed a synergistic effect of *L. plantarum* and *S. carnosus* on the protein components (Table 2). Their joint use increased the efficiency of hydrolytic decomposition of the system proteins leading to a release of free amino acids (Table 1).

Table 3 shows the composition of fatty acids in the animal-based food system and changes in their contents under the influence of *L. plantarum* and *S. carnosus*. At the initial stages, we found a decrease in the contents of lower C6–C10 and unsaturated fatty acids, especially essential omega-3 acids (α -octadecatrienoic

Table 2 Proteolytic activity of Lactobacillus plantarum

 and Staphylococcus carnosus in relation to proteins of animalbased food systems, units/mg

Name	LP	SC	LP+SC
Model protein	12	14	17
Food system protein	6	4	8

LP - Lactobacillus plantarum; SC - Staphylococcus carnosus

C18:3, eicosapentaenoic C20:5, and docosapentaenoic C22:5). However, there was an increase in *cis*-11,14,17eicosatrienoic C20:3 acid that is important for proper nutrition of mammals. Three weeks of treating the food system with *L. plantarum* and *S. carnosus* led to a decrease in unsaturated fatty acids and an increase in saturated acids by 1–5% (P > 0.95). Similar changes in contents of saturated and unsaturated fatty acids are usually observed for animal-based products subjected to long-term storage at low temperatures [22, 23].

We did not evaluate the direct lipolytic activity of *L. plantarum* and *S. carnosus* in the presence of synthetic substrates commonly used for this purpose. However, Table 3 shows that a combined action of the cultures on the system led to a more efficient breakdown of animal fats and a release of free fatty acids, compared to their individual action. Yet, this effect was not expressed clearly.

Thus, the action of *L. plantarum* and *S. carnosus* on the fat components of the food system not only transformed the fatty acid composition, but also, to a much greater extent, increased the amount of free fatty acids.

Figure 2 shows a kinetic curve of changes in the content of free fatty acids as a result of treatment with *L. plantarum* and *S. carnosus*. Longer treatment time led to a higher mass fraction of free fatty acids in all cases. We found no differences in the kinetics of free fatty acids formation under individual or joint action of the cultures, apparently due to their comparable lipolytic activity.

Table 3 Changes in the fatty acid composition of the animal-based food system treated with Lactobacillus plantarum,

 Staphylococcus carnosus and their mixture, % of total

Name of fatty acid	tty acid Initial 3 weeks		eks	4 weeks without	
	mixture	LP	SC	LP+SC	cultures
Caproic C6:0	0.06	0.05	nd	-	-
Octanoic C8:0	0.17	_	-	0.04	-
Decanoic C10:0	1.05	1.24	0.48	0.88	0.65
Decenoic C10:1	0.14	0.12	0.08	0.07	0.03
Undecanoic C11:0	0.12	0.06	0.06	_	0.02
Dodecanoic C12:0	0.75	0.79	1.28	1.14	1.23
Tridecanoic C13:0	_	-	0.07	_	0.08
Tetradecanoic C14:0	2.61	2.18	2.16	2.2	2.34
Cis-9-tetradecenoic C14:1	0.52	0.21	0.18	0.16	0.15
Pentadecanoic C15:0	_	-	0.12	_	0.12
Cis-10-pentadecenoic C15:1	0.29	0.22	0.17	0.16	0.14
Hexadecanoic C16:0	19.3	21.	22.8	22.6	23.1
Cis-9-hexadecenoic C16:1	4.23	2.24	2.55	2.45	3.18
Heptadecanoic C17:0	0.51	-	0.16	0.15	0.33
Cis-10-heptadecenoic C17:1	0.18	0.14	0.1	0.12	0.24
Octadecanoic C18:0	21.6	24.1	22.4	23.9	23.6
Cis-9-octadecenoic C18:1n9c	18.8	19.8	19.2	19.1	18.7
Trans-9-octadecenoic C18:1n9t	_	-	0.01	_	0.02
Cis-9,12-octadecadienoic C18:2n6	4.56	3.62	4.23	3.56	3.88
Cis-6,9,12-octadecatrienoic C18:3n6	3.44	3.02	3.17	2.34	2.51
Cis-9,12,15-octadecatrienoic C18:3n3	0.55	0.44	0.61	0.23	0.43
Nonadecanoic C19:0	0.07	0.16	0.41	0.28	0.25
Eicosanoic C20:0	0.35	0.48	0.49	0.61	0.56
Cis-9-eicosenoic C20:1n9	0.45	0.43	0.47	0.45	0.38
Cis-11,14-eicosadienoic C20:2n6	3.0	4.45	4.13	4.65	3.62
Cis-8,11,14-eicosatrienoic C20:3n6	2.05	3.0	3.23	3.18	3.17
Cis-11,14,17-eicosatrienoic C20:3n3	0.41	0.5	0.38	0.44	0.39
Cis-5,8,11,14-eicosatetraenoic C20:4n6	0.45	-	0.32	0.11	0.24
Cis-5,8,11,14,17-eicosapentaenoic C20:5n3	0.06	0.02	0.12	0.13	0.07
Heneicosanoic C21:0	0.11	0.11	0.06	_	0.15
Docosanoic C22:0	0.12	-	0.13	0.12	0.16
Cis-13-docosenoic C22:1n9	0.11	0.25	0.2	0.11	0.11
Cis-13,16-docosadienoic C22:2 n6	0.46	0.19	0.11	_	_
Cis-7,10,13,16,19-docosapentaenoic C22:5n3	0.05	0.03	-	0.04	0.01
Cis-4,7,10,13,16,19-docosahexaenoic C22:6n3	0.04	0.02	-	_	0.03
Tricosanoic C23:0	0.1	0.2	0.16	0.15	0.15
Tetracosanoic C24:0	0.21	-	0.12	_	0.18
Cis-15-tetracosenoic C24:1	_	0.23	0.06	_	_
Unidentified fatty acids	13.1	10.7	9.8	10.6	9.7
Mass fraction of free fatty acids	0.01	0.2	0.2	0.3	0.3

nd - not detected

The synergistic effect of L. *plantarum* and S. *carnosus* on the fat components of the animalbased food system did not manifest reliably, since the amounts of free fatty acids in the final products were approximately the same.

Thus, the action of *L. plantarum* and *S. carnosus* resulted in not only the hydrolytic decomposition of fat components, but also in their biochemical

transformation into ultimate chemical structures. The increased content of saturated acids found in our study should be considered critically, in light of current trends in the production of foods with an increased amount of unsaturated, especially polyunsaturated, fatty acids of the omega-3 family. This problem should be taken into account in further development of methods for producing animal-based foods.



Figure 2 Changes in the acid number of the animal-based food system caused by treatment with *Lactobacillus plantarum* and *Staphylococcus carnosus* (1), *Staphylococcus carnosus* (2) and without starter cultures (3)

Table 4 shows the quantitative identification of free carbohydrates in the food system treated with *L. plantarum* and *S. carnosus*. As we can see, the content of some sugars (galactose, glucose, fructose) decreased, while the content of others increased. It appears that the decrease was caused by the consumption of those sugars by the cultures themselves, whereas the increase was associated with proteolytic and carbohydrolase activity, leading to the breakdown of animal polysaccharides. The mass fraction of such polysaccharides in animal raw materials is 2-3%, but their decomposition can lead to the formation of 0.1-100 mg% free carbohydrates [18, 23].

According to Table 4, free monosaccharides formed most intensively under the joint action of *L. plantarum* and *S. carnosus*. It manifested through changes in the content of galactose, glucose, xylose, and ribose and through a higher rate of disaccharides (lactose and sucrose) formation in the food system. In fact, the amount of free lactose was almost twice as high as when the cultures were used individually. As a result, the product acquired a sweetish taste. Thus, *L. plantarum* and *S. carnosus* produced a synergistic effect on the changes in carbohydrate components of the animalbased food system. Further, treating food systems with microcultures totally changes the chemical composition of organic substances in raw materials and intermediates or those substances formed as a result. Table 5 shows changes in some minor components of the food system in the presence of *L. plantarum* and *S. carnosus*. The mass spectrometric analysis made it possible to identify over 250 organic compounds which could be considered as a result of biochemical effects that microorganisms had on protein, lipid, and carbohydrate compounds were derivatives of fatty acids. Table 5 lists the main substances identified, whose content exceeded 0.001%.

As can be seen in Table 5, the joint use of *L. plantarum* and *S. carnosus* resulted in more pronounced changes in almost all compounds, compared to their individual action. To some extent, this result indicated a synergistic mechanism of action of both cultures used to treat animal raw materials.

Given that all identified substances can affect the taste and aroma of final products, varying the use of starter cultures – both individual and joint – can make it possible to obtain products with a wide range of consumer properties [21].

Finally, the biochemical transformations of cholesterol require our special attention (Table 5). This substance is a significant component of food systems based on animal raw materials. Its high content in products is considered as an unfavorable factor leading to the development of atherosclerosis. In our case, the combined action of the cultures led to a more considerable degradation of cholesterol, which is an important advantage of using these cultures together.

CONCLUSION

Thus, the joint use of starter cultures, *Lactobacillus plantarum* and *Staphylococcus carnosus*, to treat animal-based food systems not only increased the yield of the product, but also had a synergistic effect on the protein, lipid, and carbohydrate components of the system. This may allow us to change the component composition of the system and form the desired characteristics of the food product.

Table 4 Changes in free carbohydrate contents in the animal-based food system caused by *Lactobacillus plantarum*,

 Staphylococcus carnosus and their mixture, % (g/100 g of sample weight)

Name	Initial mixture	LP	LP+SC	SC	Without starter cultures
arabinose	0.016	0.13	0.33	0.16	0.23
galactose	1.85	0.0025	0.004	0.0018	0.0015
glucose	0.03	0.002	0.003	0.002	0.001
xylose + mannose	0.03	0.008	0.05	0.022	0.028
fructose + sucrose	0.08	0.006	0.13	0.095	0.053
ribose	0.15	0.095	0.19	0.11	0.12
lactose	0	0.08	0.18	0.085	0.12

LP - Lactobacillus plantarum; SC - Staphylococcus carnosus

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Name	CAS No.	Relative intensity of	Probability of mass	Raw	Final p	product,	mg/kg	Time,
		mass spectrum main signals, units	spectrometric peak identification from the mass spectra library, %	material, mg/kg	LP	SC	LP+SC	min
2-phenyl-4-quinolinol	001144-20-3	221(999), 193(44), 220(183), 222(171), 165(134)	66	nd	nd	0.003	0.008	4.123
2,5-diphenyl-oxazole	000092-71-7	221(999), 165(617), 166(388), 892(386), 77(351)	70	nd	nd	0.003	0.002	4.174
Cyclobarbital	000052-31-3	207(999), 141(195), 81(189), 67(132), 79(122)	66	0.002	0.003	0.009	0.013	4.237
Nonanal dimethyl acetal	018824-63-0	75(999), 157(97), 41(88), 69(86), 43(83)	78	nd	0.001	0.012	0.013	4.346
8H-dinaphtho[2,3- b:2',3'-g]carbazole	003557-50-4	367(999), 368(243), 366(168), 183(106), 184(98)	72	0.001	0.001	0.004	0.01	6.603
Decanoic acid, methyl ester **	000110-42-9	74(999), 87(585), 55(249), 43(205), 143(198)	96	0.002	0.004	nd	0.006	8.933
7-methoxy-9-(3-methyl- 2-butenyl)-furo[2,3-b] quinolin-4(9H)-one	018904-40-0	215(999), 283(222), 216(133), 284(42), 172(37)	74*	0.002	0.001	0.003	0.011	10.271
Ethyl-6-amino-4-[p- chloroanilino]-5-nitro-2- pyridincarbamate	021271-60-3	351(999), 43(432), 353(338), 352(297), 270(208)	88	0.003	0.006	0.003	0.005	10.889
1-hexadecenyl methyl ester	015519-14-9	71(999), 41(330), 82(270), 43(170), 96(150)	96	nd	0.005	0.423	0.579	14.007
1,1-dimethoxy-9- octadecene	015677-71-1	31(999), 71(850), 32(730), 29(520), 41(430)	91	nd	0.046	0.061	0.067	15.112
Methyl-1-octadecenyl ether	026537-06-4	71(999), 82(370), 41(300), 43(210), 68(190)	93	0.009	nd	0.017	0.169	15.252
2-methyl-1H-indole	000095-20-5	130(999), 131(657), 77(131), 103(110), 51(83)	63	0.056	0.108	0.106	0.680	16.970
cis-11-hexadecenal	053939-28-9	55(999), 41(554), 69(436), 67(392), 81(376)	90	0.03	0.027	0.037	0.082	18.298
Cholesterol	000057-88-5	43(999), 55(886), 57(744), 105(686), 86(681)	98	0.331	0.097	0.145	0.065	22.854

Table 5 Chemical components in products with Lactobacillus plantarum and Staphylococcus carnosus (n = 6, P = 0.95)

nd - not detected

* - not detected at less than 0.001 mg/kg

** - compounds identified as methyl esters by the method

LP - Lactobacillus plantarum; SC - Staphylococcus carnosus

CONTRIBUTION

A.N. Ivankin led the project; he set the research problem and the objects of study and decided on the methods. A.N. Verevkin conducted experimental work with the strains of cultures. A.S. Efremov developed formulations and determined their physicochemical parameters. N.L. Vostrikova identified and experimentally confirmed the combined effect of the cultures on the protein components of the food system. A.V. Kulikovskii identified and experimentally confirmed the microcultures' effect on the lipid components, as well as established, through mass spectrometry, their synergistic action on the main chemical components of the food system. M.I. Baburina identified and experimentally confirmed the combined effect of the cultures on the carbohydrate components of the food system.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interests.

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Fungal microbiome of barley grain revealed by NGS and mycological analysis

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Abstract:

Introduction. Barley can be infected with a broad variety of fungi, which can cause considerable loss of crop yield and reduce the quality of grain. Modern vision on the geographical and ecological distribution and biodiversity of micromycetes has been established by traditional, cultivation-based methods. However, more recently, molecular methods have shifted microbiological research to a new level, making it possible to investigate hidden taxonomical biodiversity.

Study objects and methods. For this study, we determined the fungal biome on the surface and inside of barley grains using the traditional mycological method and the contemporary molecular method, which employed DNA metabarcoding based on NGS (nextgeneration sequencing) of the ITS2 region. We analyzed five cultivars that were collected in two subsequent crop seasons (2014, 2015). *Results and discussion*. DNA metabarcoding revealed 43 operational taxonomic units, while 17 taxa of genus or species level were recovered by the traditional method. DNA metabarcoding revealed several minor species and one predominant, presumably plant-pathogenic *Phaeosphaeria* sp., which were not detected in the agar plate-based assay. Traditionally, *Fusarium* fungi were identified by mycological assay. However, the resolution of DNA metabarcoding was sufficient to determine main *Fusarium* groups divided by ability to produce toxic secondary metabolites. The combined list of Ascomycetes consisted of 15 genera, including 14 fungi identified to species level. The list of Basidiomycota derived from DNA metabarcoding data alone included 8 genera.

Conclusion. It was found that crop season predetermines the fungal community structure; mycobiota on the surface and inside of grain was significantly different.

Keywords: Barley, seed-borne fungi, infection, next-generation sequencing, rDNA, Alternaria, Fusarium

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INTRODUCTION

Barley (*Hordeum vulgare* L.) is one of the major cereal crops. It occupies fourth place among cereals in the world and second place in Russia by production quantity and cultivation area [1]. The importance of barley has been accepted since ancient times and used in the food, feed and brewing industries due to its versatility, excellent adaptation capabilities and superior properties [2].

The increased interest in barley as a source of food and fodder has resulted in a huge number of studies of associated microorganisms. It is known that barley can be infected with a broad variety of plant-pathogenic and toxigenic fungi, many of which may persist in grains. The *Bipolaris*, *Pyrenophora*, *Phaeosphaeria*, *Alternaria*, and *Fusarium* genera are considered to be prevailing fungi in barley grain worldwide [3, 4]. Species of the last two genera are well known as mycotoxin producers, with *Fusarium* spp. being the most dangerous food and feed contaminants.

Cultivation-based methods have traditionally established modern vision on geographical and ecological distribution and biodiversity of micromycetes. These methods cannot provide accurate data on taxon composition because some microorganisms do not have specific characteristics to be identified, and some appear to be noncultivable. Thus, data on the mycobiome of many substrates, including barley grain, is likely to be incomplete.

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In recent decades, molecular methods have shifted microbiological research to a new level, making it possible to investigate hidden taxonomical biodiversity. Next-generation sequencing (NGS), implemented on various independent technical platforms, became the most promising method for conducting research projects aimed at revealing fungal or bacterial composition [5–7]. Several studies have focused on a variety of agricultural subjects [8–12]. Advances in this field led to consideration that NGS-based methods are suitable as incipient techniques for seed testing [13].

454 In Denmark, pyrosequencing of the internal transcribed spacer 1 (ITS1) of the nuclear ribosomal DNA (rDNA) has been chosen to recover the composition of fungal communities associated with wheat grain [14]. NGS revealed a significantly higher level of biodiversity than it was observed in previous culturing studies. Another appropriate 454 pyrosequencing of both ITS regions was done to study the mycobiome of barley grain in western Canada [3]. It demonstrated that geographic location and agronomical practices were the determining factors explaining the observed differences in the fungal communities associated with barley. Such studies may contribute to a better understanding of fungal species compositions in cereals. They may also lead to more accurate food-quality testing and the precise design of crop protection strategies that would reduce the level of fungal contamination of agricultural products.

The objective of this study was to revise the taxonomical variety of fungi contaminated the surface and infected barley grains harvested in the northwestern region of Russia. We hypothesized that grain mycobiome could be significantly differ on the surface and inside the grain, and the difference may depend on the crop year. In our research we used the traditional agar plate-based method and the contemporary method based on 454 pyrosequencing of ITS2.

STUDY OBJECTS AND METHODS

Sampling. Grain samples of five spring barley cultivars (Suzdalets, Krinichnyy, Moskovskiy 86, Tatum, and Belgorodskiy 110) were received in 2014 and 2015 from the State Experimental Station (Volosovo, Leningradskaya oblast, Russia, 59°31'N, 29°28'E). Small grain cereals on this station were cultivated with no fungicide treatments. The grain samples intended for fungi isolation and DNA extraction were stored separately at 4°C and –20°C respectively.

Extraction of DNA. Two representative subsamples consisting of 500 grains were picked from each sample. One subsample was placed in a 50 mL polypropylene tube and subjected to superficial sterilization. The grains were consistently washed with 20 mL of 2% sodium hypochlorite solution containing 0.1% of sodium dodecyl sulphate (SDS). They were then washed once with 5% sodium hypochlorite, two times with deionized water

(diH₂O), and finally rinsed with 98% ethanol. With each step the mixtures were actively stirred up within 2 min, and the flushing solution was then decanted without the grain. The ethanol was removed by burning at regular stirring during 10 s. After this, the grains were homogenized in sterile disposable chambers on a Tube Mill Control (IKA, Germany) grinder. The other subsample was similarly homogenized, but the step of superficial sterilization was skipped.

Further, 240 mg of the ground subsamples were transferred to a 2 mL Eppendorf tube, where DNA was extracted with an AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen, USA), according to the centrifugal protocol for plant tissues and fungal mycelium. The DNA concentrations were measured with a Qubit 2.0 (Thermo Fisher Scientific, USA) using a dsDNA HS Assay Kit. Extracted DNA was used for library preparation and subsequent universal tailed amplicon sequencing, as described for the 454 Sequencing System.

Pyrosequencing and primary data analyses. For amplicon library preparation we chose the taxonomically significant ITS2 region. which is commonly used, as well as ITS1, in DNA metabarcoding studies of fungal diversity. To a large extent, ITS1 and ITS2 have similar results when used as DNA metabarcodes for fungi [15-17]. However, the ITS2 region lacks the insertions commonly found in ITS1 and thus reduces length variation [18]. This is important, as length variation can bias community pyrosequencing toward shorter amplicons. Also, ITS2 is the best-represented fungal genomic element in the public databases [19, 20]. Therefore, in studies similar to our project, use of ITS1 obtained with fungal specific primer (ITS1F) can be helpful in eliminating plant ITS amplification, and may turn out to be the method of choice in cases of mixed plant and fungal genomic DNA [21]. However, it is necessary to take into account that ITS1F (with constricted, specific range toward exclusion of all eukarya except fungal taxa), may not be able to amplify several fungal taxa because it is hampered with a high degree of mismatches relative to the target sequences [22, 23].

The ITS2 region amplified was with eukaryote-specific ITS3 ITS4 primers and GCATCGATGAAGAACGCAGC; ITS4: (ITS3: TCCTCCGCTTATTGATATGC) [22, 24]. Multiplex identifiers (MIDs) were attached to the primers' ends to carry out in consequence the simultaneous analysis of all samples.

The amplicon library pool was sequenced with 454 pyrosequencing on the GS Junior sequencer (Roche, USA) according to the recommendations of the manufacturer [25]. The ITS2 locus reads were processed by QIIME Version 1.6.0 (Quantitative Insights into Microbial Ecology) [26]. To reduce the amount of erroneous sequences and thus increase the accuracy

of the whole pipeline, the denoising procedure was employed [27].

Next steps included assigning multiplexed reads to samples based on their specific MIDs (demultiplexing), removing the low-quality or ambiguous reads, truncating primers, and other accessorial sequences. Chimeric sequences were detected using the UCHIME algorithm with the Unite database [28-30]. All of the reads were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using the UCLUST method [31]. Representative sequences were chosen according to their abundance between similar reads. Low-abundance OTUs, which have less than four copies (singletons, doubletons and tripletons), were deleted over all of the analysis [32]. All 454 pyrosequencing data of the present investigation are available through the Sequence Read Archive (SRA) under BioProject PRJNA353503, with run accession numbers from SRR5022991 to SRR5023010 [33].

Phylogenetic and statistical analyses. Taxonomical identification of representative sequences was carried

out by the BLAST method using Genbank databases [34, 35]. Query coverage $\geq 99\%$ was recognized as significant. Query identity of $\geq 99\%$ was considered identification at the species level; identity of $\geq 98-95\%$ was considered reliable identification at the genus level. The smaller similarity Ribosomal Database Project classifier, along with the Unite database (minimum confidence at 0.9), were implemented to assign OTUs to a higher taxonomic rank [29, 30, 36, 37].

Alignment of representative sequences was carried out using MAFFT algorithm G-INS-1 [38]. A phylogenetic tree was conducted with MEGA5 using the Maximum Likelihood method, based on the Tamura-Nei model with 1000 bootstrap replicates [39–41].

Vegdist and hclust R functions were used for computing Bray-Curtis dissimilarity indices and UPGMA hierarchical clustering of OTUs, showing their coexistence in the samples [42]. Heatmaps were generated with QIIME 1.8.0 with log-transformed abundance data. OTUs were sorted by phylogenetic or hierarchical trees.



Figure 1 Maximum likelihood consensus tree and fungal taxa heat map of sterilized and non-sterilized grain samples, which were harvested in 2014 and 2015 years. Read counts of each OTU were weighted according to sum of reads in the sample and log-transformed. White corresponds to low and blue to high number of reads Kri – Krinichnyy, Suz – Suzdalets, B110 – Belgorodskiy 110, Tat – Tatum, and M86 – Moskovskiy 86

Beta diversity between samples was calculated by beta_diversity.py script in QIIME with unweighted UniFrac metric [26, 43]. To check the robustness of estimated beta diversity, jackknifed analysis, with 96 reads per sample depth and 100 replicates, was performed. The results were visualized with Principal coordinate analysis (PCoA) in a 2D scale plot.



Figure 2 Boxplots depicting relative abundance (%) of the taxonomic ranks in sterilized and non-sterilized grain samples harvested in 2014 and 2015. All taxonomical ranks are marked by different colors. The boxplots consist of square boundaries indicating the 25th and 75th percentiles. Whiskers indicate the 10th and 90th percentiles, and the line inside the box represents the median. Outliers are not displayed

Bray-Curtis, weighted and unweighted UniFrac dissimilarity indices were used for measuring the strength and significance of sample groupings with Permutational Multivariate Analysis of Variance (PERMANOVA) and Analysis of Similarity (ANOSIM) with script compare categories.py [26].

Agar plate-based method of isolation and identification of fungi. Representative subsamples (200 grains) of each cultivar were surface-sterilized by being shaken for 2 min in 5% sodium hypochlorite. Then they were rinsed twice in sterilized water. The surfacesterilized grains were placed on 90 mm Petri dishes (10 grains per dish) with potato-sucrose agar (PSA) and incubated at 24°C for 10-14 days. The isolated fungal colonies from every grain were identified by visual and microscopic observations according to Ellis, Gerlach and Nirenberg, Lawrence, Rotondo, and Gannibal, and Samson et al. [44-47]. To present data comparable to those obtained with NGS, relative abundance was calculated as the number of all isolates of a certain taxon divided by the total number of fungal isolates (%). A more conventional index of seed expertise, infection frequency, was calculated as the number of grains infected by the fungus (%).

RESULTS AND DISCUSSION

NGS-based identification of fungi. After quality filtering and removal of nonfungal and chimeric sequences, in total, 8484 fungal reads were obtained and clustered into 43 operational taxonomic units (OTUs). The number of observed OTUs in the grain samples was ranged from 10 to 27. The estimated OTU richness was higher on the surface (Chao1 = 24.3 ± 4.4 , ACE = 26.2 ± 4.9 (2014); Chao1 = 30.7 ± 4.2 , ACE = 29.7 ± 1.8 (2015)), than inside (Chao1 = 15.1 ± 1.5 , ACE = 16.8 ± 1.9 (2014); Chao1 = 19.3 ± 2.0 , ACE = 21.7 ± 1.7 (2015)) the barley grains. The rarefaction curves pointed that the diversity of some samples might be underestimated, although all rarefaction curves were beyond the linear ranges.

All of the OTUs were assigned to *Basidiomycota* and *Ascomycota* phyla (Fig. 1).

Primary Ascomycota prevail over Basidiomycota, but in non-sterilized grain, the ascomycetous read prevalence (percentage of reads) was significantly lower (Fig. 2). The 26 OTUs from Ascomycota belonged to the families Nectriaceae, Dothioraceae, Microdochiaceae, Cladosporiaceae, Pleosporaceae, Sclerotiniaceae, Didymellaceae, Phaeosphaeriaceae, and unidentified Dothideomycetes, as well as undefined groups within Helotiales and Hypocreales. Seventeen basidiomycete OTUs belonged to yeastlike fungi from Entylomataceae, Cystofilobasidiaceae, and other undefined groups within Tremellales, Cystofilobasidiales, and Sporidiobolales.

One of the most abundant OTU, otu166, assigned as Dothideomycetes, failed to be identified to a more

precise taxonomical level. It has similar characteristics (BLASTn 100% query coverage and identity) with several GenBank sequences (e.g., EU552134, AJ279448, HG935454) which can be joined together only at the rank of class. More likely, it coincides with *Epicoccum nigrum*, the one abundant *Dothideomycete* identified during mycological analysis.

Eleven minor OTUs (*Alternaria* related otu44, otu53, otu61, otu180, and otu190; *Cryptococcus* related otu29, otu60, otu218, and otu264; *Davidiella* related otu123; and *Fusarium* related otu89) had no close relation to any known species but appeared in the same samples where a major OTU of a certain genus was abundant. However, potentially such satellite OTUs represent rare and/or poorly studied species, but most likely they are technical errors or sequence variances, which can occur despite all filtering and trimming procedures.

From seven clustered OTUs that were assigned as *Alternaria*, the most abundant OTUs, otu18 and otu304, can refer to *Alternaria* and *Pseudoalternaria* sections respectively (Fig. 3) [46, 48].

From ITS2 sequences combined into six OTUs and designated as *Fusarium*, several OTUs can be readily assigned as two synapomorphic (Fig. 4) clades, similar to that described by Watanabe *et al.* [49]. Two OTUs (*F. poae* – otu224 and *Fusarium* sp. – otu259) were abundant, but four OTUs appeared as solitary sequences.

Distribution of *Fusarium*-related OTUs among clusters corresponded to the prevalent toxic secondary metabolite production. The first cluster consisted of *Fusarium* fungi that are able to produce the trichothecene group metabolites. The subcluster 1a included *F. sporotrichioides* and *F. langsethiae*, which are the main producers of type A trichothecenes (like T-2 and HT-2 toxins); the subcluster 1b included *F. culmorum* and *F. graminearum* the producers of type B trichothecenes (like DON, or NIV). Species *F. poae* (subcluster 1c) produces trichothecenes of types



Figure 3 Maximum likelihood tree of OTUs related to the *Alternaria* genus. Bootstrap values based on 1000 replicates. Bootstrap values less than 50% are not presented

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Figure 4 Maximum likelihood tree of OTUs related to the *Fusarium* genus. Bootstrap values based on 1000 replicates. Bootstrap values less than 50% are not presented

A and B, and enniatins (ENNs). Fungus *F. equiseti* (subcluster 1d) is able to produce ENNs, but according to some authors, it can also produce a small amount of type A trichothecenes [50, 51]. The subcluster 2 brought together *Fusarium* fungi that are able to synthesize ENNs: *F. tricinctum*, *F. avenaceum*, and *F. lateritium* [52].

Fourteen OTUs that were defined to the species level belonged to Bipolaris sorokiniana, Fusarium poae, Neoascochyta exitialis, Sarocladium strictum, Cystofilobasidium macerans, Udeniomyces pannonicus, Cryptococcus victoriae, Cryptococcus tephrensis, Cryptococcus wieringae, Sporobolomyces *Sporobolomyces* ruberrimus, roseus, Dioszegia hungarica, Aureobasidium pullulans, and Tilletiopsis washingtonensis.

In general, the mycobiome of nonsterilized barley grains was characterized by a greater abundance of Basidiomycetes in comparison with surface-sterilized grains. The most abundant fungi in nonsterilized grains were *Davidiella* (*Cladosporium*) spp. and *Cryptoccocus* spp. After surface sterilization, the average abundance of *Fusarium*, *Alternaria*, *Pyrenophora*, and *Phaeosphaeria*, as well as fungi from *Dothideomycetes*, increased, but the ratio of those taxa depended on the year.

Comparison of taxonomical structure and relative abundance between groups of samples combined by crop year (2014/2015) and type of treatment (sterilized/ non-sterilized) reflected significant distinctions in both cases (Fig. 5). Nevertheless, distinctions between sterilized and non-sterilized grain mycobiota (ANOSIM R = 0.64, 0.76, 0.69; P = 0.001, 0.001, 0.001; PERMANOVA pseudo F = 9.89, 17.7, 10.93; P = 0.001, 0.001, 0.001; data shown successively for Bray-Curtis, Weighted and Unweighted UniFrac community dissimilarity matrices) occurred to be more strong, than those determined in successive crop years (ANOSIM R = 0.53, 0.21, 0.37; P = 0.001, 0.02, 0.006; PERMANOVA pseudo F = 8.03, 4.97, 5.02, P = 0.001, 0.019, 0.003).



Figure 5 DNA metabarcoding data based on jackknifed betadiversity PCoA plots with an unweighted Unifrac distance metric for barley samples categorized by year (blue for 2014; red for 2015) and surface treatment (dots – non-sterilized, squares – sterilized) Kri – Krinichnyy, Suz – Suzdalets, B110 – Belgorodskiy 110, Tat – Tatum, and M86 – Moskovskiy 86

The fungal species composition of non-sterilized grains differed from the mycobiome of surfacesterilized grains primary due to a higher abundance of Basidiomycetes (Cryptococcus spp. and other Tremellales, and Cystofilobasidium macerans and other Cystofilobasidiaceae) and Davidiella (Cladosporium spp.) in the non-sterilized grains. All Basidiomycetes disappeared or became sparse after surface sterilization. The most abundant of them, Cryptococcus tephrensis (otu204) and C. victoriae (otu124), were also revealed inside grains but in fewer samples and in lesser amounts. Several OTUs, e.g., Cryptococcus wieringae (otu183), Mrakiella sp. (otu254), and Dioszegia sp. (otu303), tended to present on seed surfaces during only one year. Mycobiomes observed in two different growing seasons differed by abundance of Pyrenophora sp. in 2014 and Fusarium spp. and Phaeosphaeria sp. in 2015. The Alternaria, Bipolaris, and Epicoccum genera were relatively abundant in both sample sets. More detailed results of fungal coexistence in the samples are introduced in Fig. 6.

Agar plate-based method of identification of fungi. From 87 to 117 fungal isolates per 100 grains were obtained from each sample. As a result of two growing seasons, a total of 18 taxa of seed-borne fungi were identified (Table 1). The taxonomic position of some fungi was vague due to the lack of sporulation (Mycelia sterilia). In both years, the appearance of *Alternaria, Bipolaris, Epicoccum*, and *Fusarium* species





Figure 6 Fungal taxa heat map of sterilized and non-sterilized grain samples harvested in 2014 and 2015. OTUs are grouped according to UPGMA clusterization of Bray-Curtis dissimilarity matrix representing coexistence of OTUs within samples. Read counts of each OTU were weighted according to sum of reads in the sample, and then the proportion of the OTU dominance between samples was calculated and log-transformed. White corresponds to OTU absence in sample and red to OTU with high relative abundance across the samples Kri –Krinichnyy, Suz – Suzdalets, B110 – Belgorodskiy 110, Tat – Tatum, and M86 – Moskovskiy 86

was common. Species of the genus *Pyrenophora* were found only in the 2014 growing season. Some potentially toxigenic fungi, such as *Penicillium*, *Aspergillus*, *Cladosporium* and unidentified *Zygomycota*, were found only in a few samples. No *Basidiomycetes* were isolated and identified by agar plate-based assay.

In both years, fungi of the genus *Alternaria* predominated in barley grain samples. The members of two sections, *Alternaria* and *Infectoriae*, were determined. More precise identification was not performed, since species concept is debatable for *Alternaria* and *Infectoriae* [53–56] sections.

Contamination of the barley grains by *Fusarium* spp. varied significantly in 2014 and 2015. In 2014, the *Fusarium* infection frequency was low (0-4%) and represented by five species, of which *F. avenaceum* was the most frequent (infection frequency up to 2.5%, relative abundance of isolates up to 2.2%). In 2015, the infection of barley grains with *Fusarium* spp. was considerably higher (infection frequency 14–19%,

isolate abundance 12–17%). Eight *Fusarium* species were identified; four of them were common for both years.

Comparison of methods. In total, 43 OTUs assigned as Ascomycota (26) and Basidiomycota (17) were revealed by DNA metabarcoding. Only 14 OTUs were assigned to species level. From those species, only two were reoccurred in traditional mycological analysis. The other 12 species either were not detected among isolates grown up from grains on agar medium or were Mycelia sterilia. At the same time, the conventional mycological seed test revealed 17 Ascomycetes, including 11 species, apart from some Zygomycetes and sterile Ascomycetes. Basidiomycetes were not recovered by conventional assay. Two species (Fusarium poae and Bipolaris sorokiniana), one section (Alternaria sect. Alternaria), and three genera (Davidiella [Cladosporium], Fusarium, and Pyrenophora) were formally common for both assays. In general, the list of undoubtedly identified dominant taxa coincides with the results of the NGS mycobiome study of Canadian barley grains [3].

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Fungal taxa	Relative abundance of taxa in the sample, % isolates											
			20)14					2	015		
	Suzd	M86	Tat	Kri	B110	Average	Suzd	M86	Tat	Kri	B110	Average
Alternaria sect. Alternaria	19.0	30.9	17.2	17.1	24.7	21.8	20.3	24.8	20.2	24.5	23.7	22.7
Alternaria sect. Infectoria	17.1	11.3	15.5	26.3	16.1	17.3	18.5	23.3	22.0	19.7	19.6	20.6
Aspergillus spp.	0.0	1.0	0.0	0.0	2.2	0.6	0.0	0.0	0.0	0.0	0.0	0.0
Bipolaris sorokiniana	30.5	11.3	36.2	27.4	29.0	26.9	27.8	7.6	21.5	15.9	16.0	17.8
Cladosporium spp.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.9	0.0	0.0	0.3
Epicoccum nigrum	1.9	3.1	1.7	6.9	4.3	3.6	8.4	17.6	10.8	20.6	16.0	14.7
F. avenaceum	1.4	0.0	2.2	0.0	0.5	0.8	9.3	5.2	5.4	6.4	7.3	6.7
F. poae	0.5	0.0	0.0	0.6	0.0	0.2	3.1	4.8	0.9	2.1	2.3	2.6
F. graminearum	0.0	0.0	0.0	0.0	0.5	0.1	0.0	0.0	0.0	0.0	0.0	0.0
F. tricinctum	0.0	0.0	0.9	0.0	0.0	0.2	3.1	3.3	4.0	3.0	2.3	3.1
F. sporotrichioides	0.0	0.0	0.4	0.0	0.0	0.1	0.9	0.0	2.2	0.0	2.7	1.2
F. incarnatum	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.1
F. equiseti	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.1
F. langsethiae	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.1
F. culmorum	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.2
Total Fusarium	1.9	0.0	3.4	0.6	1.1	1.4	16.7	13.8	13.0	11.6	15.5	14.1
Penicillium spp.	1.0	10.3	0.9	0.0	0.0	2.4	0.0	0.0	0.0	0.0	0.0	0.0
Pyrenophora spp.	21.0	25.8	22.4	17.1	7.5	18.8	0.0	0.0	0.0	0.0	0.0	0.0
Zygomycota sp.	0.0	0.0	0.0	1.1	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0
Mycelia sterilia	7.6	6.2	2.6	3.4	15.1	7.0	8.4	12.4	11.7	7.7	9.1	9.9

	Table 1 Mycologica	l identification of fu	ngi in sterilized	l barley grains h	arvested in 2014 and 2013
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Barley cultivars: Kri-Krinichnyy, Suz-Suzdalets, B110-Belgorodskiy 110, Tat-Tatum, and M86-Moskovskiy 86

The predominant OTUs from *Alternaria* were identified as *Alternaria* and *Pseudoalternaria* sections when *Alternaria* and *Infectoriae* sections were fixed during mycological analysis. In both cases, taxa were identified to the section level. Such precision is sufficient for the majority of practical purposes, e.g., for tests of seed, food, or feed-grain quality. The big section *Infectoriae* and lately described section *Pseudoalternaria* are morphologically similar and phylogenetically close groups [46]. This obviously can be the cause of errors, if identification is based on morphological features.

Both methods similarly reflected a very low abundance of *Fusarium* spp. in 2014 and a higher quantity in 2015. Traditional mycological analysis revealed nine *Fusarium* species. DNA metabarcoding results were more limited; only one OTU was identified as a certain species, *F. poae*, but the others were assigned to a clade level. Phylogenetic resolution derived from ITS2 is not useful in defining *Fusarium* species. Recently, *Fusarium*-specific primers targeting translation elongation factor 1 (TEF1) were evaluated and successfully applied to analyze *Fusarium* communities in soil and plant material [57].

The taxonomy of *Fusarium* fungi is confusing and various classification systems have been proposed [58]. For *Fusarium*, chemotaxonomy is considered a supplement to traditional morphology-based taxonomy. Several fungal genes involved in trichothecene and enniatins biosynthesis have been defined and used for

development of molecular assays aimed at identification. In spite of the ITS sequences used in our analysis, the results strongly suggested the division of fungi based on their ability to produce metabolites. In the future, this will provide an opportunity to predict the severity of grain contamination by some mycotoxins according to the number of certain identified OTUs.

Fusarium avenaceum, F. poae, F. tricinctum, and *F. sporotrichioides* were the most abundant representatives of the genus. They are the typical pathogens of barley in northwestern Russia [59, 60]. Most likely, multi-copy otu259 discovered by DNA metabarcoding is associated with *F. avenaceum*, which occurred frequently on the barley grain.

Both methods revealed pathogenic fungi from *Pleosporaceae: Bipolaris* and *Pyrenophora*. Those fungi have different patterns of appearance through the cropping seasons. DNA metabarcoding demonstrated higher sensitivity. *Pyrenophora* sp. colonies were not recovered in 2015 at all, but several respective reads were obtained for 7 out of 10 samples.

Davidiella (Cladosporium) associated reads were abundant in DNA metabarcoding assay in non-sterilized samples but only single colonies were detected in the agar plate-based test. Underestimation of relative abundance of the fungus in the latter case can be result of two reasons: rapidly spreading colonies suppress or mask slowly growing fungi, and infected individual grains contain not uniform quantity of fungal biomass that appear as insufficient correlation between the number of infected grains and the amount of fungal DNA in the whole sample.

Four fungal genera revealed by only DNA metabarcoding contained agents of cereal diseases (Neoascochvta, Botrvtis, Microdochium, and *Phaeosphaeria*). The first three taxa were represented by solitary reads. Phaeosphaeria (otu106 and otu215) were found in 14 of 20 samples. In 2015, in surface-sterilized samples, the relative abundance of Phaeosphaeria reads varied between 11 and 36%. Sequences of otu106 had the closest similarity (99%) with representative sequences of Parastagonospora avenae (Septoria avenae or Stagonospora avenae), widespread fungus causing leaf blotch of barley and some other cereals, and *Parastagonospora poagena*, a recently described fungus from Poa sp. [61, 62]. Less abundant OTU, otu215, had a similarity of 98%, with several Phaeosphaeria species and with some unidentified endophytes.

CONCLUSION

DNA metabarcoding, based on high-throughput sequencing, is a sensitive and powerful method of grain mycobiome analysis that provides large amounts of data. However, at this time, not all fungi can be identified to species level by molecular markers, especially by rDNA sequences. In spite of universality, rDNA has a limitation as a taxonomic marker. The resolution of the ITS sequence-based method is not enough to differentiate many fungal species. For instance, many Fusarium species have nonorthologous copies of ITS2. Many other important plant pathogenic and toxigenic fungi also can be identified up to genus level, but that is not always informative in the framework of mycological seed expertise. Erroneous and chimerical sequences, as well as the lack of reference sequences of many species, still limits wide application of NGS-based technologies in biodiversity studies.

The most complete and credible results can be obtained when several approaches are implemented simultaneously. Combining the results of DNA metabarcoding and traditional culture-plating assay allowed us to revise the diversity of fungi colonizing on the surface of and inside barley grains in Leningradskaya oblast (northwest Russia).

Fungal species diversity of barley grain revealed by DNA metabarcoding formally exceeded the traditional microbiological culture-based agar plating: 43 operational taxonomic units (OTUs) vs. 17 taxa of genus or species level. DNA metabarcoding assay allowed seven ascomycete taxa to be added to the total list. Of those additional taxa, only Phaeosphaeria was abundant internal fungus. Seventeen OTUs belonging mainly to surface-seed-borne, yeastlike Basidiomycetes were completely outside the scope of traditional analysis. Meanwhile, routine mycological analysis, in contrast to DNA metabarcoding, resulted in precise identification of practically important Fusarium species. On the other side, due to DNA analysis, one Alternaria taxon was reidentified as Alternaria section Pseudoalternaria instead of section Infectoriae.

CONTRIBUTION

Authors are equally related to the writing of the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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Antimicrobial activity of mono- and polynuclear platinum and palladium complexes

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Abstract:

Introduction. Infectious diseases remain a serious threat to humanity worldwide as bacterial pathogens grow more diverse. Bacteria, fungi, and parasites develop resistance to clinically approved antimicrobials, which reduces the efficacy of available drugs and treatment measures. As a result, there is an ever growing demand for new highly effective pharmaceuticals. This review describes mono- and polynuclear platinum and palladium complexes with antimicrobial properties. We compared several groups of antibacterial agents: antibiotics, antioxidant biologically active substances, antimicrobial nanoparticles, nanocomposite materials, biopolymers, micellar systems, and plant extracts.

Study objects and methods. The review covered relevant articles published in Web of Science, Scopus, and Russian Science Citation Index for the last decade. The list of descriptors included such terms as mononuclear and binuclear complexes of platinum, palladium, and antimicrobial activity.

Results and discussion. Chelates of platinum, palladium, silver, iridium, rhodium, ruthenium, cobalt, and nickel are popular therapeutic agents. Their antimicrobial activity against pathogenic microorganisms can be enhanced by increasing their bioavailability. Metalbased drugs facilitate the transport of organic ligands towards the bacterial cell. The nature of the ligand and its coordination change the thermodynamic stability, kinetic lability, and lipophilic properties of the complex, as well as the reactivity of the central atom. Polynuclear platinum and palladium complexes contain two or more bound metal (coordinate) centers. Covalent bonding with bacterial DNA enables them to form a type of DNA adducts, which is completely different from that of mononuclear complexes. *Conclusion.* Metal-based drugs with functional monodentate ligands exhibit a greater antimicrobial effect compared to free ligands. Poly- and heteronuclear complexes can increase the number of active centers that block the action of bacterial cells. When combined

with other antibacterial agents, they provide a synergistic effect, which makes them a promising subject of further research.

Keywords: Antimicrobial activity, antibacterial activity, antitumor activity, mononuclear complexes, polynuclear complexes, platinum (II), palladium (II), platinum (IV)

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INTRODUCTION

Infectious diseases represent a serious problem worldwide. The growing antimicrobial resistance of various pathogens reduces the efficacy of existing drugs and preventive treatment, thus fuelling the neverending search for new drugs. Living organisms are in constant contact with a huge number of chemical compounds. Some of them are beneficial, e.g. proteins, lipids, carbohydrates, biologically active substances, mineral components, etc., while others are toxic. People in industrial regions are especially vulnerable to the negative impact of xenobiotics.

The antioxidative system of living organisms consists of the enzymes of oxidismutase, peroxidase,

and catalase. It helps to destroy bacteria and substances absorbed by leukocyte cells. Antioxidants provide protection against the damage that results from the controlled production of reactive oxygen intermediates followed by lipid peroxidation, protein damage, and DNA rupture. Thus, antioxidants reduce the risk of chronic diseases, including cancer and heart diseases.

Enzymes and oxygen are responsible for regulated oxygenase and dioxigenase oxidation of biosubstrates in the organism. Biosubstrate comes in direct contact with oxygen only in the presence of enzymes. Therefore, oxidation processes can be controlled. In case of direct contact of the substrate with reactive oxygen intermediates, the redox process proceeds according

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to the radical mechanism, and its rate depends on the concentration of free radicals in the cell.

Radiation exposure causes violation of the redox transformations of complexing ions in various biological complexes. Various radicals and other reactive oxygen intermediates form as a result of the activation and decomposition of water molecules.

Induced cytochrome enzyme system ensures the oxidative transformation of xenobiotics. It triggers the activation mechanism of the genes responsible for protein synthesis. Transcription of the corresponding part of the chromosome starts when the xenobiotic binds to the receptor protein in the cell. The resulting mRNA leaves the nucleus and becomes the template for the synthesis of the protein component of the monooxygenase. Drugs, polycyclic aromatic hydrocarbons, food components, e.g. flavonoids. xanthines. and indole derivatives, can exhibit monooxygenase-inducing properties. The intake of xenobiotics increases the number of monooxygenases, which leads to immunological exhaustion [1].

This review features mono- and polynuclear platinum and palladium complexes with antimicrobial properties. It contains a comparative analysis of various classes of antibacterial agents, e.g. antibiotics, antioxidant biologically active substances, antimicrobial nanoparticles, nanocomposite materials, biopolymers, micellar systems, and plant extracts.

STUDY OBJECTS AND METHODS

The review presents platinum and palladium complexes with antibacterial properties, various coordination structure, and different methods of ligand coordination. The list included mono- and polynuclear complexes with the central atom oxidation state of (+2) and (+4). The polynuclear complexes contained both mono- and polydentate bridging and terminal ligands. For comparison, we examined the main antibacterial agents – antibiotics, antioxidant biologically active

substances, antimicrobial nanoparticles, and nanocomposite materials, as well as such biopolymers as polysaccharides, peptides, micellar systems, and plant extracts.

The review was based on highly relevant and recent publications retrieved from the Web of Science, Scopus, and Russian Science Citation Index bases. We limited the search to mononuclear and binuclear complexes of platinum and palladium and antimicrobial activity.

RESULTS AND DISCUSSION

Antibiotics. Antibiotics are natural substances of microbial, plant, and animal origin and products of their chemical modification that are capable of suppressing the growth of bacteria, lower fungi, protozoa, viruses, or cancer cells, when administered in low concentrations $(10^{-3}-10^{-2} \ \mu\text{g/mL})$. Science knows several thousands of natural antibiotics, and almost all of them are heterocyclic compounds. Synthetic and semi-synthetic antibiotics are more active and stable than natural ones.

Antibiotics can be divided into four main types according to the mechanism of action: 1) those that inhibit the synthesis of bacterial cell walls; 2) those that inhibit template (ribosomal) protein synthesis; 3) those that inhibit nucleic acid synthesis; 4) those that inhibit the functioning of the cytoplasmic membrane (Fig. 1). Antibiotics, antiseptics, bacteriophages, disinfectants, preservatives, and other antimicrobials are used in all industries. However, large doses of antibiotics and long treatment sessions may cause allergic or direct toxic reactions that affect kidneys, liver, gastrointestinal tract, central nervous and hematopoietic systems, etc.

The European system for surveillance and control of antimicrobial resistance has identified seven types of clinically significant bacteria that shape the antimicrobial resistance in Europe: *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, *Enterococcus faecium*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*.



Figure 1 Antibiotics: mode of action

Strains of microorganisms isolated from various plant and animal raw materials demonstrate antibacterial properties, e.g. *Bacillus safensis, Bacillus endopheticus,* and *Bacillus subtilis* [2]. Bacteriocins of lactic acid bacteria strains of *Lactobacillus delbrueckii* B2455, *Lactobacillus paracasei* B2430, and *Lactobacillus plantarum* B884 are known to possess an antimicrobial potential [3].

As a rule, antimicrobial activity is determined by the optical density of culture fluid by using the method of serial dilutions, as well as the disk-diffusion method or diffusion E-test. The list of quantitative indicators that describe antibacterial activity includes: minimum inhibitory concentration (MIC); minimal inhibitory concentrations that inhibit 50% and 90% of bacteria (MIC₅₀ and MIC₉₀, respectively); minimal bactericidal concentration that causes the complete death of bacterial cells (MBC).

Antioxidant biologically active substances. Scientists pay much attention to the antioxidant activity of organic and organometallic compounds against toxic active forms of oxygen and nitrogen. Antioxidants prevent oxidative reactions by stabilizing free radicals. However, the necessary amount of antioxidants can be obtained only with the regular use of biologically active additives. Plant-based bioflavonoids are popular food additives, e.g. rutin, quercetin, dihydroquercetin, eriodiktiol, resveratrol, etc. [4]. There are complex compounds that protect DNA from damage in the presence of hydrogen peroxide [5].

The growing prevalence of multiresistant bacterial pathogens has become a worldwide problem in the early XXI century. Infectious diseases remain a serious problem worldwide. When bacteria, fungi, and parasites become resistant to antimicrobials, it reduces the efficacy of drugs and preventive treatment. More and more microorganisms can withstand vaccines and antibiotics. For instance, methicillin-resistant Staphylococcus aureus is resistant to vancomycin [6]. The World Health Organization has already emphasized the need to develop new antimicrobial molecules because conventional antibiotics are growing helpless, especially in fighting the so-called ESKAPE pathogens with their gradually increasing antibiotic resistance: Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa, and Enterobacter [7].

Fungal infections also cause high morbidity and mortality, especially in immunocompromised HIV and cancer patients. The growing cancer incidence is another global health concern as it remains one of the most common causes of death worldwide. The recent advances in cancer treatment, e.g. chemotherapeutic drugs, have significantly improved the prognosis and survival of cancer patients [7].

Antimicrobial nanoparticles and nanocomposite materials. Nanoparticles can target bacteria as an

alternative to antibiotics. Nanotechnology can be especially useful in the treatment of bacterial infections. Nanoparticles cover antibacterial coatings of implantable devices to prevent infection and promote wound healing. They are used in treating diseases as antibiotic delivery systems. In bacteria detection systems, they facilitate microbial diagnostics. They also can control bacterial infections in antibacterial vaccines [8]. Metal nanoparticles have a pronounced wound healing effect.

Nanocomposite materials of silver, gold, platinum, and iron possess high antimicrobial activity when stabilized by arabinogalactan, which is a natural polysaccharide, as well as by other metal nanoclusters. A biologically active complex called Fullerene C60/Tween 80 affects the main pathogenesis of wound process [9]. There have been studies of the sorption activity of *Acetobacter xylinum* cellulose nano-gel films in various biological media in comparison with other sorbents.

Antibacterial bimetallic surfaces of implant biomaterials have also become focus of scientific attention [10]. The research featured platinum and silver nanoparticles that were 1.3–3.9 nm thick and 3–60 nm wide. To create an antimicrobial surface, they were subjected to magnetron sputtering on a titanium substrate, both separately and together. Sequential sputtering of silver and platinum nanoparticles increased the antimicrobial activity, if compared to co-sprayed silver and platinum samples or pure silver patches (Fig. 2).

Researchers have synthesized gold and platinum nanoparticles coated with a pyrimidine-based ligand [11]. The nanoparticles interacted with DNA due to hydrophobic forces and demoinstrated a good antioxidant activity. In addition, they possessed antimicrobial properties against *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas fluorescens*, *Shigella sonnei*, *Staphylococcus aureus*, *Aspergillus niger*, *Candida albicans*, *Candida tropicalis*, and *Rucoropus mucis indica*.

Antimicrobial nanoagents can be used in dentistry, medical devices, and food industry [12].

Antimicrobial nanoparticles and peptides can become new non-antibiotic antimicrobials that kill bacteria in biofilms. Biofilms can be produced by several species or one strain of bacteria. A biofilm is a template



cosputtered Pt/Ag nanopatches consecutively sputtered Pt/Ag nanopatches

Figure 2 Antibacterial activity of silver and platinum particles [10]

coating of one or more strains of bacteria that adhere to biological or non-biological surfaces. Biofilms increase the resistance of microorganisms to antimicrobial agents by producing extracellular polymeric substances.

Many bacterial pathogens have developed antibiotic resistance, resulting in infections that cannot be treated with conventional antibiotics. New non-antibiotic antimicrobial agents, e.g. silver nanoparticles or new antimicrobial proteins, can bind and oxidize thiol groups, block DNA replication, alter the expression of bacterial genes and denaturing enzymes, induce reactive oxygen species, or damage bacterial membranes. Antimicrobial proteins, e.g. antimicrobial peptides, and natural enzymes, e.g. those derived from insects and bacteria, also demonstrate antibacterial properties [2, 3]. As a result, they can be used in biomedicine and food industry as antibacterial agents. The antimicrobial properties of peptides are not as strong as those of conventional antibiotics, but sufficient enough to kill pathogenic microorganisms. The mechanisms of their action remain unclear, but they are believed to target bacterial membranes and intracellular molecules.

Chronic infections lead to inflammation and deplete immune defense, thus contributing to the proliferation of cancer cells. Cisplatin (CDDP) has been approved by the Food and Drug Administration (FDA) as an antitumor drug, which is now widely used to treat various types of cancer. Cisplatin owes its antitumor properties to the fact that it affects DNA directly [13]. DNA alkylation suppresses the biosynthesis of nucleic acids and kills the cell. However, cisplatin has no targeted effect: it spreads in all biological fluids and body tissues, causing renal function impairment, anaphylactic reactions, leukopenia, thrombocytopenia, anemia, and neuropathy [14]. The antiproliferative effect that cicplatin produces on rapidly dividing cells explains its toxic impact on the functional state of organs and tissues.

As a result, scientists around the world have been trying to develop more effective antitumor platinumbased drugs with fewer complications. Currently, it is one of the most urgent tasks of bioorganic chemistry and biotechnology. The introduction into the internal sphere of a complex of powerful antiproliferative and functionally active ligands is another strategic direction in the search for methods of highly effective agents. Structural analogues of clinically tested platinum complexes have been subjects of numerous studies in the recent decades. Most of them feature monofunctional platinum (II) complexes that carry only one labile ligand, each complex binding to DNA only once [15].

The nature of the ligand and its coordination type affect the reactivity of the central atom. Coordination changes not only the thermodynamic stability and kinetic lability of the complex, but also its lipophilic properties. It either stabilizes or destabilizes the oxidative state of the central atom. **Biopolymers: polysaccharides and peptides. Micellar systems.** Metals can produce complex biologically-active biopolymers with antimicrobial and antitumor properties.

Galactan-containing polysaccharides are known for their high biological activity and immunomodulatory effect. Arabinogalactans contain numerous galactose and arabinose residues, which allow them to interact with asialoglycoprotein receptors. This valuable property makes it possible to use these polysaccharides to deliver substances that are unable to pass through the outer membrane into the cell. For instance, Starkov *et al.* used arabinogalactan to deliver platinum into tumor cells [16]. Platinum has an antitumor effect as part of cisplatin, which is widely used in cancer treatment [14]. Starkov *et al.* also proved the antitumor effect of the equimolar platinum-arabinogalactan complex based on the interaction of cis-diamine(cyclobutane-1,1-dicarboxylate-O,O')platinum (II) with a polysaccharide [17].

Popova and Trifonov analyzed research results published over the past 15 years which featured the synthesis and biological properties of analogues and derivatives of amino acids with tetrazolyl fragments [18]. They concluded that tetrazolyl analogues and derivatives of amino acids and peptides have a great potential for medical chemistry. Tetrazoles are polyazitous heterocyclic systems which include four endocyclic nitrogen atoms. They are able to exhibit the properties of acids and bases, as well as form strong hydrogen bonds with proton donors and, less often, with proton acceptors. They are metabolically stable and can penetrate biological membranes. Another promising area is the synthesis of linear and cyclic peptides based on modified amino acids with a tetrazolyl fragment. Finally, some tetrazole-containing amino acids and peptides possess a high biological activity and can become a source of new drugs [18].

Porphyrins are tetrapyrrole compounds that form metal porphyrins when interacting with metal compounds, and metal porphyrins can easily enter into electrophilic substitution reactions. In addition, free and metal-bound porphyrins are easily reduced to produce mono- and dianionic compounds. Their nucleophilic properties allow them to interact with proton donors. Simulated solutions of porphyrin compounds help study photo-oxygenation.

Platinum-bound porphyrins can inhibit multiresistant bacteria, e.g. *Staphylococcus aureus* [19]. Tetra-platinum (II) porphyrin increased its hemolytic activity when exposed to light. Lopes *et al.* proved that platinized porphyrins had a good potential for wastewater treatment, biofilm control, and bioremediation since they can be used for microbial photodynamic inactivation [19].

Proline derivatives are known to possess antibacterial activity. Thioproline is an antioxidant, while phenylproline derivatives inhibit the *Staphylo*- *coccus aureus* sortase SrtA isoform [20]. Gram-positive bacteria produce surface proteins that promote the attachment of the bacterial cell to the host and prevent phagocytosis. During catalysis, sortase enzyme sorts surface proteins on the bacterial cell wall. Surface proteins then bind covalently to the bacterial cell wall through the catalyzed *S. aureus* SrtA transpeptidase reaction. Deactivation of SrtA genes of Gram-positive microorganisms inhibits the fixation of surface proteins and reduces the virulence of the bacterium. Antibiotics are not the only *S. aureus* SrtA inhibitors: peptides, plant extracts, and low-molecular-weight organic compounds have the same properties [20].

Therefore, biopolymers and micellar systems with their ability to penetrate biological membranes can deliver metal complexes into cells.

Complex platinum and palladium compounds. Drugs based on organic ligand complexes exhibit a greater antimicrobial effect compared to organic pharmaceuticals. Complexation produces a synergistic effect between the organic ligand and the complexing agent. Chelates of platinum, iron, iridium, rhodium, ruthenium, palladium, cobalt, and nickel have a reputation of effective therapeutic agents.

Metal-containing active centers with a stable, inert, and non-toxic nature are quite rare in biological systems. They owe their activity to the bioavailability of the complexes. Metal complex-based drugs facilitate the transport of organic ligands towards the bacterial cell. Palladium complexes proved highly effective against resistant forms of microorganisms. For instance, tetracycline palladium (II) complex appeared sixteen times more effective against tetracycline-resistant bacterial strains of *E. Coli* HB101/pBR322 than traditional drugs [6].

There are a huge number of pharmacologically active heterocyclic compounds. Advanced medical chemistry has made it its main task to study the antimicrobial and antitumor properties of platinum and palladium complexes with heterocyclic ligands.

Benzothiazole derivatives are one of the most popular pharmacologically known heterocyclic compounds. Benzothiazole and its analogues demonstrate a wide range of biological properties, e.g. antitumor, antimicrobial, anticonvulsant, antiviral, antituberculous, antimalarial, anthelmintic, analgesic, anti-inflammatory, antidiabetic, fungicidal, etc. [21]. Thiazole nuclei that can be coordinated to metal atoms are often used as an ambidentate ligand in biologically active complexes.

Thiosemicarbazone and its derivatives can be used as synthetic antiviral agents. They are heterocyclic ligands and contain nitrogen, sulfur, and oxygen donor atoms. Platinum (II) and palladium (II) complexes with thiosemicarbazones exhibit anti-tuberculosis activity against *Mycobacterium tuberculosis* [22].

Suleman *et al.* described Schiff-base complexes that contained donor atoms of nitrogen, sulfur, and oxygen and possessed antimicrobial and antitumor activity. The

antibacterial activity of these multi-dentate ligands and their complexes demonstrated great prospects pharmacy and agricultural chemistry. Coordination compounds of transition metals owe their unique configuration and chemical lability to their specific electronic and steric properties, which make them sensitive to the molecular environment [23].

The antimicrobial and antitumor properties of these complexes depended on the electron-donor and acceptor substituents in the aromatic ring. Bioligands modified by hydrophilic groups appeared to increase the solubility of compounds [24].

Platinum (II)complexes obtained from functionalized aroylaminocarbo-N-thioyl prolineates demonstrated antibacterial and antifungal also properties [25]. Sulfur and oxygen atoms allowed aroylaminocarbo-N-thioyl ligands to coordinate bidentally. Non-electrolyte complexes had a squareplanar configuration.

Mawnai *et al.* synthesized complexes with N-coordinated pyridylpyrazolyl ligands that formed a six-membered metallocycle [26]. They conducted *in vitro* studies of the antibacterial activity of ligands and their complexes. The research featured both Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus aureus* and *Bacillus thuriengiensis*) bacteria. The cationic nature of the complexes made them more effective against the Gram-negative bacteria.

Bakr *et al.* synthesized organometallic platinum and palladium complexes with heterocyclic derivatives of pyrazolone [5]. Pyrazolone derivatives had a fivemembered ring with an additional keto group, which allowed them to form chelates. They studied the biological activity of azo-compounds to use them as antitumor, antioxidant, and antimicrobial agents. They also assessed their nuclease activity against DNA. They performed an MTT lab-test on four human cancer cell lines to study the antitumor activity of the compounds in question. The cell lines included hepatocellular carcinoma (HePG-2), colorectal cancer (HCT-116), human prostate carcinoma (PC-3), and breast carcinomas (CMC-7) [5].

As a rule, researchers employed standard methods to study the antimicrobial activity of the abovementioned compounds, e.g. the cut-plug method. Some experiments featured strains of pathogenic bacteria, e.g. Escherichia Staphylococcus aureus, Bacillus coli, subtilus, Salmonella typhi, and Proteus spp, or such malicious fungi as Candida albicans and Aspergillus niger [5]. An in vitro anntioxidant analysis of pyrazolone derivatives and their metal complexes made it possible to compare the results of erythrocyte hemolysis. The palladium complexes demonstrated a greater antioxidant activity in comparison with platinum complexes. The free ligand had a more prominent increase in the antioxidant activity, compared to metal complexes. This result could be explained by a greater ability to charge transfer of the condensed ring system. It increased the ability of the heterocycle to stabilize unpaired electrons of the azocompound, thus binding free radicals.

Chitosan is an antimicrobial agent that can destroy bacteria, filamentous fungi, and yeast. Chitosan is a copolymer of 2-amino-2-deoxy-D-glucopyranose and 2-acetamido-2-deoxy-D-glucopyranose combined with β (1 \rightarrow 4), which gives it high biocompatibility and biodegradability. Chitosan is widely used in food industry, agriculture, and medicine.

The antimicrobial activity of chitosan and its derivatives depend on pH, type of microorganisms, molecular weight of the biopolymer, and the degree of its deacetylation. If a chemical change occurs in the structure of the amino- and hydroxyl groups of the glucosamine chains of the biopolymer, it can affect not only the solubility and stability of chitosan, but also its antimicrobial activity. Berezin et al. described the synthesis of water-soluble conjugates of chitosan with tetrazoles. They bound tetrazoles by the chlorohydroxypropyl groups of N-(3-chloro-2hydroxypropyl) chitosan, while the other part of the groups interacted with the amino groups of the polymer, which led to intra- or intermolecular crosslinking [27]. The antimicrobial properties increased as a result of the complexation of chitosan with various metals.

Barbosa et al. developed new platinum (II) and palladium (II) complexes with biopolymer amphiphilic Schiff-bases to increase the biological activity of chitosans. They performed the binding by fixing chitosans in templates of various molecular weights. The chitosans were modified with salicylic aldehyde and glycidol [24]. They introduced salicylaldehyde to obtain the complexing Schiff-base sites in the chitosan template. Glycidol made it possible to increase the water solubility of the resulting biopolymer complexes. The new complexes underwent spectral and thermal testing for antimicrobial and antitumor activity. When compared to the free ligand, the complexes demonstrated a higher antibacterial efficacy against gram-negative bacteria Pseudomonas syringae than against Fusarium graminearum fungi. They also demonstrated a high antitumor effect on MCF-7 breast cancer cells, with certain selectivity for non-tumor cells (Balb/c 3t3 clone A31) depending on the concentration and molar mass. In higher concentration, all complexes synthesized with different molecular weights of the polymer template decreased the viability of MCF-7 cancer cells [24].

Bobinihi *et al.* synthesized dithiocarbamide ligands based on primary amines, N-phenylaniline, 4-methylaniline, and 4-ethylaniline [28]. S,S-binding resulted in bidentate coordination, which led to the formation of squared complexes of platinum (II) [Pt (L) 2] and palladium (II) [Pd (L) 2]. They exhibited antimicrobial activity against gram-negative bacteria

(Escherichia coli, Klebsiella pneumonia, and Pseudomonas aeruginosa), gram-positive bacteria (Bacillus cereus and Staphylococcus aureus), and fungi (Candida albicans and Aspergillus flavus).

The mechanisms of the antitumor effect changed naphthalenbenzimidisole was when introduced as a ligand into the platinum-metal system. The antiproliferative activity, drug resistance, and toxicity increased. Liang et al. invented a synthesis method for naphthalene benzimidisole-platinum (II) complexes [29]. They studied their antiproliferative activity for eight cancer cell lines, namely Hela, HepG2, SKOV3, NCI-H460, BEL-7404, SMMC-7721, U251, and A549. Unlike cisplatin, the naphthalenbenzimidisole complexes did not show resistance to A549-CDDP. The mechanism of the antitumor effect appeared due to the covalent binding to DNA and an increase in the expression level of intracellular type I. An in vitro experiment showed that several complexes proved sensitive and selective to cell lines SMMC-7721 and U251 and possessed low toxicity to normal HL-7702 cells.

Antimicrobial activity depends on the alkyl chain length of N-substituted imidazolium salts, where long alkyl chain compound with 8–16 carbon atoms reached the lowest values of the minimum inhibitory concentration. While alkyl chains under six carbon atoms are usually inactive, the alkyl chain length affects the functioning of the bacterial membrane [30, 31]. When a long hydrocarbon chain integrates with a lipid bilayer of the cell membrane, cell contents may start leaking out [32]. The antimicrobial activity of imidazolium salts depends on such factors as hydrophobicity, adsorption, critical micelle concentration, and the transport rate in aqueous media.

Meng *et al.* synthesized a number of platinum (II) complexes with substituted 3-(2'-benzimidazolyl) coumarins (1-benzopyran-2-one) [33]. The complexes exhibited a high cytotoxic activity *in vitro* against cisplatin-resistant cancer cells, namely SK-OV-3/DDP with a low IC₅₀.

Choo et al. described a wide range of organometallic drugs with N-heterocyclic carbene (NHCs) ligands [34]. The new complexes were insoluble in most solvents except dimethyl sulfoxide. Complexes with several conjugated rings are highly hydrophobic and do not affect the activity of Gram-negative bacteria. Inhibition of the growth of Gram-positive bacterial strains occurs at low micromolar concentrations of the synthesized complexes. The different susceptibility of Grampositive and Gram-negative bacteria results from their morphological differences, namely the permeability of the outer layer of bacteria. The difference in susceptibility can be explained by their morphological differences between Gram-positive and Gram-negative bacteria. Gram-positive bacteria have a lower permeability of the outer peptidoglycan layer, while the outer membrane of Gram-negative bacteria contains
structural lipopolysaccharide components. They make the cell wall impervious to lipophilic solutions. As a result, porins, membrane transport proteins, form a selective barrier for hydrophilic solutions [34]. The part of the channel protein that is responsible for transmembrane transport opens and closes depending on the hydrophilicity of the complex.

The synthesis of platinum (IV) antitumor drug precursors relies on the fact that the oxidation state of platinum (IV) leads to a greater stability than their platinum (II) analogues. The stability of platinum (IV) precursors results from their resistance to reduction, inertness to ligand exchange, and reactivity [35].

There have been successful attempts to synthesize antimicrobial platinum complexes with coumarin derivatives as heterocyclic biologically active ligands [36]. They inhibited the cyclooxygenase enzyme by coumarin complexes of platinum (IV) with cisplatin and oxaliplatin centers.

Oxygen atoms allow carboxylate ligands of $\text{RCO}_2^$ to possess electrodonor properties. Their coordination is monodentate, bidentate, and even tetradentate. The carboxylate platinum and palladium complexes are analogues of biologically active compounds. The acidoligand and synthesis conditions proved to affect the formation of the internal coordination sphere. The system of hydrogen bonds and/or $\pi - \pi$ -stacking interactions between aromatic ligand segments also produced a certain effect on the processes of selforganization of complexes into supramolecular structures [37].

Carboxylate metal complexes often take the form of polynuclear compounds due to the oligomerization of oxo- and hydroxo-functional groups, thus developing M-O-M structural units. There are platinum (IV) carboxylate complexes with anticancer activity [35, 38].

Al-Khathami et al. synthesized several Schiff bases with various primary aromatic amines derived from pyridine-2-carboxaldehyde as ligands for platinum (II) complexes [39]. They studied their antimicrobial activity in vitro using the cut-plug method in nutrient media. Microorganisms were plated in wells filled with the test solution of ligands and complexes with subsequent incubation. Some complexes and ligands proved to have inhibitory effect on such pathogenic human bacteria as Escherichia coli, Bacillus subtilus, Salmonella typhimurium, Klebsiella pneumoniae, Staphlococcus aureus, Pseudomonas aeruginosa, and Candida fungus. Studies of DNA binding showed that the electron-withdrawing groups facilitated the binding of platinum (II) complexes containing the Schiff base pyridyl ligands (Fig. 3). The complexes with an electron-withdrawing group demonstrated the highest antimicrobial effect. The complex with a nitro group proved effective against bacteria, but not against fungi. The acetyl group increased antimicrobial activity against almost all strains. Due to the hydroxyl group, free



Figure 3 Methods of binding a mononuclear complex with a protein [39]

ligands possessed a higher antimicrobial activity against gram-negative bacteria, compared to their platinum (II) complexes.

Platinum complex compounds are not the only platinum group metals with pronounced antimicrobial and antitumor properties. Gold, silver, iridium, rhodium, and ruthenium complexes demonstrate similar activities. The cytotoxicity of gold complexes usually consists in the inhibition of thiol-containing enzymes. When gold binds with thiol groups, the reductases and proteases of cancer cells become potential targets for gold complexes (Fig. 4). Inhibition of the activity of these enzymes can disrupt the redox state of the cell and increase the production of reactive oxygen species (ROS), thus causing cellular oxidative stress and leading to its own apoptosis. This mechanism differs from that of platinum-based drugs [40].

Polynuclear platinum and palladium complexes. Binuclear and polynuclear platinum complexes have recently proved biologically active and antimicrobial. Bridging ligands contribute to the formation of cyclometallic complexes. Polynuclear compounds exhibit properties different from those of free ligands and monomeric complexes.

Johnstone *et al.* studied non-classical platinum (II) complexes with trans-geometry or a monofunctional



Figure 4 Binding of silver with thiol groups [40]

coordination center, as well as polynuclear platinum (II) compounds, platinum (IV) prodrugs, photoactivated platinum (IV) complexes, and other precursors [41].

Ligands and complexes differ in chemical nature, size, and geometric shape, which affect their DNA-binding properties. A detailed study of the method of binding polynuclear complexes of platinum with DNA produced a mixed result. The complexes were able to interact directly with DNA due to covalent binding, electrostatic forces, or intercalation [42]. Groove binding proved to be the cause of cell apoptosis [43].

Complexes owe their activity to the formation of new adducts with DNA. As a result, there are three important aspects to their binding: DNA pre-association, formation of DNA adducts, and induced conformational changes in DNA [44]. Multinuclear platinum complexes contain two or more bound platinum centers that can covalently bind to DNA and, therefore, are capable of forming a completely different kind of DNA adducts compared to cisplatin and its analogues. Multicore complexes represent a completely new paradigm of biologically active complexes, in particular, for platinum-based anticancer agents.

In our previous research, we proved that the bonds of bridged halide ligands had a greater lability, compared with the terminal ones [45]. This fact made it possible to introduce polynuclear platinum complex compounds into the biosystem. Their aquatization resulted in a break of bridging bonds with the formation of monomeric complexes.

P,N- and S,N-bidentate ligands have the properties of both soft and hard bases. As a result, they can direct organization of the metal coordination sphere (Fig. 5), as well as form bimetallic and polynuclear systems [43].

In our previous studies, we also described a method for the synthesis of binuclear complexes of divalent platinum. According to this method, amino acids (glycine, alanine, and valine) bound with two central atoms simultaneously via two donor atoms, i.e. bridges $[(NH_3)_2Pt(\mu-N,O-L)_2Pt(NH_3)_2](NO_3)_2$ [46]. The coordination of amino acids led to the formation of chelates, while the presence of a biogenic ligand in the internal coordination sphere reduced the overall toxicity



Figure 5 Binding of DNA with binuclear complex [43]

of the platinum complex. The compounds showed cytotoxic activity^I.

Popova and Trifonov synthesized antimicrobial binuclear platinum (II) complexes with tetrazole and 5-methyltetrazole with the composition of cis- $[{Pt(NH_3)_2(L-H)Cl}_3]Cl$ [18].

Lunagariya *et al.* studied the antibacterial activity of platinum (II) binuclear complexes based on pyrazolo [1,5-a] pyrimidine with neutral tetradentate ligands. The general formula was $[Pt_2LCl_4]$ [42]. The research featured five test organisms: two gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and three gram-negative (*Escherichia coli, Pseudomonas aeruginosa*, and *Serratia marcescens*). It also included an *in vitro* study of anti-tuberculosis activity against *Mycobacterium tuberculosis* H₃₇Rv strain.

Antibacterial actions include several phases of inhibition: cell wall synthesis, cell membrane functions, protein synthesis, nucleic acid synthesis, and folic acid synthesis. Chelation makes it possible to increase the values of the minimum inhibitory concentration of the complexes. This effect can be explained by the Tweedy's chelation theory: chelation allows the complex to penetrate the cell membrane. The complexes are toxic partially because the metal-ligand bond is strong. The toxicity differs from the type of substituent present in the synthesized compounds (Fig. 6) [42]. Active substituents in ligands have a high lipophilicity, which allows them to penetrate the complexes through the cell membrane. Complexes with a high-electronic substituent NO₂⁻ in its phenyl ring exhibit a greater antibacterial and anti-tuberculosis activity. Nitro groups act as chemical isosteres for oxygen atoms in the heterocyclic base of thymidine. However, they also participate in the "strong" O-H bond. As a result, the bond exhibits greater DNA-binding and antimicrobial activity than other complexes. The phenyl group is replaced with donor substituents, e.g. methoxy- or methyl group, and a hydrogen atom in the para position. Subsequently, the antibacterial activity against P. Aeruginosa and E. coli decreases, while acceptor chloro-, nitro-, and fluorosubstituents increase their efficacy against S. Marcescens and B. Subtilis [42].

Rubino *et al.* synthesized binuclear platinum (II) complexes with fluorinated heterocyclic ligands: 5-perfluoroalkyl-1,2,4-oxadiazolylpyridine and 3-perfluoroalkyl-1-methyl-1,2,4-triazolylpyridine [47]. Chlorine atoms served as bridges between the two platinum atoms. The complexes showed antimicrobial activity against *Escherichia coli*, *Kocuria rhizophila*, and two strains of *Staphylococcus aureus*. Azolate-bridged polynuclear platinum complexes formed DNA adducts as a result of additional electrostatic interaction.

¹ Salishcheva OV, Moldagulova NE, Proskynov IV. Investigation of the biological activity of organometallic complexes of platinum. XXI Mendeleev Congress on General and Applied Chemistry; 2019; St. Petersburg, St. Petersburg, 2019. p. 334.



Figure 6 Coordination of tetradentate pyrazolo-pyrimidine in the binuclear platinum (II) complex [42]

Icsel *et al.* obtained mono- and binuclear palladium (II) and platinum (II) complexes with ligands $L_1 = 5,5$ -diethylbarbiturate and pyridine derivatives $L_2 = 2$ -phenylpyridine, 2,2'-bipyridine and 2,2'-dipyridylamine. The general formula was $[M(L_1-N)_2(L_2-N,N')]$ and $[M_2(\mu-L_1-N,O)_2(L_2-N,C)_2]$ [48]. The complexes appeared to have similar DNA binding mechanisms.

There have been much fewer medical studies concerning palladium (II) complexes for medicinal use. Palladium (II) and platinum (II) complexes have different chemical properties because palladium compounds have a greater lability of the ligandcomplexing bonds. As a result, hydrolysis processes get accelerated, and the amount of dissociation products increases, e.g. aqua- or hydroxo-complexes, which are unable to fulfill their biological functions. To eliminate this factor, large heterocyclic and chelate ligands have to be introduced into the internal sphere.

Rubino *et al.* synthesized antibacterial palladium complexes with aromatic nitrogen, sulfur, and oxygencontaining ligands. They described the synthesis of binuclear platinum (II) and palladium (II) complexes with the 2,2'-dithiobis-benzothiazole (DTBTA) ligand $[Pd_2(\mu-Cl)_2(DTBTA)_2]Cl_2$. The research included an *in vitro* analysis of their antitumor activity against human breast cancer (MCF-7) and hepatocellular carcinoma (HepG2), as well as against *Escherichia coli* and *Kokuria rhizophila*. The complexes proved to have a greater antimicrobial effect against gram-positive bacteria than cisplatin. The low activity against gram-negative bacteria was explained by the fact that these bacteria have an additional outer membrane, which can interfere with the absorption of both compounds.

Terbouche *et al.* studied palladium (II) and ruthenium (III) binuclear complexes with phenylthiourea derivatives, namely their antibacterial properties, antioxidant activities, and stability (Fig. 7) [49]. They used the spectrophotometry method to assess the formation constants of the new Schiff-base alkali metal complexes and the systems formed by these chelates and cholesterol.

Chakraborty et al. described the synthesis and characteristics of binuclear palladium (II) complex



Figure 7 Tridentally coordinated bis-[1-(2-[(2-hydroxynaphthalen-1-yl)methylidene]amino}ethyl)-1-ethyl-3-phenylthiourea] ligand in a binuclear palladium (II) complex [49]

[(3,5- dimethylpyrazole)₂Pd₂(μ -3,5-dimethylpyrazolate)₂ (2,6-dipicolinate)] [50]. It was a dimer connected by two 3,5-dmpz units. One palladium atom contained two protonated 3,5-Hdmpz ligands and the other – one bidentate 2,6-dipicolinate, which made the complex asymmetric. The central nucleus of Pd₂N₄ consisted of six elements. It was a boat-like structure with palladium atoms located at the tops. The molecules assembled in an elongated zigzag one-dimensional network formed by 3,5-Hdmpz-carboxylate (2,6-dipic 2-) hydrogen bonds. The complex demonstrated antimicrobial activity against *Bacillus subtilis*, *Escherichia coli*, and *Aspergillus niger*. The minimum inhibitory concentration was 100 µg/mL.

Another study featured pyrazolate binuclear Palladium (II) complex $[Pd_2(\mu-dppz)_2(Hida)_2] \cdot CH_3$ OH·2H₂O (dppz = 3,5-diphenylpyrazolate) with monoprotonated iminodiacetate (Hida). It demonstrated antimicrobial activity against *Bacillus subtilis* [51]. The donor atoms of oxygen and nitrogen coordinated the pyrazolate ligand.

A binuclear pyrazolate square-planar palladium complex $Pd_2Cl_4L_2$ (L = 5-methyl-5-(3-pyridyl)-2,4imidazolidenedione ligand) with cis- and transconfigurations also showed antimicrobial activity [52]. The trans-isomer appeared more stable in the liquid and gaseous phase than the cis-isomer. The pyridinetype nitrogen atoms provided for the square-planar geometry around the metal center. Each palladium atom was coordinated by one nitrogen atom and three chlorine atoms, one serving as terminal and two as bridging ligands (Fig. 8). The initial mononuclear complex and the binuclear palladium complex were tested for antibacterial activity against six types of microorganisms: Staphylococcus aureus (ATCC 6633), Staphylococcus saprophyticus (ATCC 15305), Escherichia coli (Lio), Proteus vulgaris (Lio), Serratia marcescens (PTCC 1330), and Bacillus cereus (ATCC 7064). Bacterial growth was studied by disk diffusion, while the minimum inhibitory concentration of the



Figure 8 Binuclear pyrazolate square-planar palladium complex $Pd_2Cl_4L_{2of}$ (*trans*-configuration) with bridging chloride ligands [52]

chemicals was determined by *in vitro* dilution. The microorganisms were cultured in harvest broth and nutrient agar (Oxoid Ltd.). The agar culture medium included 0.5% of peptone, 0.3% of beef or yeast extract, 1.5% of agar, 0.5% of NaCl, and distilled water; pH = 6.8 at 25°C [52].

The compounds inhibited the metabolic growth of bacteria to varying degrees. The binuclear complex had a higher activity compared to the free ligand, while the ligand activity became more pronounced when coordinated with the metal. The increased activity of metal chelates could be explained by Tweedy's chelate theory: the polarities of the ligand and the complexing agent are restored by balancing the charges throughout the whole chelate ring. As a result, the lipophilic nature of the metal chelate increases and facilitates its penetration through the lipid layer of the bacterial membrane [53].

Plant extracts. Natural products or their extracts possess antimicrobial properties, e.g. grape skin or essential plant oils, e.g. of citrus fruits, wormwood, mint, and ginger [54–57]. When used in combination with nanoparticles, various functional essential oils produce a synergistic effect against multidrug resistant microbial pathogens (Fig. 9) [58].

CONCLUSION

Malicious microorganisms keep mutating. They grow ever more resilient to drugs, which triggers a never-ending search for new antimicrobial agents. Drugs based on organic ligand complexes exhibit an antimicrobial effect comparable to that of antibiotics. The complexation leads to a synergistic effect between the organic ligand and the complexing agent. Chelates of platinum, palladium, silver, iron, iridium, rhodium, ruthenium, cobalt, and nickel are therapeutic agents. Complexes with enhanced bioavailability have a better antimicrobial effect against pathogenic microorganisms. Metal-based drugs facilitate the transport of organic ligands towards the bacterial cell.

The reactivity of the central atom depends on the nature of the ligand and the coordination method. Coordination changes not only the thermodynamic stability and kinetic lability of the complex, but also the lipophilic properties that ensure the ability of the complex to penetrate the cell membrane. It stabilizes or destabilizes the oxidative state of the central atom. When complexes with functional multi-dentate ligands enter the internal sphere, it enhances the antimicrobial effect. The presence of a biogenic ligand in the coordination sphere reduces the general toxicity of platinum and palladium complexes. Drugs based on complexes with functional multi-dentate ligands exhibit a greater antimicrobial effect compared to free ligands. Inhibition of bacterial growth occurs at lower concentrations of metal complexes.

Active metal centers with a stable, inert, and nontoxic nature are of great value for biological systems. Polynuclear and heteronuclear complexes increase the number of active centers that block the action of bacterial cells and improve the formation of cross-links between different molecules. These valuable properties



Figure 9 Antimicrobial effect of nanoparticles used with functional essential oils [58]

encourage researchers to synthesize new complexes with antibacterial and antitumor properties. Due to their ability for covalent binding to bacterial cell DNA, polynuclear platinum and palladium complexes contain two or more bound metal centers that can form a completely different kind of DNA adducts, as compared to mononuclear precursors.

The biological activity of structural analogues of clinically approved platinum complexes has been focus of scientific attention in the recent decades. A further synthesis of complex antimicrobial compounds used in combination with other agents may help to build up a rich bank of substances with a great antimicrobial potential. In the long term, further studies of their antimicrobial action and the way it changes under various factors will make it possible to promptly overcome local or global outbreaks of infectious diseases, such as the current pandemic.

CONTRIBUTION

Authors are equally related to the writing of the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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Production of enriched lamb in biodegradable packaging

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Abstract:

The paper describes an environmentally safe technology for biofortifying lamb with target components in required concentrations packed in biodegradable film. To address the problem of micronutrient deficiency, we developed a biologically safe method of enriching lamb with organic iodine and selenium supplements. Introducing selenium and iodine feed supplements to the animals' diet increased the average daily growth of their live mass in experimental groups by 3.43, 6.72, and 14.92% in groups I (iodine), II (selenium), and III (iodine and selenium), respectively, compared to the control group (feed only). The analysis of immune-physiological status showed an increase in phagocytic number in experimental groups: by 5.1% (P > 0.95), 9.4%, and 14.5% in groups I, II, III, respectively. In addition, the highest phagocytic activity and phagocytic intensity were observed in animals of group II and group III, indicating their higher resistance to adverse environmental factors, compared to the control. There was an increase in iodine, selenium and zinc content in the lamb meat from the experimental groups grown on enriched diets. We recorded a higher concentration of the micronutrients in the lambs receiving "Yoddar-Zn" and "DAFS-25" supplements together (Zn – 980 µg/100 g; Se – 53.9 µg/100 g; I – 77.6 µg/100 g). We found that the contents of zinc, selenium, and iodine in 100 g of *m. Longissimus dorsi* were 8.2%, 77.0%, and 51.7%, respectively, of the required content in the daily diet. Thus, we can use this raw material to produce functional meat products. Packaging lamb in sodium alginate-based biodegradable film helped reduce moisture loss during storage.

Keywords: Lamb, environmentally-friendly technology, feed supplements, essential trace elements, iodine deficiency, biodegradable film, polysaccharides, sodium alginate

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INTRODUCTION

The project "Fundamentals of the Russian state policy for healthy nutrition of the population until 2020" aims to create a set of measures to meet health needs of different groups of the population in accordance with medical requirements and people's traditions, habits, and economic status [1]. Fortification, or the process of *in vivo* optimization of raw materials and later a final product, is a significant tool for functional and specialized nutrition, especially in the light of diet personification trend [2].

According to V.A. Tutelyan, academician of the Russian Academy of Sciences, human health is largely determined by nature, level, and structure of nutrition, which is reported to have a number of serious disorders.

Malnutrition is the main factor that causes irreparable damage to health, several times more severe than environmental pollution. It is the cause why 70% of the Russian population is reported to lack vitamin C, 40% have â-carotene and vitamin A deficiency, nearly a third of the population are vitamin B deficient, and absolutely everyone is iodine and selenium deficient.

The shortage of essential substances in nutrition is one of the most important issues in Russia. Many regions lack vital trace elements, such as selenium and iodine, in soil and water and consequently in livestock products [5].

According to WHO, sheep farming, a supplier of raw material for the meat processing industry, is now the third largest in the world. The use of lamb for the

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production of functional products is highly promising due to its contents of biologically active substances, such as complete animal protein, bioactive peptides, minerals (zinc, iron, selenium), vitamins, and fatty acids [2].

One of the ways to obtain high quality lamb meat that can provide people with essential trace elements is *in vivo* optimization of the meat chemical composition by adding essential nutrients into lamb diets [6]. The main advantage of *in vivo* lamb enrichment is the elimination of negative effect (overdose), since the supplement has already been "approved" by animals [7].

Meat processing companies today are extremely interested in innovative technologies that increase production profitability. It could open the floodgates to the global market, which is timely in view of the sanctions imposed on Russia.

In this regard, introducing organic trace elements into protein-carbohydrate complexes for agricultural animals' diets is one of the safest and inexpensive methods to obtain enriched meat and dairy raw materials [7, 8]. In humans and animals, iodine is found in inorganic compounds (iodides) and organic (about 75% of total iodine) covalently related forms (thyroglobulin), iodized amino acids (monoiodthyrosine and diiodothyrosine), and iodine-containing hormones (thyroxine and triiodothyronine). Inorganic iodine bound by chemical covalent bond (by amino acid residues - tyrosine, histidine) is easily organized and absorbed by internal secretion organs (thyroid system). Iodotyrosines are synthesized in thyroid follicles as part of thyroglobulin. "Iodine organification" occurs in the thyroid gland, where the enzymatic binding of inorganic iodine to amino acids of the protein thyroglobulin (iodization) - occurs every second. As a result of aromatic electrophilic substitution, iodide (J⁻) is embedded into the molecule of aromatic amino acid (tyrosine), forming a strong covalent bond with carbon (C-J). In addition to the thyroid gland, "iodine organification" is also carried out in the mammary and salivary glands, as well as other tissues and organs, though to a smaller degree [9].

Due to some circumstances, about 12% of total lamb production in Russia is industrially processed. The rest is sold mainly in unpacked carcasses, which results in mass loss and quality degradation [8].

Polyethylene, Saran (polyvinylidene chloride), and viscose polymer film materials protect the product from external influences, which improves the sanitary condition of meat and reduces its mass loss and bacterial seediness, promotes color preservation and prevents fat oxidation [3]. However, they not only increase the cost of the product but also make the environmental situation worse, as plastic waste takes too long to decompose. A solution to the problem can be to create environmentally friendly types of biodegradable packaging materials based on polysaccharide – sodium alginate [4]. This could ensure financial stability of processing enterprises,

through transporting refrigerated raw materials to remote regions as well.

Our aim was to develop a progressive technology for growing lamb enriched with organic form of iodine and selenium and packed in a biodegradable sodium alginate film.

STUDY OBJECTS AND METHODS

The experiment was conducted at Saratov State Vavilov Agrarian University. We formed four groups of Edilbay lambs (10 in each) aged 4.5 months by the analog method. Feed supplements were added to the diet once a day, as directed by the guidelines, namely "Yoddar-Zn" (100g/t of feed) and "DAFS-25" (1.6 mg/kg of premixed feed). The control group received only feed in a daily amount of 250–300 g per head in addition to the main diet. The first experimental group received feed and "Yoddar-Zn". The second experimental group had feed and selenium-based "DAFS-25". The third experimental group received feed, as well as "Yoddar-Zn" and "DAFS-25" supplements.

The researchers of Volga region Research Institute of Manufacture and Processing of Meat-and-Milk Production and Vavilov Saratov State Agrarian University developed feed supplements containing essential micronutrients further enriched with proteincarbohydrate complex and Coretron mineral feed based on "Yoddar-Zn" (Specifications 10.91.10-252-10514645-2019) and "DAFS-25" (Specifications 10.91.10-253-10514645-2019). Feed supplements were added to the diet as directed by the use instructions once a day together with grain feed (barley groats) in the amount of 10% of their quantity.

Our study objects were lambs, *m. Longissimus dorsi* lamb carcasses in sodium alginate biodegradable film (experimental) and without packaging samples (control). The control and experimental samples were stored in the refrigerator chamber at -1° C and relative humidity of 85%.

The protein-carbohydrate complex included pumpkin oil cake (20%), which is the by-product of oil processing. Due to cold pressing, the pumpkin oil cake preserves the bulk of nutrients, vitamins, and trace elements, biologically active components contained in seeds, and up to 8-12% of pumpkin oil. Pumpkin oil cake is an important source of protein (up to 45% raw protein). In addition to protein, pumpkin oil cake includes sugars, phytosterol, resins, organic acids, carotenoids, thiamine, riboflavin, phosphoric and silica acid salts, potassium, calcium, iron, magnesium. A significant zinc content in pumpkin oil cake, as well as in the oil (containing glycerides linolenic, stearic, palmitic, and oleic acids) produces its favorable impact on the body's numerous functions. Following the research findings, we applied for the Patent of the Russian Federation "Feed supplement for young sheep" (application registration number: Intellectual Product 2019140759 dated 09.12.2019).

Table 1 MRM ion impact parameters and electrical field (ESI)

 spray ionization conditions with positive ion registration

Solute	Precursor ion, m/z	Daughter ions, m/z	Fragmentor Voltage (Frag), V	Disso- ciation energy (CE), V
3-iodine-L-	364.0	134.9	112	30
tyrosine (MIT)	364.0	261.9	112	13
3,5-diiodine-L-	489.9	387.8	116	17
tyrosine (DIT)	489.9	260.9	116	30

Coretron is a fine grey powder consisting of amorphous silica of biogenic origin (100%). It is used in the production of feed and premixes for farm animals and birds. Coretron prevents feed particles from caking. It has insecticidal properties, stabilizes humidity, adsorbs and excretes mycotoxins, and is a source of water-soluble silicon needed to improve calcium assimilation and provide stable functioning of animals' smooth muscles of the intestines and stomach. An important advantage of Coretron is that it eliminates product tracking, mold and mycotoxins formation, and destroys adult insect and larvae species during transportation and storage. In addition, it ensures systematic reduction of helminths in the digestive tract and saturates the body with amorphous silicon. It does not contain genetically modified ingredients. The quantity of iodine and selenium is evaluated depending on their content in the feed. Feed supplements do not violate intestinal normal flora and do not have toxic and sensitizing effects.

The main authenticity criteria for supplements based on iodized milk proteins is the presence of iodotyrosines and the degree of iodization. Obtaining organic iodine is based on enzymatic iodization of amino acid residues of cow's milk whey proteins. Until the present, the existing methods have stood on the voltammetric determination of inorganic iodine. The Federal Research Center of Food Systems named after Gorbatov (Russian Academy of Sciences) developed and certified a control method for the content of iodotyrosines in biologically active supplements (Instruction 103.5-132-2012) and State Standard 33422-2015^I. The method allows identifying organic iodine (iodotyrosines) [10, 11]. Determination of iodotyrosines in complex food matrices containing the organic form of iodine requires the use of highefficiency liquid chromatography technique with mass spectrometric detection (HPLC - MS/MS). The determination of organic iodine was carried out on an Agilent Technologies 6410 Triple Quadrupole system.

The conditions for recording analytical signals in the Multiple Reaction Monitoring (MRM) mode are presented in Table 1.

Confirming the presence of certain solutes requires the use of mass spectrometric identification since food and raw materials consist of organic substances that affect measurement, such as HPLC or GC. HPLC methods with classical detectors of different types (spectrophotometric, diode matrix, fluorescent, refractometric) and GC (flame ionization, electron gripping) are not selective in the determination of organic iodine and do not allow the necessary sensitivity in the study of food samples and raw materials. Voltammetry cannot be applied in the determination of iodotyrosines, as it is for analyzing inorganic iodine only. The possibility of chromatographic separation and identification of iodotyrosines in case of presence of organic impurities almost completely eliminates errors during analysis. It allows recommending HPLC - MC/MC as a reliable and highly accurate method of iodotyrosines determination in meat and meat products.

M. Longissimus dorsi from lamb carcasses was coated with sodium alginate biodegradable film (E401). Sodium alginate is used as enterosorbent: it exhibits sorption activity against heavy metals and radionuclides, as well as significantly reduces cholesterol levels in blood. In medicine, sodium alginate is used as a drug (antacid) to treat gastrointestinal diseases. Alginates have antisclerotic, immune-modulating and antimicrobial properties, improve carbohydrate metabolism, reduce lipids in the blood, and normalize thyroid function, as it contains iodine. Due to its natural origin, sodium alginate has a high safety level for humans, certified for the production of baby food.

Calcium chloride is a dietary supplement (E509) that refers to emulsifiers and is a drug that complements the lack of calcium in the body. It is generally used together with other hydrocolloids: carrageenans, pectin, and most often with sodium alginate, which needs its ions to form biodegradable food coatings for meat raw materials.

The mechanism for alginate gels formation involves the joint binding of calcium ions between single line polygaluronate sequences. The chains of macromolecules bound in such a configuration have pores or cavities corresponding to the size of the Ca^{+2} ion radius. Gelling is intense when filling pores with calcium ions.

To produce biodegradable film, we prepared a homogeneous 2% solution that was constantly mixed at 150–200 rpm. Alginate film formed as a result of spaying 0.05% calcium chloride (pH below 3.6). Calcium chloride, interacting with sodium alginate, forms a thin stable, thixotropic, transparent protective coating. To accelerate the formation of the coating outer layer, it was fixed by the flow of air in the refrigerating chamber.

The mineral composition of lamb muscle tissue was assessed for the presence of micronutrients (Se, Zn, I). Macro- and micronutrients were determined by atomic

¹ State Standard 33422-2015. Meat and meat products. Determination of iodotyrosines using high performance liquid chromatography with mass spectrometry detection. Moscow: Standartinform; 2016. 10 p.

Element	Content, µg/g					
	Control group	Group I (iodine)	Group II (selenium)	Group III (iodine, selenium)		
Arsenic (As)	0.002 ± 0.0004	0.003 ± 0.0007	0.003 ± 0.0005	0.002 ± 0.0005		
Cadmium (Cd)	< 0.00048	0.001 ± 0.0003	0.0005 ± 0.00015	< 0.00048		
Lead (Pb)	0.008 ± 0.0016	0.007 ± 0.0013	0.01 ± 0.002	0.01 ± 0.002		

Table 2 Content of toxic elements in lamb grown on enriched diets

absorption spectroscopy (State Standard 53182-2008^{II}, State Standard 31660-2012^{III}, State Standard 30178-96^{IV}, State Standard 33422-2015^V).

Immuno-physiological parameters of blood were analyzed by standard methods: the number of red and white blood cells was counted in Goryaev's chamber, haemoglobin was determined by Sahli's method, total serum protein was refractometrically measured by McCord, phagocytic number, phagocytic activity, and phagocytic intensity Mancini method.

Toxic elements - lead, cadmium and arsenic - were determined according to Methodological Guidelines 4.1.986 "Methods of measuring the mass fraction of lead and cadmium in food and food raw materials by electrothermal atomic absorption spectrometry"VI established by the Scientific Council for Analytical Methods 450×. Statistical processing of the results on the dynamics of changes in hematological and biochemical parameters of blood serum was carried out according to standard procedures, using the Microsoft Excel application 2010 (Microsoft Corp. USA) and the StatPlus 2009 Professional 5.8.4 for Windows statistical data analysis package (StatSoft Inc., USA), with the Student t criterion applied to assess the validity of differences between experimental and control samples. Based on the arithmetic mean and standard deviation, we determined the standard error of the arithmetic mean and the boundary of its confidence interval, taking into account the coefficient t(n, p) at a significance level of 95% (P = 0.05) and number of measurements. The significance of differences between the average values in the experimental and control tests was assessed by the *P*-value in the variant of a two-sample unpaired *t*-test

with unequal variances. The differences were considered significant at $P \ge 0.05$. In addition, we observed the inequality *t*, *t*(*n*, *p*) at *n*= (*df*+1) (where *df* is the number of degrees of freedom), P = 0.05, where

$$t = \frac{\left|x_1 - x_2\right|}{\left(s_1^2 + s_2^2\right)^{1/2}}$$

where x_1 and x_2 are arithmetic mean values, s_1 and s_2 are their standard errors for two experimental data samples [12].

RESULTS AND DISCUSSION

High productivity of small cattle is impossible without rational and full-value feeding based on the knowledge of physiological state, level of productivity, intended use and need in energy, nutrients, minerals, vitamins and other biologically active substances. Highly important is to balance diets in minerals and micronutrients, especially in the regions where their content is low.

To optimize the chemical composition of lamb *in vivo*, we studied the efficiency of feed enriched with organic forms of iodine, selenium, and zinc ("Yoddar-Zn" and "DAFS-25"), when rearing small cattle.

At the initial stage, we performed a sanitary examination of lamb meat from the animals grown on enriched diets. The content of toxic elements in the lamb under study is presented in Table 2.

The findings showed that the content of toxic elements in the lamb from experimental groups complied with the requirements of the Technical Regulations of the Customs Union "On meat and meat products safety" (TR CU 034/2013^{VII}).

The study of "DAFS-25" and "Yoddar-Zn" effects, as well as their combined effect on the lambs' resistance, showed that the hematological indicators of the animals were within physiological norms. At the same time, blood morphological composition and biochemical parameters showed intergroup differences (Table 3).

We detected that hemoglobin was higher (P > 0.99) in the lambs of the experimental groups. The concentration of total protein during the same period was slightly lower, which might be driven by more intensive protein exchange processes and better growth energy. No reliable differences were established in the groups in terms of blood cells (Table 3).

¹¹ State Standard 53182-2008. Foodstuffs. Determination of trace elements. Determination of total arsenic and selenium by hydride generation atomic absorption spectrometry (HGAAS) method after pressure digestion. Moscow: Standartinform; 2010. 16 p.

^{III} State Standard 31660-2012. Foods. Anodic stripping voltammetric method of iodine mass concentration determination. Moscow: Standartinform; 2012. 15 p.

^{IV} State Standard 30178-96. Raw material and food-stuffs. Atomic absorption method for determination of toxic elements. Moscow: Standartinform; 2010. 8 p.

^v State Standard 33422-2015. Meat and meat products. Determination of iodotyrosines using high performance liquid chromatography with mass spectrometry detection. Moscow: Standartinform; 2016. 10 p.

^{v1} MUK 4.1.986-00 Metodika vypolneniya izmereniy massovoy doli svintsa i kadmiya v pishchevykh produktakh i prodovol'stvennom syr'e metodom ehlektrotermicheskoy atomno-absorbtsionnoy spektrometrii [MG 4.1.986. Methods of measuring the mass fraction of lead and cadmium in food and food raw materials by electrothermal atomic absorption spectrometry]. Moscow: Federal Center for State Sanitary and Epidemiological Supervision of the Ministry of Health of Russia; 2000. 32 p.

^{VII} TR TS 034/2013. Tekhnicheskiy reglament Tamozhennogo soyuza "O bezopasnosti myasa i myasnoy produktsii" [TR CU 034/2013. Technical Regulations of the Customs Union "On meat and meat products safety"]. 2013. 108 p.

Group		Lambs 4.5 mont	ths old $(n = 25)$			Lambs 7.5 mor	ths old $(n = 25)$	
Indicator	Control group	Group I (iodine)	Group II (selenium)	Group III (iodine, selenium)	Control group	Group I (iodine)	Group II (selenium)	Group III (iodine, selenium)
Erythrocytes, mln/µL	14.30 ± 0.03	14.60 ± 0.02	13.90 ± 0.03	14.00 ± 0.01	10.01 ± 0.01	10.40 ± 0.02	10.07 ± 0.02	10.70 ± 0.02
Leukocytes, thousand/µL	4.00 ± 0.02	3.9 0 ± 0.01	4.10 ± 0.02	4.20 ± 0.02	8.21 ± 0.01	8.25 ± 0.01	8.26 ± 0.01	8.28 ± 0.02
Hemoglobin, g/L	130.00 ± 0.01	139.00 ± 0.01	134.00 ± 0.01	129.00 ± 0.01	128.00 ± 0.04	131.20 ± 0.07	132.70 ± 0.03	133.20 ± 0.09
Total protein, g	85.30 ± 0.09	84.90 ± 0.03	85.30 ± 0.05	85.30 ± 0.04	69.20 ± 0.02	64.00 ± 0.07	62.10 ± 0.04	61.00 ± 0.01
Phagocytic number, unit	1.33 ± 0.02	1.38 ± 0.02	1.30 ± 0.02	1.32 ± 0.02	1.38 ± 0.02	1.45 ± 0.01	1.51 ± 0.02	1.58 ± 0.02
Phagocytic activity,%	56.80 ± 0.03	53.40 ± 0.03	52.90 ± 0.03	56.10 ± 0.03	55.70 ± 0.01	57.70 ± 0.02	59.20 ± 0.03	59.70 ± 0.02
Phagocytic intensity, unit	2.33 ± 0.03	2.17 ± 0.03	2.26 ± 0.03	2.20 ± 0.03	2.34 ± 0.01	2.46 ± 0.05	2.55 ± 0.02	2.70 ± 0.02

Table 3 Immuno-physiological blood indicators of lambs on different diets

The lambs of the experimental groups had a higher phagocytic number compared to the control, whose diet included only feed: 5.1% (P > 0.95), 9.4% (P > 0.95), and 14.5% (P > 0.99) in groups I, II, III, respectively. In addition, the meat of group II and group III had increased phagocytic activity and phagocytic intensity. This indicated their higher resistance to adverse environmental factors compared to the control group.

Thus, selenium and iodine feed supplements in the diet of lambs in the early post-embryonic period stimulate their development and increase resistance.



Control group



Group II (selenium)

Meat quality is largely determined by the histological structure of animal muscle tissue and depends on the size of muscle fibers, as well as condition and structure of connective and adipose tissues. To assess the meat quality, we studied the changes in the histological structure of *m. Longissimus dorsi* carcasses of the four lamb groups depending on their diet.

The muscle tissues of all the studied lambs grown on various diets had morphological features characteristic of the beginning of rigidity. We detected no significant differences in muscle tissues in the course of autolysis.



Group I (iodine)



Group III (iodine and selenium)

Figure 1 Microstructure of lamb tissue (m. Logissimus dorsi) grown on different diets

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Age, months	Mass growth, g (n = 10)					
	Control group	Group I (iodine)	Group II (selenium)	Group III (iodine and selenium)		
4.5-7.5	111.67 ± 0.12	115.50 ± 0.22	119.17 ± 0.32	128.33 ± 0.22		

Table 4 Average daily growth of living lamb mass $(M \pm m)$

Table 5 Chemical composition of lamb meat obtained from animals on different fattening diets

Group	Moisture, %	Dry	Protein, %	Fat, %	Ash, %	Energy
		substance, %				value/kg, kJ
Control	71.12 ± 0.22	28.88	18.23 ± 0.12	9.60 ± 0.10	1.05 ± 0.02	8167.60
Group I (iodine)	$71,01 \pm 0,31$	28.98	18.13 ± 0.11	9.84 ± 0.13	1.01 ± 0.01	8239.19
Group II (selenium)	70.85 ± 0.25	29.15	18.06 ± 0.14	10.11 ± 0.17	0.98 ± 0.01	8329.87
Group III (iodine and selenium)	69.96 ± 0.23	30.04	17.94 ± 0.12	11.17 ± 0.14	0.95 ± 0.01	8718.02

The micro-structural analysis revealed that the use of "DAFS-25" and "Yoddar-Zn" in raising young sheep did not cause any negative changes in muscular fibers and surrounding connective tissue, endomysium and perimysium. This finding indicated that they can be used for fattening animals in industrial production.

The average daily growth of live mass among lambs aged 4.5–7.5 months showed that the animals in the experimental groups had higher weight than those in the control group. In particular, the differences were 3.83 g (3.43%) in group I ($P \ge 0.999$), 7.50 g (6.72%) in group II ($P \ge 0.999$), and 16.66 g (14.92%) in group III ($P \ge 0.999$) (Table 4).

In our opinion, the mass growth was due to the diet enrichment with organic selenium and iodine.

The chemical composition of lamb is shown in Table 5.

The meat of the experimental groups had a better composition. With equal protein and ash content, it contained less water and had higher caloricity (Table 4).

Mineral substances are a structural material for tissues and organs. They are a part of organic substances participating in respiration, hemotogenesis, digestion, absorption, synthesis, flow, and release of metabolism products from the body. They are interrelated with the activity of many biologically active substances and generally affect metabolism and numerous physiological functions of the organism. Iodine, selenium, and zinc are of high importance in the metabolism of the organism.

The content of zinc, iodine, and selenium in lamb was directly dependent on their content in the diets, with a higher content recorded in lamb of group III (Fig. 2).

Soft enzymatic hydrolysis, chromatography and mass spectrometry were used to identify and assess the site of iodine incorporation into proteins. We found no changes in hydrolysis of lamb proteins involving proteolytic enzymes, unlike acid hydrolysis. There were no changes in hydrolysis products. Figures 3, 4 show the chromatograms of the lamb samples with iodotyrosines.

Iodotyrosine determination can be performed using diode array or spectrophotometric detectors. However, when the target iodotyrosines are extracted from compounds such as food products, the finished samples may contain foreign organic impurities. In some cases, while using diode array or spectrophotometric detectors, mistakes may occur due to the presence of crosssignal substances. Determining separate iodotyrosines may also be difficult due to incomplete separation of chromatographic peaks.

We should take into account that a column is not capable of separating iodotyrosines in the presence of organic compounds as well as it works without foreign impurities. Liquid chromatography with mass spectrometric detection (HPLC – MC/MC) is a more reliable method to determine iodotyrosines in food products than HPLC with diode array or spectrophotometric detectors. Its advantage is high sensitivity toward the components under examination.



Figure 2 Zinc, selenium, and iodine contents in 100 g of lamb depending on the diet



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Figure 3 Chromatogram of a lamb sample with iodthyrosines (total ion current and MRM transitions of monoiodotyrosine)



Figure 4 Chromatogram of a lamb sample with iodotyrosines (total ion current and MRM transitions of diiodotyrosine)

The selective ion detection mode eliminates falsepositive results in case of cross-signal substances present in samples. The monitoring mode of daughter ions allows determining of the compounds when their molecular masses match.

In the course of analysis, we tested the method of ionization by spraying in the electric field (ESI) with pre-column derivatization of iodotyrosines by a butanol:acetylchloride mixture (4:1). As shown in Figs. 3, 4, the chromatograms of the lamb samples with an iodotyrosine content are presented in the mode of monitoring daughter ions on the three-quadrupole mass detector Agilent 6410. The confirming ions for these substances were different; solutes were chromatographically separated with a fine-grained column with C18 phase (Agilent Eclipse XDB C18, 4.6×50 mm, 1.8μ m).

Thus, we identified organic iodine and determined its quantity in the form of iodotyrosines. It was due to its covalently bound form that organic iodine was able to exhibit many biological properties, including through iodine-containing hormones, thyroxine and



Figure 5 Loss of moisture in experimental and control lamb groups during storage

triiodothyronine, involved in the regulation of all metabolic processes in the human body.

We found that the use of mineral supplements in the diets of small cattle is promising and relevant. It allows obtaining lamb enriched with organic trace elements.

The duration of lamb storage under refrigerator conditions is limited by a number of factors, including temperature, humidity, carcass contamination, postmortal biochemical processes, as well as the presence of eco-toxicants and residues of medicines in meat [13]. To eliminate the negative impact of refrigeration on meat quality and extend the shelf life, we covered the cuts with biodegradable film based on sodium alginate.

Sodium alginate film is homogeneous in structure, flexible, and transparent. It has structural resistance and barrier effect against air oxygen and microorganisms. Biodegradable package does not lose its structureforming properties after refrigeration; there is no need to remove it before use. An important advantage of bio-film is sensory acceptability and low cost [14].

Loss of meat mass due to moisture evaporation during cooling and freezing is not only a quantitative characteristic. The surface of meat after draining becomes porous, with temperature burns resulting in the deterioration of commercial appearance. Meat easily absorbs foreign odors and oxidation processes accelerate. Moisture losses during lamb cooling in biodegradable film are presented in Fig. 5.

Moisture loss during storage of the control samples (lamb without coating) was 3.71% higher than in the

experimental group (lamb in biodegradable film). This confirmed that biodegradable film provides dense adhesion to the surface of raw materials, which prevents moisture exchange and, therefore, minimizes moisture loss [15].

CONCLUSION

The use of selenium and iodine supplements, namely "Yoddar-Zn" and "DAFS-25", in small cattle diets stimulated their development, increased their resistance and productivity, and enriched lamb meat with organic zinc, selenium, and iodine. This has a big medical and social importance for preventing micronutrient deficiency in the population.

We found that the hematological indicators of animals grown on enriched diets were within physiological norms. The content of hemoglobin was higher in the experimental groups (P > 0.99). The concentration of total protein during the same period was slightly lower, which might be driven by more intensive protein exchange processes and better growth energy. No reliable differences were established in the content of red blood cells in the groups (Table 3).

We detected a higher phagocytic number in the experimental groups. Also, the meat of group II and group III exceeded the control group for phagocytic activity and intensity. This indicated their higher resistance to adverse environmental factors compared to the control group.

We determined organic iodine and its quantity in the form of iodotyrosines. It was due to its covalently bound form that organic iodine was able to exhibit many biological properties, including through iodinecontaining hormones – thyroxine and triiodothyronine – involved in the regulation of all metabolic processes in the human body. Monitoring trace elements showed that zinc, iodine, and selenium contents in lamb meat directly depended on their amounts in the lambs' diet. We detected their higher concentration in lamb meat of the 3^d experimental group (iodine and selenium diet).

The microstructural analysis revealed that the use of "DAFS-25" and "Yoddar-Zn" in raising young lambs did not cause any negative changes in muscular fibers, endomysium and perimysium. Thus, they can be used for fattening animals in industrial production.

CONTRIBUTION

Authors are equally related to the writing of the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest

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Toxicity of apple juice and its components in the model plant system

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Abstract:

Introduction. In view of the ongoing research into the negative effects of fruit juice on human health, we aimed to study the subchronic toxicity of apple juice, a model mixture based on its components, and ethanol on biomass growth, cellular oxidative enzymes, and chromosomal abnormalities in *Allium cepa* roots.

Study objects and methods. Our objects of study included clarified apple juice and its components such as fructose, glucose, sucrose, D-sorbitol, and malic acid. After treating *Allium cepa* roots with apple juice and a model mixture in different concentrations, we analyzed their toxic effects on biomass growth, malondialdehyde levels, as well as the nature and frequency of proliferative and cytogenetic disorders in the plant tissues.

Results and discussion. The incubation in an aqueous solution of apple juice at a concentration of 1:5 inhibited the growth in root mass by 50% compared to the control (water). The mitotic index of cells decreased with higher concentrations of juice, reaching zero at a 1:5 dilution. The fructose and model solutions in the same concentrations appeared less toxic in relation to cell mitosis and root mass growth. Although malondialdehyde levels increased in the onion roots treated with juice and model solutions, they were twice as low as in the control due to the juice's antioxidant activity. Adding 1% ethanol to the 1:2 diluted juice abolished the effect of acute toxicity on root growth and reduced malondialdehyde levels by 30%.

Conclusion. The study revealed a complex of interdependent biomarkers of apple juice responsible for its subchronic toxicity in *Allium cepa* roots. These data can be used to create biological response models based on the approaches of systems biology and bioinformatics.

Keywords: Juice, fructose, Allium cepa, biotesting, toxicity, cytogenetic analysis, biomarker

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INTRODUCTION

New approaches to food testing are becoming increasingly urgent today, in view of continuously growing production and consumption of various foods. These approaches are primarily meant to ensure food safety by identifying possible toxic effects that food products and related additives may have on human health [1–4]. Any food component can have a negative effect on the human body. Excessive consumption can lead to the accumulation of toxic metabolic products. Some components can cause allergic reactions and modulate adaptation reactions [5]. Such studies are primarily based on *in silico* and *in vivo* methods of testing various types of food products.

Fruit juice is an integral part of the human diet and, undoubtedly, a complex food system. It contains physiologically active substances (vitamins, minerals, antioxidants, enzymes, and amino acids) that regulate a variety of metabolic processes and increase the body's resistance to infections. In addition, epidemiological studies have proved that fruits and vegetables reduce the risk of chronic diseases [6, 7]. Clinical studies also

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confirm that fruit juice can have beneficial effects on blood parameters, cholesterol, and heart function, as well as prevent cancer and Alzheimer's disease [8–11].

However, the benefits of fruit juice are not as evident as they may seem [12]. As we know, natural mutagens, such as pyrolysidine alkaloids and some flavonoids, account for about 1% of dry matter in almost all higher plants. Moreover, vitamins C, E, and A can have mutagen-potentiating effects [13]. Recent studies have shown that fruits and juices can contribute to the development of cancer and asthma in children [7, 14–16].

Sugars contained in fruit juice and their potentially adverse metabolic effects have long been in the center of scientific debates. Fructose, in particular, is one of the main carbohydrate components of fruit juice. As early as the 1980s, it was considered responsible for several metabolic abnormalities [17, 18]. This carbohydrate can be "toxic", especially when consumed with sweetened drinks. Moreover, it can participate in the pathogenesis of noncommunicable diseases such as obesity, diabetes, or arthritis [19, 20]. Sucrose, another carbohydrate component of fruit juice, has also shown negative mutagenic effects [21]. In the USA and Europe, a half of sugar consumption accounts for sweetened products with a thick consistency (yogurt, candy and chocolate bars, ice cream, etc.) and the other half, for sweetened fizzy drinks and fruit juices. The negative health effects of fructose have encouraged European countries to impose taxes on sweetened drinks [22].

Quality control is an equally important aspect of fruit juice safety. The past decades have seen a significant increase in the demand for juice, partly due to continuous improvement of its sensory (color, smell, texture, and taste) and technological (convenient packaging, long shelf life) characteristics. As a result, juice composition has undergone a number of changes, with added microelements and synthetic substances (acidity regulators, stabilizers, thickeners, and sweeteners). The technology of juice production (e.g., heat treatment) also affects juice properties. Although the use of these additives is strictly regulated, scientists are increasingly emphasizing a need for rigorous research into the mechanisms of their toxic manifestations [23, 24].

Studies have shown that food additives can lead to cancer and change the functioning of various organs [25–27]. Children are especially vulnerable to their toxic effects that can provoke allergies and other diseases if manufacturers do not follow strict regulations [28]. Although several types of food additives can be used in juice in various combinations, there have been no studies into their integrated toxic effect on the human body. Moreover, as chemically active agents, these additives or their oxidation products can interact with natural organic or inorganic juice compounds and cause especially dangerous mutagenic and carcinogenic effects [29]. In this regard, *in vivo* studies of subchronic toxicity of fruit juice components are becoming increasingly urgent. Modern food scientists aim to develop models in which the processes of detoxification and metabolism of toxic compounds are similar to those in the human body. At the same time, they strive not to use laboratory animals [4].

We find biotesting quite effective when using plants, in particular *Allium cepa* roots (Allium test). This test has been successfully used to study toxicity, cytotoxicity, and genotoxicity of various agents, including food additives, as well as to determine genotoxic effects of medicinal plant extracts [23, 30–32]. The Allium test is simple, economical, well reproducible, highly sensitive, applicable in a wide pH range (3.5–11.0), and just as efficient as other biotests. We believe that this test can be reliably used to assess subchronic toxic effects of various juice components, both individually and in combination with each other. Similar studies in animals may not produce objective results. The components under study may be present in the animals' basic diet, compromising the results.

Our aim was a comparative study of subchronic toxic effects that apple juice, its components, and ethanol have on biomass growth, oxidative enzyme activity at the cellular level, as well as the nature and frequency of proliferative and cytogenetic disorders in *Allium cepa* roots.

STUDY OBJECTS AND METHODS

To model the composition of apple juice, we used the following materials: glucose (SIGMA-ALDRICH, lot. SLBZ9363, Germany), fructose (SIGMA-ALDRICH, lot. SLCC1647, Germany), sucrose (SIGMA-ALDRICH, lot. BCCB2955, Germany), D-sorbitol (SIGMA-ALDRICH, lot.BCBT4918, Germany), malic acid (SIGMA-ALDRICH, lot.MKBS7851, Germany), and clarified apple juice (10.5% carbohydrates) purchased from a retail outlet.

For biotesting, we used small 5-7 g *Allium cepa* L. onions of Stuttgart variety with a diameter of 2.5–3 cm, with their dry scaly outer layers removed. The roots were preliminarily germinated in 15 cm³ test tubes with bottled water in a thermostat (23–25°C) for two or three days in complete darkness. The bulbs with a sprouted root length of at least 1 cm were selected for further experiments. Prior to treating them with juice solutions and other compounds listed above, we measured the average root mass in the control group.

Then, the control samples were incubated in water, while the test samples were incubated in aqueous solutions in a thermostat $(23-25^{\circ}C)$ in complete darkness for 1, 2, or 3 days, depending on the purpose of the experiment. After incubation, the roots were cut off, wiped with filter paper, and weighed [33]. EC₅₀ was determined as a concentration of material that reduced the test function (growth in root mass) by 50% compared

to the control, taking into account the average mass of the roots before treatment (except when they were treated with fructose).

For cytogenetic analysis, the cells of the root apical meristem were stained with a 2% aceto-orcein solution (1 g of orcein diluted in 50 cm³ of 45% CH₃COOH). For long-term storage, the roots were placed in a 70% ethanol solution used as a preservative. Instant squash preparations were obtained to analyze the division of apical meristem cells, using an Axioskop 40 light microscope (Zeiss). In particular, we determined the mitotic index (ratio of dividing cells to total cells) and the chromosome aberration index (number of chromosomal aberrations related to total cells).

The intensity of lipid peroxidation in root tissues was determined based on the amount of malondialdehyde (MDA) interacting with 2-thiobarbituric acid and expressed in µmol/g (MDA in fresh mass) [34]. We placed 0.2–0.9 (\pm 0.0001) g into a 15 cm³ polymer tube, added 1 cm³ of trichloroacetic acid (Merck, Germany) at a concentration of 200 g/dm³ and then another 3 cm³ of the same solution after stirring the mixture. The tubes were centrifuged at 1000 g and 4°C for 15 min. Then, we transferred 1 cm³ of the upper liquid layer into another tube and added 4 cm³ of thiobarbituric solution – 0.5 g thiobarbituric acid (Diaem, Russia) and 100 cm3 of trichloroacetic acid (200 g/dm³). The tubes were tightly closed and placed for 30 min in a water bath at 95°C, followed by cooling in an ice bath. Next, the tubes were centrifuged for 10 min at 1000 g and 20°C. The solutions were spectrophotometrically detected on a Cary WinUV 100 spectrophotometer (Varian, USA) at 600 and 532 nm.

Statistical processing was performed in Microsoft Excel and Statistica (version 12). The Student's criterion and Fisher transformation were used for comparative analysis of percentages.

RESULTS AND DISCUSSION

After a three-day sprouting, the onion roots were treated with apple juice diluted with water for 2 days

 $\begin{array}{c} 120 \\ 100 \\ \hline \\ 100$

Figure 1 Growth in root mass after treatment with apple juice in different concentrations (n = 10). * $P \le 0.15$

to determine the degree of juice dilution that causes subchronic toxicity. According to the Allium test, toxicity was determined by the changes in root mass after exposure to juice solutions, compared to the control. As we can see in Fig. 1, a decrease in root mass was observed at ten times dilution and EC_{50} was recorded at five times dilution ($P \le 0.15$).

The cytological analysis of the root meristem cells showed that higher juice concentrations decreased the mitotic index more intensively (Fig. 2) than the growth in root mass (Fig. 1). As we can see, the level of proliferation for meristematic cells, when treated with a 1:20 diluted solution of apple juice, was half the control values, and their division almost stopped in the roots with a 50% delay in mass growth (EC₅₀).

As we know, plants grow due to two main processes, cell division and extension. Like all eukaryotes, plant cells enter the cell cycle in response to external mitogenic stimuli. This process is regulated by a large number of compounds, such as phytohormones, ARGOS proteins, CLE peptides, transcription factors, cyclins, and cyclin-dependent protein kinases. Decreased cell proliferation during stress or after treatment with abscisic acid may result from activated expression of genes that encode protein inhibitors of cyclin-dependent protein kinases, ICK/KRP. However, the mechanisms that control differentiation can function independently of the cell cycle [35]. It appears that the subchronic amounts of apple juice triggered similar processes in our study and, therefore, the inhibition of cell proliferation did not significantly affect the growth in root mass.

The percentage of chromosomal aberrations in dividing cells in relation to total stained cells was quite low, about 0.4%, both in the control and the test samples treated with 1:20 and 1:10 diluted juice. We found no effect dependent on the amount. Neither could we determine this indicator in the test samples treated with a higher concentration of juice (1:5 and 1:2 dilution) due to the absence of dividing cells.

The abnormalities detected in both the control and the test samples included the adhesion of chromosomes to each other, their leading during anaphase, as well



Figure 2 Mitotic index of root meristem cells after treatment with apple juice in different concentrations (n = 10). $*P \le 0.05$



Figure 3 Chromosomal aberrations in onion root meristem cells: adhesion in metaphase (a), leading in anaphase (b), disorganization in anaphase (c), and a micronucleus in telophase (d)

as disorganization and disordered separation during metaphase and anaphase. However, these abnormalities were not distributed evenly among the samples. For example, aberrations (Fig. 3) and anaphase leading were almost ten times as high in the test samples. Also, micronuclei were detected during telophase and interphase in the samples treated with tenfold diluted juice.

With the data at hand, we had to understand which of the juice components was responsible for the identified toxic effects and to what extent. Carbohydrates are a major component of apple juice, with up to 10% of fructose, glucose, and sucrose (in 100 g juice). Taking into account published data on the negative effects of glucose on plant growth and development, we conducted several experiments to determine their toxicity for onion roots [36, 37]. We started with fructose, as its content in apple juice is two times as high as that of glucose and sucrose.

As we can see in Fig. 4, higher concentrations of fructose delayed the growth in root mass, but only a 10% concentration of this carbohydrate revealed a significant difference. After treatment with 10 and 15% fructose solutions, the roots died, becoming thin, soft, and slightly mucous. In the Allium test, this finding probably indicated acute toxicity of fructose in the given concentrations. Thus, the concentration of fructose should not exceed 10%.

Fructose at concentrations of 2 and 5% decreased the mitotic index in the test samples by only 17 and 33%, respectively, compared to the control (Fig. 5). Like in the previous test, the comparative cytogenetic analysis did not reveal a significant increase in the number of chromosomal aberrations, compared to the control. However, we observed some redistribution in their spectrum. For example, higher concentrations of fructose in the test samples caused more disorders such



Figure 4 Growth in root mass after fructose treatment (n = 7). * $P \le 0.1$

as chromosomal bridges, fragmentation, and segregation (up to 20%), compared to the control.

According to the results, the subchronic toxicity of fructose, one of the main components of apple juice, is mainly associated with a weak mitosuppressive effect in the root meristem cells.

Then, we prepared a model aqueous solution from the main chemical components of apple juice. Their concentration ratios corresponded to those in juice [38]. In particular, 100 mL of the model solution contained 7 g fructose, 2 g glucose, 1 g sucrose, 0.5 g D-sorbitol, and 0.3 g malic acid. Prior to that, we had measured the pH of the study objects to make sure that its range was acceptable for the Allium test (Table 1). Next, we analyzed the subchronic toxicity of the resulting model solution and apple juice in *Allium cepa* roots after two days of germination and two days of treatment.

According to the results, the growth in root mass after treatment with juice was 40% lower than after using the model solution (Table 2), despite the same degree of dilution (1:5, P < 0.05). The cytological analysis showed that the mitotic index of the root meristem cells after treatment with the 1:10 and 1:5 model solutions did not differ much from the control. However, treatment with the 1:10 and 1:5 diluted juice, just like in the previous experiment (Fig. 2), reduced the mitotic index ten times and led to an almost complete halt in cell division. Thus, the chemical components of the model solution, which make up



Figure 5 Mitotic index of root meristem cells after fructose treatment (n = 7). $*P \le 0.05$

Table 1 pH of study objects

Study object	pН
Juice	3.88
Model solution	2.62
Juice:water = 1:5	4.23
Juice:water = $1:10$	5.11
Model solution:water = $1:5$	3.78
Model solution:water = $1:10$	4.63

the bulk of juice solids, were not responsible for the subchronic toxicity associated with violation of mitosis in the roots. Obviously, this effect was caused by other juice compounds with antiproliferative activity of natural origin.

Based on the data, we can conclude that these compounds (one or more) are present in juice in small quantities and have high biological activity. We need further studies to identify these substances and better understand the mechanisms of potential juice toxicity.

MDA is known to reflect the degree of lipid peroxidation resulting from the oxidation process. The higher its concentration, the more damaged are lipids in the walls of plant cells. In our study, the treatment of onion roots with the 1:10 and 1:5 diluted model solutions produced a dose-dependent increase in MDA, with its maximum levels twice as high as in the control samples (Fig. 6). However, apple juice in the dilutions of 1:10 and 1:5 increased this indicator by only 11%. Apparently, these results are indicative of the juice's antioxidant activity.

To study toxic effects, we treated the onion roots, which had germinated for two days, with the 1:10 and 1:2 diluted juice for only one day. As we can see in Table 3, a day of incubation brought about a slightly higher (10%) decrease in mitotic indices in these test samples than in those treated for two days, compared to the control (Fig. 2). Thus, the toxic effect was recorded as early as after the first cycle of cell division, while the decrease in root mass growth was more likely to be cumulative.

Table 2 Root mass growth, mitotic activity, and chromosomal aberrations of onion root meristem cells after two days of treatment with apple juice and model solution (n = 10)

Test variant	Growth in root mass, g/onion	Mitotic index, %	Chromoso- mal aberra- tions, %
Control Juice:water = 1:10 Juice:water = 1:5 Model solution:	$\begin{array}{c} 0.273 \pm 0.024^a \\ 0.164 \pm 0.031^b \\ 0.091 \pm 0.013^c \\ 0.184 \pm 0.027^d \end{array}$	$\begin{array}{c} 10.30\pm 0.35^a\\ 1.25\pm 0.14^b\\ 0.99\pm 0.13^b\\ 7.44\pm 0.32^a \end{array}$	$\begin{array}{c} 0.21 \pm 0.05^{a} \\ 0.18 \pm 0.05^{a} \\ nd \\ 1.10 \pm 0.13^{b} \end{array}$
water = 1:10 Model solution: water = 1:5	$0.158\pm0.010^{\text{d}}$	$9.15\pm0.32^{\text{a}}$	$0.40\pm0.07^{\text{b}}$

a,b; a,c; a,d; c,d P < 0.05; b,c P < 0.1

nd - not detected



Figure 6 MDA concentration in onion roots treated with apple juice and model solutions

Food additives are commonly studied for toxicity separately from those food products which they are part of. We believe that such practice does not allow scientists to objectively determine the patterns of toxic manifestations. Therefore, our further experiments attempted to evaluate the effect of ethanol on the previously detected toxicity of *Allium cepa* roots, which had been germinated for two days and then incubated with apple juice for another two days. We chose this food additive due to the fact that 1 and 2% aqueous ethanol solutions delay the growth of *Allium cepa* roots within EC₅₀ [29, 32]. In addition, ethanol may be part of some juice-containing products.

Table 4 shows that 1% ethanol increased the average mass of the roots treated with the 1:2 diluted juice by a factor of five. We believe that this effect can be associated with the activity of lipid oxidation enzymes and its regulation. Indeed, raising ethanol concentration to 2% not only decreased their activity, but made it lower than the control values (Fig. 4). However, in the 1:2 diluted juice samples, MDA was almost 1.7 times as high as in the control (Fig. 7), which we had expected from the previous results (Fig. 3). Thus, 1% ethanol appeared to slow down the destruction of cell wall lipids caused by the juice components, which had a positive effect on the root growth. The question is, why is it that a higher concentration of ethanol (2%) did not cause a similar effect? Probably, despite lower lipid oxidation, the total toxicity of 2% ethanol was so high that it prevented the roots from growing and developing.

Table 3 Root mass growth, mitotic activity, and the frequency of chromosomal aberrations of onion root meristem cells after a day of treatment with apple juice (n = 5)

Sample	Growth in	Mitotic	Chromoso-
	root mass,	index, %	mal aberra-
	g/onion		tions, %
Control	$0.113\pm0.018^{\text{a}}$	$8.31\pm0.31^{\rm a}$	$0.42\pm0.07^{\rm a}$
Juice:water = 1:10	$0.099\pm0.018^{\mathrm{a}}$	$4.57\pm0.25^{\rm b}$	$0.17\pm0.05^{\text{b}}$
Juice:water = 1:2	$0.057\pm0.011^{\text{a}}$	$1.25\pm0.12^{\circ}$	$0.13\pm0.04^{\text{b}}$

^{a,b; a,c; b,c} $P \le 0.05$

Table 4 Root mass growth, mitotic activity, and the frequency of chromosomal aberrations of onion root meristem cells after two days of treatment with apple juice and ethanol (n = 9)

Sample	Growth in root	Mitotic	Chromoso-
	mass,	index, %	mal aberra-
	g/onion		tions, %
Control	$0.226\pm0.044^{\rm a}$	$6.52\pm0.26^{\rm a}$	$0.76\pm0.09^{\rm a}$
Ethanol (1%)	$0.140\pm0.022^{\text{b}}$	$3.45\pm0.21^{\text{b}}$	$0.46\pm0.08^{\rm b}$
Ethanol (2%)	$0.132\pm0.039^{\circ}$	$3.81\pm0.20^{\text{b}}$	$0.84\pm0.09^{\rm a}$
Juice:water = 1:2	$0.012\pm0.004^{\text{d}}$	$0.04\pm0.02^{\circ}$	$0.00\pm0.00^{\rm c}$
Juice:water = 1:2	$0.064\pm0.030^{\text{e}}$	nd	nd
+ ethanol (1%)			
Juice:water = 1:2	$0.017\pm0.010^{\rm f}$	$0.03\pm0.02^{\circ}$	nd
+ ethanol (2%)			

a,c; a,d; a,e; a,f; b,e; b,f; c,e; c,f; c,d $P \le 0.05$; a,b $P \le 0.1$; e,f $P \le 0.15$ nd – not detected

Another observation we made was that adding 1 and 2% ethanol to the juice did not increase the proliferative activity of the meristem cells. This result was quite predictable since treating roots with ethanol solutions decreased the mitotic index of the meristem cells, compared to the control.

CONCLUSION

Our study showed that apple juice manifested subchronic toxicity when it was diluted with water in a ratio of 1:5 (~ 2% soluble solids). The toxicity caused a 50% delay in the growth of Allium cepa roots, compared to the control. At the same time, it sharply inhibited the division of meristem cells, with their mitotic index decreasing by a factor of 18 and the MDA concentration increasing by 11%. To identify the mechanisms of these disorders, we treated the roots with the main compounds of juice dry solids - fructose, glucose, sucrose, D-sorbitol, and malic acid - and compared the above indicators. We found that in contrast to the 1:5 diluted juice, 2% fructose decreased the mitotic index by only 17%, compared to the control. The model solution containing 1.4% fructose, 0.4% glucose, 0.2% sucrose, 0.1% D-sorbitol, and 0.06% malic acid showed a 40% higher growth in root mass compared to the 1:5 diluted juice (P < 0.05), the same mitotic index of meristem



Figure 7 MDA concentration in onion roots treated with apple juice and ethanol

cells as the control, and a doubled concentration of MDA compared to the control.

Thus, the subchronic toxicity of apple juice primarily manifested through its antiproliferative activity in the meristem cells. However, the above juice components were not involved in that activity. What they were responsible for was an increased level of lipid oxidation in the root tissues, which was restrained by the natural antioxidants present in the juice.

In addition, we analyzed the contribution of a food additive (ethanol) to the potential toxicity of apple juice, using the Allium test. We found that 1% ethanol in the 1:2 diluted juice reduced the concentration of MDA in the roots by 30%, with no effect of acute toxicity in relation to their growth.

The above effects of, and relationships between, various biomarkers of apple juice and its components can form a basis for more detailed large-scale research into its safety. Our findings can also be used to study the toxic potential of juice depending on manufacturing technology or food additives, as well as to create new juice-based products.

CONTRIBUTION

All the authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Antioxidant and anti-diabetic activity of pomegranate (*Punica granatum* L.) leaves extracts

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Abstract:

Introduction. This study aimed to evaluate the antioxidant and anti-diabetic activity of aqueous and hydroalcoholic extracts of pomegranate (*Punica granatum* L.) leaves *in vitro*, as well as to determine the content of polyphenols, flavonoids, and flavonols. *Study objects and methods.* The antioxidant activity was determined by the DPPH test using the free radical 1,1-diphenyl-2-picrylhydrazyle and the FRAP method, as well as by measuring total antioxidant capacity and the hydrogen peroxide scavenging activity.

Results and discussion. The content of total polyphenols varied between 4.43 ± 0.3 and 12.66 ± 1.6 mg EAG/g. The highest content of flavonoids was observed in the hydroalcoholic extract of *P. granatum* leaves (P < 0.05). The flavonol contents in the hydroalcoholic and aqueous extracts were 7.68 ± 0.6 and 9.20 ± 2.8 mg EQ/g, respectively. The IC₅₀ of the antioxidant potential of the hydroalcoholic and aqueous extracts was 32.4 ± 1.109 and 35.12 ± 4.107 mg/mL, respectively. According to the DPPH test, the aqueous extract was the least active (IC₅₀ = 14.15 \pm 1.513 mg/mL). The highest percentage of hydrogen peroxide trapping was found in the aqueous extract ($45.97 \pm 6.608\%$). The inhibition of α -amylase showed an IC₅₀ of between 9.804 ± 0.67 and 19.011 ± 9.82 mg/mL in the aqueous and hydroalcoholic extracts, respectively. The inhibition of glucose uptake by yeast recorded a high inhibitory capacity at 50 mg/mL of glucose.

Conclusion. We found that the antioxidant and anti-diabetic activity of *P. granatum* leaves extracts was due to the presence of bioactive compounds such as flavonoids, which is why they are effective in preventing diabetes and its complications.

Keywords: Punica granatum L., plant extracts, antioxidant activity, anti-diabetic activity, flavonoids

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INTRODUCTION

The pomegranate (*Punica granatum* L.) is a shrub that belongs to the *Lythraceae* family. It is between 5 and 10 m tall and is characterized by deciduous fruiting leaves. The pomegranate is used to prevent cancer, cardiovascular disease, diabetes, dental conditions, and erectile dysfunction, as well as against ultra violet radiation. Pomegranate leaf extracts contain high total phenols, tannins, and triterpenoids [1]. Numerous studies have demonstrated the *in vitro* antioxidant activity and polyphenol content of pomegranate. According to Amjad *et al.*, the antioxidant activity of pomegranate leaves is directly related to the presence of phenolic compounds and antioxidant components which act as hydrogen donors, contributing to the concentration of total phenols [2]. These authors demonstrated that pomegranate n-butanol, ethyl acetate, hydroethanol, and aqueous leaf extracts contained ellagic acid, an efficient free radical scavenger [2].

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Vinodhini *et al.* reported that the aqueous extract of pomegranate leaves had the greatest antioxidant activity and contained significant levels of total phenols and flavonoids [3]. The leaf extracts showed antioxidant activity *in vivo* by protecting yeast cells against oxidative stressing agent H_2O_2 . The authors found pomegranate a good source of natural compounds with health benefits, which makes it possible to use it in diets to reduce oxidative stress.

In the study by Bekir *et al.*, the methanolic extract of pomegranate leaves displayed high antioxidant, antiinflammatory, anti-cholinesterase, and antiproliferative activities [4]. These results showed that pomegranate leaves could be a potential source of active molecules intended for applications in pharmaceutical industry.

The aim of the study was to evaluate the antioxidant and anti-diabetic activity of aqueous and hydroalcoholic extracts of pomegranate leaves *in vitro*.

STUDY OBJECTS AND METHODS

The pomegranate (*Punica granatum* L.) leaves were collected in September 2017 in Chlef, Algeria. The collected samples were dried at room temperature away from sunlight and then powdered using an electric mortar.

Preparation of aqueous extract. The aqueous extract of pomegranate leaves was prepared according to the method described by Diallo *et al.*, with some modifications [5]. 15 g of powdered leaves in 150 mL of boiling water was heated for 15 min and filtered through filter paper. The filtrate was placed in an oven at 40°C until obtaining a dry extract and stored at 4°C.

Preparation of hydroalcoholic extract. The hydroalcoholic extract of pomegranate leaves was prepared by maceration of 15 g of powdered leaves in 100 mL of a hydroalcoholic solution (70%) at room temperature away from light, with maximum agitation for 72 h [6]. Then the mixture was filtered through filter paper. The filtrates were placed in an oven at 40°C. The dry extract was stored in a refrigerator at 4°C.

Total polyphenols were determined spectrophotometrically following the Folin-Cioclateu method [7]. For this, 0.2 mL of each leaf extract was mixed in a test tube with 1.0 mL of Folin-Cioclateu reagent and 0.8 mL of a 7.5% sodium carbonate solution (Na₂CO₃). After incubation in the shade and at room temperature for 30 min, absorbance was measured at 760 nm. The results were expressed in milligram equivalent of gallic acid per gram of extract (mg EAG/g extract) from a calibration curve prepared using gallic acid as a standard.

Flavonoid levels were measured using the method described by Mbaebie *et al.* [8]. For this, 1.0 mL of each extract was added to 1.0 mL of a 2% ethanol solution of aluminum chloride (AlCl₃) and then incubated for an hour at room temperature. Absorbance was measured by a UV-visible spectrophotometer at 420 nm. The concentrations of flavonoids in the extracts were calculated from the calibration curve and expressed in

milligram equivalent of quercetin per gram of extract (mg EQ/g extracted).

Flavonols were determined according to the method described by Kosalec *et al.* [9]. For this, 0.3 mL of the extract was mixed with 0.3 mL of aluminum chloride (AlCl₃) and 0.45 mL of sodium acetate. The mixture was vigorously stirred and then incubated for 40 min. Absorbance was measured at 440 nm. The quantification of flavonols was based on a calibration curve made by quercetin. The content of flavonols was expressed in milligram equivalent of quercetin per gram of extract (mg EQ/g).

Total antioxidant capacity. Determination of total antioxidant capacity is a technique based on the reduction of molybdate Mo (VI) to molybdenum Mo (V) in the presence of an antioxidant with the formation of a green complex (phosphate/Mo (V)) at acidic pH [10]. The phosphomolybdate reagent was prepared from a mixture of 0.6 M sulfuric acid (H₂SO₄), 28 mM sodium phosphate (Na₃PO₄), and 4 mM ammonium molybdate $((NH_4) 6Mo_7O_24 \cdot 4H_2O)$. 1.0 mL of this reagent was added to 100 µL of each extract with concentrations of 10, 25, 50 and 100 mg/mL. The tubes were incubated at 95°C for 90 min. After cooling, absorbance was measured at 695 nm. Total antioxidant capacity was expressed in milligrams of ascorbic acid equivalent per gram of extract (mg Eq AA/g extract) from a calibration curve of ascorbic acid.

Ferric Reducing Antioxidant Power (FRAP). The FRAP method involves measuring the ability of a sample to reduce the tripyridyltriazine ferric complex to tripyridyltriazine at a low pH. This ferrous tripyridyltriazine complex has an intense blue color measured by a spectrophotometer at 593 nm [11]. The FRAP reagent was prepared by mixing a 300 mM sodium acetate buffer (pH 3.6), a solution of 10 mM TPTZ in 40 mM HCl and 20 mM FeCl₃ in a ratio of 10:1:1 (v/v/v). 200 μ L of each extract (10, 25, 50 and 100 mg/mL) was added to 3 mL of the FRAP reagent. After incubation in the dark at 37°C for 30 min, absorbance was measured at 593 nm against the blank [11].

Hydrogen peroxide scavenging activity. The scavenging capacity of hydrogen peroxide is based on the reduction of the H_2O_2 concentration by scavenger compounds, the absorbance value of the latter at 230 nm also reduces [12]. A 40 mM hydrogen peroxide solution was prepared in a 50 mM phosphate buffer (pH 7.4). 4.0 mL of each extract with a concentration of 10 mg/mL was mixed with 0.6 mL of the H_2O_2 solution. After 10 min incubation, absorbance was measured at 230 nm. Ascorbic acid was used as a positive control [13]. The percent inhibition was calculated using the following equation:

where A is absorbance of the control and experimental samples.

DPPH test. To prepare a 0.004% solution of DPPH, 250 μ L of extracts at concentrations of 10, 25, 50, and 100 mg/mL or standard (ascorbic acid) was added to 1 mL of the DPPH solution. After incubation in the dark at room temperature for 30 min, absorbance was measured at 517 nm against a blank sample that contained pure methanol [14]. The antioxidant activity evaluated with the DPPH method was expressed in percentage according to the following formula:

% antioxidant activity =
$$[(A \text{ control} - A \text{ sample}) / A \text{ control}] \times 100$$
 (2)

where A is absorbance of the control and experimental samples.

Inhibition test of α -amylase enzymatic activity. The inhibition test of α -amylase enzymatic activity followed the method of Daksha *et al.* [15]. For this, two solutions had been prepared, namely a 1% starch stock solution and a 1% amylase solution in a 0.1 M phosphate buffer at pH 7.2, both solutions preserved at 4°C. The reaction mixture contained 2.0 mL of a phosphate buffer, 1.0 mL of each extracts (aqueous and hydro-alcoholic) at concentrations of 10, 25, 50, and 100 mg/mL, 1 mL of amylase, and 1 mL of starch. The mixture was incubated for an hour. The enzymatic reaction was stopped by the addition of 0.1 mL of the iodide indicator. All experiments were performed in triplicate. Absorbance was measured at 565 nm.

The inhibitory activity of each extract was calculated according to the following formula:

% inhibition activity = (A sample – A control /
A sample)
$$\times$$
 100 (3)

where A is absorbance of the control and experimental samples.

To determine effects of the extracts on glucose uptake by yeast, we prepared yeast cells according to the method described in [16]. 1 g of commercial baker's yeast was washed by centrifugation (4200 rpm, 5 min) in 5 mL of distilled water until the supernatant liquid was clear. Then a 10% suspension (v/v) was prepared in distilled water. Different concentrations of plant extracts (10 to 100 mg/mL) were added to 1 mL of glucose solution (10, 25 and 50 mg/mL) and incubated together for 15 min at 37°C. Then, 100 µL of the yeast suspension was added, followed by a vortex and a new incubation at 37°C for 60 min. After one hour, the tubes were centrifuged (2500 rpm, 5 min) and glucose was estimated in the supernatant by the iodine reagent [17]. Metformin was taken as a standard antidiabetic drug. Absorbance was measured at 540 nm and all experiments were performed in triplicate. The percentage increase in glucose uptake by yeast cells was calculated using the following formula [18]:

% inhibition of glucose uptake = (A sample – A control /
A sample)
$$\times$$
 100 (5)

where A is absorbance of the control and experimental samples.

The data presented in our study were analyzed using XL Stat Pro 7.5 statistical software. The experiments were performed in triplicate. The results were presented as mean values and a standard deviation. ANOVA test was conducted to determine any significance differences. P < 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

Table 1 demonstrates total phenolic, flavonoid and flavonol contents of the pomegranate (*Punica* granatum L.) extracts. The hydroalcoholic extract showed a significantly (P < 0.05) higher content of total phenolic compounds compared to the aqueous extract, with values of 12.66 \pm 0.10 and 4.43 \pm 0.01 mg EAG/g extract, respectively (Table 1). These results were not consistent with those found by Sinha *et al.*, namely 9.85 \pm 0.82 and 14.78 \pm 2.10 mg EAG/g extract for the pomegranate aqueous and hydroalcoholic extracts, respectively [19].

The hydroalcoholic extract showed a significantly (P < 0.05) higher content of flavonoids than the aqueous extract (24.78 ± 1.59 and 8.76 ± 0.90 mg EQ/g, respectively). These results were closer to those reported by [19], namely 12.7 ± 0.23 and 26.08 ± 1.24 mg EQ/g for the aqueous and methanolic extracts, respectively. According to quantitative analyses, pomegranate leaves contained a higher amount of flavonoids compared to phenolic compounds. These results were confirmed by [19], where pomegranate leaf extracts showed a lower content of total polyphenols and a higher content of flavonoids compared to parameters.

Our results indicated that the aqueous extract was richer in flavonols compared to the hydro-alcoholic extract; with contents of 9.20 ± 2.80 and 7.68 ± 0.60 mg EQ/g of extract, respectively (Table 1). The statistical analyses did not show any significant difference between the two extracts (P > 0.05).

Table 2 shows the antioxidant capacity of the pomegranate extracts. The aqueous extract of pomegranate leaves had a significantly higher (P < 0.05) total antioxidant capacity with an IC₅₀ value of 12.404 ± 0.136 mg/mL, while the hydroalcoholic extract showed a significantly lower (P > 0.05) antioxidant capacity with an IC₅₀ of 18.719 ± 1.001 mg/mL.

Table 1 Total phenolic, flavonoid and flavonol contents

 of *pomegranate* L. extracts

Extract	TPC,	TFC,	TFLC,
	mg GAE/g	mg QE/g	mg QE/g
Aqueous extract	$4.43\pm0.01^{\rm b}$	$8.76\pm0.90^{\rm b}$	$9.20\pm2.80^{\rm a}$
Hydroalcoholic	$12.66\pm0.10^{\text{a}}$	$24.78\ \pm 1.59^{a}$	$7.68\pm0.60^{\rm a}$
extract			

Values with different lowercase letters mean they are significantly different (P < 0.05) (a > b > c)

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Extract	Total antioxidant	FRAP,	Hydrogen peroxide	DPPH,
	capacity, mg/mL	mg/mL	scavenging, %	mg/mL
Aqueous	$12.404 \pm 0.136^{\rm a}$	$35.12\pm4.107^{\mathtt{a}}$	$45.97 \pm 6.608^{\rm b}$	$14.15 \pm 1.513^{\circ}$
Hydroalcoholic	$18.719 \pm 1.001^{\mathrm{b}}$	$32.4\pm1.109^{\mathrm{a}}$	$43.57 \pm 10.145^{\rm b}$	$9.40\pm1.586^{\mathrm{b}}$
Ascorbic acid	/	55.531 ± 1.133^{b}	85.663 ± 5.024^{a}	$2.27\pm0.012^{\rm a}$

Table 2 Antioxidant activity of pomegranate extracts

Values with different lowercase letters mean they are significantly different ($P \le 0.05$) (a $\le b \le c$)

In a study of three local varieties of *Piper betle* leaves by Dasgupta *et al.*, the Kauri variety showed the highest total antioxidant capacity expressed in milligrams of ascorbic acid equivalent per milligram of extract [20].

According to the FRAP test results, the antioxidant potential of iron was almost the same for both hydroalcoholic and aqueous extracts, with IC₅₀ of 32.4 ± 1.109 and 35.12 ± 4.107 mg/mL, respectively (Table 2).

While there were no significant differences (P > 0.05) between the hydroalcoholic and aqueous extracts, there was a significant difference (P < 0.05) between the extracts and ascorbic acid, which showed a reducing power with an IC₅₀ of 55.531 ± 1.133 mg/mL.

These results were not consistent with those recorded by [19], namely IC₅₀ of 348.68 \pm 24.69 and 293.63 \pm 15.29 mg/mL for the aqueous and methanolic extracts of pomegranate leaves, respectively.

The percentage of hydrogen peroxide scavenging activity of the hydroalcoholic and aqueous extracts was $43.57 \pm 10.145\%$ and $45.97 \pm 6.608\%$, respectively. There was no significant difference between the extracts (*P* > 0.05) (Table 2).

Compared to the extracts, ascorbic acid showed a significantly higher (P < 0.05) percentage, namely $85.663 \pm 5.024\%$.

According to the DPPH test results, the hydroalcoholic extract was significantly the most potent extract (P < 0.05) with an IC₅₀ of 9.40 ± 1.586 mg/mL, followed by the aqueous extract with an IC₅₀ of 14.15 ± 1.513 mg/mL (Table 2).

Compared to the extracts, the standard antioxidant (ascorbic acid) showed a significantly higher (P < 0.05) antioxidant activity, with an IC₅₀ of 2.27 ± 0.012 mg/mL (Table 2).

These results were in agreement with the data reported by Bekiretal, where the methanolic extract of pomegranate leaves showed a greater antioxidant activity than the ethanolic extract, with an IC₅₀ of 5.62 ± 0.23 mg/L and 9.25 ± 0.72 mg/L, respectively [4]. The study also revealed comparable antioxidant activity between the methanolic extract and quercetin (2.86 \pm 0.09 mg/L). The dichloromethane extract showed lower antioxidant activity (IC₅₀ = 71.57 \pm 3.65mg/L). However, the extract obtained with hexane had the lowest DPPH activity with an IC₅₀ value of 263.44 \pm 12.72 mg/L.

According to Fig. 1, the aqueous extract showed an α - amylase inhibitory concentration of 9.804 ± 0.67 mg/mL. This value was significantly lower (P < 0.05) than that for acarbose and hydroalcoholic extracts, with IC₅₀ values of 17.179 ± 4.26 and 19.011 ± 9.82 mg/mL, respectively. On the other hand, there was no significant difference between the IC₅₀ of acarbose and the IC₅₀ of the hydroalcoholic extract (P > 0.05).

These inhibition results were not in agreement with those found by Kam *et al.*, who recorded IC_{50} inhibitory concentrations of 0.19 and 0.65 mg/mL for aqueous and alcoholic extracts of pomegranate, respectively [21].

This inhibitory power can be explained by the fact that the hydroalcoholic and aqueous extracts have compounds that bear functional groups close to those of the substrate (starch), which occupies the active site of the enzyme.

Figure 2 demonstrates the inhibition of glucose uptake by yeast. At a concentration of 10 mg/mL of glucose, metformin showed a significant difference from the extracts (P < 0.05), with an IC₅₀ of 5.442 ± 0.047 mg/mL. However, we found no significant







Figure 2 Inhibition of glucose uptake by yeast, IC₅₀, mg/mL

difference between the hydroalcoholic and aqueous extracts (P > 0.05), with IC₅₀ values of 7.267 ± 0.644 and 6.975 ± 0.394 mg/mL, respectively.

At a concentration of 25 mg/mL of glucose, there was no significant difference (P > 0.05) between the aqueous extract, metformin, and hydro-alcoholic extract, with IC₅₀ values of 7.297 ± 0.76, 5.353 ± 0.11, and 8.509 ± 2.94 mg/mL, respectively (Fig. 2).

At a concentration of 50 mg/mL of glucose, there was a significant difference between metformin and the extracts (P < 0.05) and no significant difference (P > 0.05) between the extracts. The IC₅₀ values of metformin, aqueous and hydroalcoholic extracts were 5.499 \pm 0.073, 8.379 \pm 2.4, and 8.937 \pm 2.892 mg/mL, respectively (Fig. 2).

Based on these results, metformin showed a higher inhibition capacity than the aqueous and hydroalcoholic extracts.

According to the results, the antioxidant property varied according to the extraction solvent. The antioxidant properties of plant extracts can be explained by various factors: the presence of natural ascorbic acid (vitamin C), α -tocopherol (vitamin E), β -carotene (a precursor of vitamin A), flavonoids, and other phenolic compounds [22, 23].

These phenolic compounds are capable of acting as antioxidants that can neutralize free radicals by donating an electron or a hydrogen atom [24, 25].

The antioxidant capacity of phenolic compounds is also attributed to their ability to chelate ionic metals involved in the production of free radicals. For example, when attaching a ligand (phenolic compound) to Fe⁺³ in the FRAP test, polyphenols can reduce iron to Fe⁺² [26].

Antioxidants act as "sensors" of free radicals, fighting against radical oxidation. Antioxidants of phenolic type react according to a mechanism proposed by Sherwin in 1976: an antioxidant formally yields a hydrogen radical, which may be an electron transfer followed, more or less rapidly, by a proton transfer [27].

Polyphenolic compounds are increasingly being used in therapeutics [28]. Many studies suggest that polyphenols participate in the prevention of cardiovascular diseases. They inhibit the oxidation of low density lipoproteins and platelet aggregation involved in the phenomenon of thrombosis that can lead to occlusion of the arteries [29]. These compounds show antioxidant activities: they have anti-inflammatory, antiatherogenic, antithrombotic, analgesic, antibacterial, and antiviral effects and can act as anticarcinogens, antiallergens, or vasodilators [30, 31].

Flavonoids also perform many biological functions that are attributed in part to their antioxidant properties. These compounds not only inhibit free radicals, but also neutralize oxidative enzymes and chelate metal ions responsible for the production of reactive oxygen species [32].

As for tannins, they are defined as sources of plant origin because they can precipitate proteins, inhibit digestive enzymes, and decrease the use of vitamins and minerals. On the other hand, tannins are also considered as "health promoting" components in plant-derived foods and beverages. For example, tannins have been reported to have anti-carcinogenic and antimutagenic potential, as well as antimicrobial properties.

The antioxidant activity of pomegranate leaves is due to their richness in phenolic compounds (tannins, flavones, glucosides). In fact, the work by Kang *et al.* suggested that polar polyphenolic molecules present in the plant's extract contributed to the increase in antiradical activity [33].

As for anti-diabetic activity, Patel *et al.* reported that pomegranate extract regulates post-ponderal glucose by its inhibitory effect on α -amylase [34].

Flavonoids have a high nutritional value because they are part of our usual diet, which could be explained by their rapid metabolism, elimination, and relatively low bioavailability [35].

The reaction mechanisms of α -amylase enzyme inhibition remain unclear. However, flavonoids in foods can interact with starch and react with nitrous acid derived from the oral cavity in the stomach before being transported to the intestine [36]. This review mainly deals with: (a) the inhibition of α -amylase activity by flavonoids, suggesting the mechanisms of inhibition, and (b) the suppression of starch digestion by flavonoids by forming starch-flavonoid complexes through hydrophobic interactions.

The inhibition potential for flavonoids and tannins is correlated with the number of hydroxyl groups in their B cycles. These compounds inhibit α -amylase by forming hydrogen bonds between its hydroxyl groups and the residues of the active site of this enzyme. Flavonoids or flavonoid-rich foods can reduce the risk of diabetes by modulating glucose uptake and insulin secretion [37].

The transport of glucose through the yeast cell membrane occurs by facilitated diffusion, a passive mechanism without energy input. Glucose transport is continued if intracellular glucose is effectively reduced or used [38].

Scientific evidence shows that apical or luminal GLUT 2, facilitating the intestinal transport of glucose, is the major route of glucose uptake and thus an attractive target for some plant-based inhibitory agents [39].

Calystegine, a compound found in the pomegranate, exerts an antidiabetic effect by acting on the absorption of glucose by a competitive mechanism because of their structural analogy with glucose [40].

CONCLUSION

Our study demonstrated that pomegranate (*Punica granatum* L.) leaf extracts are rich in phenolic compounds which play a very important role in the scavenging of free radicals, it makes a significant contribution to the justification of the antioxidant and

anti-diabetic activity. It gives the extracts a power to protect the body against stress and manifestations linked to diabetes. The hydroalcoholic leaves extract was effective in preventing diabetes due to its high flavonoid. Therefore, there is a need for further *in vivo* studies to better understand the mechanism of their action.

CONTRIBUTION

M. Cheurfa and A. Azouzi performed the extraction

and chemical characterization. A. Mariod, A. Azouzi, and M. Cheurfa performed the biological experiments and wrote the manuscript. M. Cheurfa and M. Achouche analyzed the data. All the authors revised the manuscript for publication.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

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Yeast-rich mannan fractions in duck cultivation: prospects of using

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Abstract:

Introduction. Due to the trend of avoiding antibiotics and acquiring eco-friendly products, the use of environmentally safe preparations is becoming increasingly relevant in poultry farming.

Study objects and methods. We used Salmonella enteritidis and Campylobacter jejuni isolated from poultry carcasses. At the first in vitro stage, we studied the ability of mannan oligosaccharides, isolated from the cell walls of Saccharomyces cerevisiae yeast, to adsorb bacterial pathogens. At the second stage, we studied the influence of fraction on the activity, colonization and microflora composition of ducklings' intestines. At the third stage, we determined the antagonistic activity of Bifdobacterium spp. (Bifdobacterium lactis, Bifidobacterium longum, Bifidobacterium bifidum) and Lactobacillus spp. (Lactobacillus fermentun, Lactobacillus salivarius, Lactobacillus acidophilus) against Salmonella enteritidis and Campylobacter jejuni isolates. The experiment was conducted on the ducklings of Star 53 H.Y. cross. Their diet was supplemented with probiotics, prebiotics, and their combination.

Results and discussion. In vitro studies showed the ability of mannan oligosaccharides isolated from the cell walls of *Saccharomyces cerevisiae* yeast to adsorb *Salmonella enteritidis* and *Campylobacter jejun. In vivo* experiment showed the ability of mannan oligosaccharides to prevent colonization of poultry intestines by bacterial pathogens with type I fimbriae.

Conclusion. The reisolation rate of ducks infected with *Salmonella enteritidis* was 53.6% lower, and those infected with *Campylobacter jejuni*, 66.2% lower than the control. Mannan oligosaccharides added to the diet did not affect the concentration of lactobacilli, enterococci, and anaerobic bacteria in the ducks' intestines. A combined use of *Bifidobacterium* spp. and mannan oligosaccharides improved the preservation of poultry stock by 8.7%, which made it an effective way to prevent poultry salmonellosis.

Keywords: Prebiotics, probiotics, mannan oligosaccharides, microorganisms, bacterial pathogens, *Salmonella* spp., *Campylobacter* spp., poultry, ducks, productivity

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INTRODUCTION

In the world production of poultry, the share of waterfowl meat is 7.2%, specifically duck meat -4.2%, goose meat -3%. Their share in the gross production of poultry meat tends to increase. In industrial poultry farming, the problem of controlling bacterial infections of waterfowl is of genuine concern. *Salmonella* and *Campylobacter* are considered the most common etiological zoonotic factors worldwide, with productive poultry being the main source of infection.

In recent years, there has been an increase in the relative number of infections caused by *Salmonella* spp. and *Campylobacter* spp. The microorganisms are widespread in most warm-blooded and farm animals, including poultry. Ducks' infection with salmonella can be detected at the age of about 14 days, and by the end of cultivation the whole flock can be found infected. Experimental studies showed that a small dose (less than 40 CFU) of *S. enteritidis* is sufficient to fully colonize the poultry intestines. This can lead to complete flock's

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infection in 48 h [5–7]. Microorganisms can colonize the intestinal tract of poultry in large quantities, often at above 10^6 – 10^8 CFU/g of intestinal contents. The highest concentrations of bacterial pathogens are known to be present in the intestinal mucosa [4].

Poultry products can be contaminated at many stages of the "from farm to table" food chain, but the strategic one is the stage of primary poultry production. Following biosafety guidelines of GMP/HACCP significantly reduces the colonization of poultry by bacterial pathogens and, later, the contamination of carcasses during processing. The European Food Safety Agency's monitoring (2008–2018) showed that about 86% of poultry carcasses in Europe were contaminated with *Campylobacter* and *Salmonella* bacteria.

In poultry production, main methods of infection control are taken at the stage of the cultivation in farms. Environmentally safe methods that ensure poultry quality and safety hold promise. Effective systems of poultry cultivation, feeding, and maintenance are reqired to control the spread of *Salmonella* and *Campylobacter* in poultry products. Bio-safety measures, decontamination of dropping and water are potentilly productive. Antibacterial drugs in treating of bacterial infections in poultry are considered a risk factor contributing to the development of antibioticresistant strains.

Following the trend of avoiding antibiotics, the search for new control methods is becoming increasingly important in poultry farming. The application of antimicrobial alternatives is highly potential. They include feed additives that are inhibitors of bacterial pathogens, as well as probiotics, prebiotics, bacteriophages, bacteriocins, which in combination prevent antibiotic-resistant strains of microorganisms and inhibit their proliferation [9–12].

Consequently, natural alternative antibacterial preparations are a way to reduce poultry gut colonization by pathogenic microflora. This is the most acceptable natural alternative to salmonella and campylobacter control that is economically viable and does not pose a risk to human health, animals, or the environment [3, 9]. Effective protection of poultry against pathogens, naturalness and safety, growth promotion, and economic effectiveness are the criteria for new alternatives to antibiotics [11, 13].

One of the requirements for probiotics use is the competitiveness of antagonistic microflora found in them. In order to prevent intestinal colonization by bacterial pathogens, probiotics are recommended for use from the first day of the birds' life. Prebiotics promote the development of birds' own symbiotic microflora, which can inhibit pathogens and reduce their adhesion to enterocytes.

Research suggests that some natural compounds have biological activity against salmonella proliferation, but few have shown efficacy in experiments on animals. "Actigen" prebiotic (Alltech) is a concentrated pure fraction of mannan oligosaccharides isolated from the cell walls of *Saccharomyces cerevisiae* yeast. The main advantage of these complex carbohydrates is their ability to adsorb certain strains of bacteria that have type I fimbriae (mannose-sensitive) and prevent intestinal colonization by pathogens. Besides, the industrial experiment proved the influence of combined use of mannan oligosaccharides and probiotics on intestinal microbiocenosis and duck productivity [14, 15].

We aimed to develop a method for preventing bacterial infections and increasing duck productivity using probiotics and prebiotics. The method was based on the study of adsorbing capacity of mannan oligosaccharides (MOS) and antagonistic properties of *Bifidobacterium* spp. and *Lactobacillus* spp. against *Salmonella enteritidis* and *Campylobacter jejuni*. We also aimed to analyze a combined effect of the cultures on gut microbiocenosis (activity and colonization) and on productivity of ducks.

STUDY OBJECTS AND METHODS

We used Salmonella enteritidis and Campylobacter jejuni isolated from poultry carcasses of Ukrainian farms. The studies were carried out in 2014–2018 at Sumy National Agrarian University, Sumy. The poultry carcasses were subjected to a detailed examination for pathomorphological changes. The liver, muscles, cloaca contents, ovaries, and various segments of the ovoid were aseptically assembled to be screened for salmonellosis and campylobacteriosis. Isolation and identification of microorganisms was carried out using tests recommended by "Bergey's Manual" (1997) [35].

At the first stage (*in vitro*), we studied the ability of mannan oligosaccharides isolated from the cell walls of *Saccharomyces cerevisiae* yeast to adsorb bacterial pathogens. In our experiments we used 27 strains of *Salmonella enteritidis* and 13 strains of *Campylobacter jejuni* isolated from ducks' chilled carcasses (liver, muscles, cloaca).

We used the daily agar culture of bacteria with 1% red blood cells of guinea pigs. Salmonella (1.5×109 CFU/mL) was used as an antigen. Erythrocytes were derived from the blood of a pre-selected donor (guinea pigs). Blood was placed in flasks containing sodium citrate and filtered through a cotton gauze filter to remove fibrin and small blood clots. Blood was centrifuged with sodium chloride isotonic solution four times (1500 rpm, 10 min). Then we introduced it into a 10% suspension of phosphate buffer solution (pH 7.0-7.2). The washed red blood cells were stabilized with 0.2% acrolein (acrylic aldehyde) solution in the phosphate buffer (1:1) and incubated in water bath at 37°C for 30-40 min while stirring periodically. Erythrocytes were washed three times by centrifuging with phosphate buffer at 5000 rpm. To improve the sorption properties of red blood cells, we treated them with tannin, combining equal parts 5% of frozen stabilized red blood cells and tannin solution (1:30 000). The mixture was left in the thermostat at 37°C for 40 min, then it was washed twice with phosphate buffer solution (pH 7.2–7.4) and then twice with sodium chloride isotonic solution (pH 7.2–7.4). To sensitize the antigen, we used a 1% red blood cell suspension. Suspensions were left for 24 h at 4°C to exclude spontaneous hemagglutination.

The degree of agglutination of the salmonellas isolated was determined by combining prepared suspended microorganisms and the aqueous solution of mannan oligosaccharides (0.2, 0.3 and 0.4 g/L) in a ratio of 1:1. *E. coli O2* test culture was used as a positive control of the agglutination level of the pathogen. One-percent red blood cell suspension in phosphate buffer solution (pH 7.2-7.4) was used as a negative control [16–18].

At the second stage, we studied the influence of fraction (Aktigen, Alltech Inc.) on the activity, colonization and species composition of the microflora of young ducks' intestines. Sixty male ducklings aged 30 days were used in the study. Each experiment involved one control and two experimental groups (50 heads in each). First experimental group was infected with Salmonella enteritidis, and the other group with Campylobacter jejuni (1×104 CFU/mL per os). Ducklings were kept in sterile boxes on the floor and fed by standards. They had free access to feed and water. In experimental groups, the birds received a prebiotic fraction of MOS (0.4 kg/t) together with the feed. Ten days after the infection we determined the concentration of salmonellas, campylobacil, lactobacil, bifidobacterium, and total concentration of anaerobic bacteria using dilution plate counting.

At the third stage, we determined the antagonistic activity of Bifidobacterium spp. (1.0×109 CFU/mL): Bifidobacterium lactis, Bifidobacterium longum, Bifidobacterium bifidum and Lactobacillus spp. (1.0×10⁹ CFU/mL): (Lactobacillus fermentun, Lactobacillus salivarius, Lactobacillus acidophilus against Salmonella enteritidis and Campylobacter jejuni isolates. Suspensions of bacterial probiotic cultures in a concentration of 1×109 m.c/cm3 were sown on Petri dishes and incubated for 24 h at 37°C. After that, suspensions with microorganisms (Salmonella enteritidis and Campylobacter jejuni) in a concentration of 1×109 m.c/cm3 were inoculated by streaking. The dishes with inoculation were incubated at 37°C for 24-72 h. We recorded the diameter of zones with no growth of test cultures. To control microbial growth, we used Preston-agar for Campylobacter, "Salmonella different agar" for Salmonella, as well as MPA and MPB for probiotics.

We used the *Star 53 H.Y. cross* ducklings to determine the effectiveness of probiotics, prebiotics, and their combination. The birds were randomly divided into 4 groups, 123 birds in each. Each group included 3 flocks, 41 birds in each (12 flocks in total). The control

group received the main diet only. Three experimental groups received three different supplements in addition to the main diet: bifidobacteria $(1.5 \times 10^9 \text{ CFU/mL})$, mannan oligosaccharides ("Actigen" prebiotic), and a combination of *Bifidobacterium* spp. and *Lactobacillus* spp. $(1.5 \times 10^9 \text{ CFU/mL})$ in a ratio of 1:1 and the fractions of mannan oligosaccharides (0.4 kg/t of feed). These supplements were mannan-rich fractions isolated from

Ingredients	Starter	Grower
Wheat	55.00	62.00
Full fat whole soya	12.00	12.00
Soybean neal	23.00	20.00
Limestone	0.72	0.50
Di-calcium phosphate	1.65	1.85
Soybean oil	4.50	5.00
Salt	0.20	0.20
Sodium bi-carbonate	0.18	0.16
DL Methionine	0.50	0.40
L-Lysine	0.37	0.30
Threonine	0.25	0.13
Vitamin-mineral premix	0.50	0.50
Nutrient analysis, %, or as indicated		
Metabolic Energy, kcal/kg	3000	3125
Crude Protein	24.10	22.00
Lysine	1.42	1.35
Methionine+Cysteine	1.10	0.93
Calcium	1.05	0.85
AVAILABLE PHOsphorous	0.50	0.42
Vitamin-Mineral Premix ¹		
Copper, mg	15.00	15.00
Iodine, mg	1.00	1.00
Iron, mg	30.00	30.00
Manganese, mg	112.00	112.00
Selenium, mg	0.40	0.40
Zinc, mg	105	105
Synergen ² , g	158	158
Vitamin A (IU)	13.00	12.00
Vitamin D ₃ (IU)	4.75	4.50
Vitamin E (IU)	70.00	50.00
Vitamin K, mg	3.00	2.75
Thiamin (B_1) , mg	3.00	2.50
Riboflavin (B_2) , mg	10.00	8.00
Niacin, mg	55.00	50.00
Pantothenic Acid, mg	17.00	15.00
Pyridoxine (B_6) , mg	5.00	4.50
Biotin, mg	0.30	0.25
Folic Acid, mg	2.00	1.70
Vitamin B ₁₂ , mg	200.00	185.00
Vitamin C, mg	200.00	200.00
Choline, mg	475.00	450.00

¹Vitamin-Mineral Premix manufactured by Target Feeds, Shropshire, UK

²Synergen (g) is a commercial enzyme product by Alltech, Inc.
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Figure 1 Research scheme

the cell wall of *Saccharomyces cerevisae* yeast. The main diets were prepared at a commercial feed mill and consisted mainly of wheat and soybean flour, as shown in Table 1 [19, 20].

The birds were given starter diets from hatch till day 20, grower diets – from day 21 to 49. Feed and water was provided throughout the whole study period. Initially,

the room temperature was maintained at 30° C for 10 days, and then gradually decreased every second day by 1°C. During the experiment, the lighting regime was the following: for 16 h – light, 8 h – darkness, which lasted 49 days. All conditions were the same for all the four groups. The birds were weighed when hatched, on days 21 and 49. We also measured feed intake to

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estimate feed conversion rates and body weight gains. The intact parts of the cecum were withdrawn from 10 randomly caught birds aged 49 days immediately after euthanasia. The contents of the cecum were placed in sterile test tubes. Then, the tubes were instantly frozen in liquid nitrogen, lyophilized and stored at - 80°C for further analysis.

No principles of the bioethics code were violated during the experiments [21].

The general scheme of our experimental and practical studies is shown in Fig. 1.

Bacteriological analysis. We tested the laying houses for *Campylobacter* spp. before placing the birds there and on day 21. According to the methods described in BS EN ISO 10272:2006, the swabs were placed in 50 mL of isotonic solution and kept at 260 rpm for one minute [23, 24]. The suspension (0.1 cm³) was then transferred to two dishes with breeding ground (Preston agar base for *Campylobacter*) and incubated in microaerobic atmosphere (85% N₂, 10% CO₂ and 5% O₂) at 40.5 ± 1°C. Then we examined them in 44 ± 4 h for typical and/or suspicious *Campylobacter* spp colonies.

Then, *Salmonella enteritidis* was isolated from the material. The serotyping of *Salmonella spp*. was carried out according to the methods with some modification according to the data [28, 32–34].

Statistical analysis. Weight gains and feed conversion rates were studied for statistical group differences using the Student's T-test. The results of the microbiological analysis were logarithmic and evaluated for the statistical difference between the indicators that were measurable.

RESULTS AND DISCUSSION

The aim of our research was to study effects of mannan oligosaccharides fractions and probiotics on *Salmonella enteritiadis* and *Campylobacter jejuni*.

In vitro experiments showed that 0.2–0.4% aquatic fractions of mannan oligosaccharides could adsorb all the *Salmonella* strains and *E. coli* O_2 test cultures (positive control).

We detected the most active and pronounced ability to adsorb bacterial pathogens in *in vitro* experiments with 0.4% aqueous fraction of mannan oligosaccharides. We recorded the beginning of the adsorption process within 2 min. The active process was manifested in the form of finely-divided sediment and clearing of the supernatant. In 8–10 min we observed significant sedimentation (Fig. 2 a–d).

The formation of the sediment illustrates the adsorption process that occurred in the test tube. The same process can occur in the gut in animals and poultry.

Intestinal colonization by pathogens begins with the binding of cells to the epithelium of the intestinal mucosa [17]. Pathogens, including most types of *Salmonella*, *E. coli*, and *Campylobacter* attach to the gut



Figure 2 Absorption of *Salmonella enteritidis* with 0.4% concentrated pure fraction of mannan oligosaccharides *in vitro*: a - in 2 min; b - in 4 min; c - in 6 min; d - in 10 min

via receptors (fimbriae) specific to certain carbohydrates containing mannose, which localize on the surface of intestinal mucosal epithelium cells [14].

When entering the intestines of poultry with feed, mannan-rich fractions bind to receptors of bacterial cells that have type I fimbriae (mannose-sensitive). Fractions of mannan oligosaccharides are not broken down by digestive enzymes and are held firmly on the surface of bacteria. Bacteria with blocked receptors cannot gain a foothold on the surface of epithelial cells – they transit through the gastrointestinal tract [13]. Thus, we found that the active concentration of mannan-rich fractions could successfully adsorb *Salmonella*, a pathogen that can cause foodborne diseases.

The following experiment examined the effects of fractions rich in mannanooligosaccharides on the activity, colonization, and species composition of microflora in ducks' intestines.

At the second (*in vivo*) stage, we determined the effect of mannan-rich fractions on the number of bacteria in the gut of experimentally infected ducks aged 30 days by type I fimbriae bacterial strains (*C. jejuni* and *S. enteritidis* strains). In experimental groups of birds that received prebiotic MOS fractions with feed, the level of bacteria with type I fimbriae decreased. The effect of mannan oligosaccharide-rich fractions on the concentration of intestinal microflora of ducks infected with *S. enteritidis* is shown in Fig. 3.

The effect of mannan-rich fractions on the concentration of intestinal microflora of ducks infected with *C. jejuni* is shown in Fig. 4.

The results showed that mannan oligosaccharides could regulate intestinal microflora due to their selective ability to inhibit *Salmonella* spp. and *Campylobacter* spp. proliferation, preventing pathogenic colonization of the intestines and minimizing its toxic effect on the poultry. Concentration of *Salmonella* spp. in the ducklings' gut was lower by 3.69 log CFU/g and *Campylobacter spp.* by 3.27 log CFU/g compared to the control, respectively. Metabolites of functional oligosaccharides did not affect the levels of intestinal colonization by pathogenic bacteria (coliforms and

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Figure 3 Effects of mannan oligosaccharide-rich fractions on the concentration of intestinal microflora of ducks infected with *S. enteritidis* log CFU/g



Figure 4 Effects of mannan oligosaccharide-rich fractions on the concentration of intestinal microflora of ducks infected with *C. jejuni* log CFU/g

anoerobeds). They did not prevent *Lactobacillus* spp. and *Bifidobacterium* spp. proliferation either, which contributed to the colonization of beneficial bacteria in the birds' intestines.

Regulation of intestinal microbiocenosis can potentially have a positive effect on immune response mechanisms, i.e. to strengthen immunity and enhance the poultry population.

The effect of mannan-rich fractions on the ducklings' gut microflora infected with *S. enteritidis* is shown in Fig. 4a. The reisolation rate of *S. enteritidis* and *C. jejuni* in the test group, which received prebiotic mannan-rich fractions with feed, decreased by 53.6 and 66.2%, respectively, compared to the control group (Figs. 5a, 5b).

Bifido- and lactobacteria also displayed antagonistic activity against *Campylobacter jujuni* and *Salmonella enteritidis* isolates. It makes them possible to be used for the prevention of infectious diseases caused by sensitive strains of pathogens to prebiotic drugs. *Bifidobacterium* spp. and *Lactobacillus* spp. suppressed the growth of microorganisms to different extents (Table 2). Twelve isolates (92.6%) of *Campylobacter* spp. were susceptible to bifidobacteria. The inhibition zone of campylobacter was 5.1 ± 0.3 mm. Ten *Campylobacter jujuni* isolates showed a moderate level of antagonistic activity - 76.9%, with the inhibition zone of 5.1 ± 1.0 mm.

Twenty four isolates (88.9%) of *S. enteritidis* were susceptible to bifidobacteria; the inhibition zone of *S. enteritidis* was 5.5 ± 0.4 mm. The antagonistic activity of lactobacilli against *S. enteritidis* showed a moderate level: 22 isolates (81.5%) had inhibition zone of 4.9 ± 0.5 mm. Bifidobacteria were more active against *Campylobacter* spp. and *Salmonella* spp. It makes it possible to use probiotics to prevent and treat infectious diseases caused by susceptible strains of pathogenic microorganisms to the drug. To improve the ducks' productivity, we studied the effect of mannan-rich fractions. The experiment plan is given in Table 3.

To solve the problem of bacteriosis prevention and increase of birds' productivity, we also studied the effect of a combined use of mannan oligosaccharides and probiotic bifidobacteria and lactobacilli. Kasjanenko S.M. et al. Foods and Raw Materials, 2020, vol. 8, no. 2, pp. 337-347



Figure 5 Effects of mannan-rich oligosaccharides on the reisolation rate of salmonellas from the intestines of poultry infected with *S. Entertitidis* (a) and campylobacteria from the intestines of poultry infected with *C. Jejuni* (b)

Table 2 Susceptibility of *Bifidobacterium* spp. and *Lactobacillus* spp. $(M \pm m)$, %

Microorganism	Inhibitio	n zone, mm	Control of growth	Control of growth on Salmo-
	Bifidobacterium spp.	Lactobacillus spp.	on Preston agar	nella agar M1078, HiMedia
C. jujuni	5.3 ± 0.2	5.1 ± 0.3	+	
S. enteritidis	5.5 ± 0.4	4.9 ± 0.5		+

(+) – signs of growth, P < 0.05

The first stage of the experiment included 20320 ducks (Star 53 Y.Y.) divided into four groups: one control and three experimental ones. The experiment was carried out three times (81280 ducks in total). Probiotics were added to the diet of the ducks with water (10 cm³ per 4 kg duck weight) once a day from the first day until the end of the fattening period (49 days).

We added mannan-rich fractions to the base diet – 400 g/t of feed. We added bifidobacteria and mannanrich fractions to the duck diet once a day until the end of fattening period (49 days). The analysis showed higher results during all periods of the birds' life compared to the control groups (Table 4).

At the age of 21 days, the average growth rate of ducks receiving probiotics with mannan-rich fractions was 87.3 g vs. to 83.6 g in the control group. We noticed a similar trend at the age of 21 days with an average daily growth of ducks from 101.4 g to 107.6 g. The experimental group III after 21 days exceeded the control group by 7.6%.

On day 21, the body weight of ducks receiving probiotics, mannan-rich fractions, and their mix exceeded that in the control group by 1.1, 1.9 and 3.6%, respectively. The body weight was 1273 ± 67 g, 1283 ± 42 g, and 1305 ± 34 g, respectively.

In 49 days, the body weight of the ducks receiving mannan-rich fractions, as well as their mix was 3415 ± 95.5 , 3459 ± 87.4 , and 3547 ± 24.3 g, respectively, which exceeded the weight of the ducks of the control group by 3.1, 4.4 and 7.1 % (Table 4). In addition, a similar trend was detected with average daily gain in

duck weight. In 49 days, it was 59.2, 59.7, and 61.3 g for experimental groups, exceeding that in the control group by 1.2, 2.1, and 4.7 %, respectively. The ducks receiving the mix of probiotics and mannan-rich fractions gained weight more intensively compared to the birds having the other diets (Table 5).

CONCLUSION

In vitro studies showed the ability of prebiotic mannan-rich fractions isolated from the cell walls of *Saccharomyces cerevisiae* yeast to adsorb type I fimbriae bacterial pathogens (*S. enteritidis* and *C. jejuni*) and prevent colonization and proliferation of pathogenic microorganisms on the surface of ducks' intestinal epithelial cells.

We studied the influence of fractions rich in mannan oligosaccharides on activity, colonization, and species composition of duck gut microflora.

 Table 3 Bifidobacteria and mannan-rich fractions in the duck's diet (n = 20320)

Groups	Diet
Control	Base diet "Starter" from day 1 to day 20 of life
	Base diet "Grower" from day 21 to day 49
Experimental group I	Base diet + probiotics from day 1 to day 49
Experimental	Base diet + mannan-rich fractions from
group II	day 1 to day 49
Experimental	Base diet + probiotics + mannan-rich fractions
group III	from day 1 to day 49

Ta	ble 4	Effect of	of experimenta	l diets on	duck growt	h (M	± m)
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Indexes	Groups					
	Control (base diet)	Experimental group I (diet with probiotics)	Experimental group II (diet with mannan- rich fractions)	Experimental group III (diet with probiotics and man- nan-rich fractions)		
	Days 0-21					
number of birds on day 1	20320	20320	20320	20320		
average body weight of ducks, g	$1259\pm45*$	$1273\pm67*$	$1283\pm42\texttt{*}$	$1305 \pm 34*$		
average body weight of ducks,%	100	101.1	101.9	103.6		
average daily gain of ducks, g	83.6 ± 8.4	84.8 ± 8.1	85.7 ± 9.5	87.3 ± 8.2		
average daily gain of ducks,%	100	101.4	102.5	107.6		
safety of poultry,%	91.3	92.4	93.46	104.4		
	Days 22-49					
number of birds on day 22	18552	18703	18991	19537		
average body weight of ducks, g	$3312 \pm 35.3*$	$3415\pm95.5*$	$3459\pm87.4*$	$3547 \pm 24.3*$		
average body weight of ducks,%	100	103.1	104.4	107.1		
average daily gain of ducks, g	58.5	59.2	59.7	61.3		
average daily gain of ducks,%	100	101.2	102.1	104.7		
number of birds on day 49, heads	16537	17353	18060	19127		
number of birds on day 49, %	89.14	92.78	95.10	97.9		
cost of feed	97644.3	103216.7	104128.4	105331.7		
feed consumption per 1 kg of growth for 49 days, kg	2.01	1.91	1.88	1.86		
feed consumption per 1 kg of growth for 49 days, %	100.0	91.52	93.53	95.02		

*The values in the column for each treatment stage that does not share the overall upper index vary significantly (P < 0.05). Each value is an average of n = 3 flocks per diet with 36, 30 and 30 birds in the flock for each growing period, respectively. Comparisons between the groups were made using the Tukeys HSD test, P < 0.05 we considered statistically significant

Table 5 Average body weight of ducks receiving probiotics and mannan-rich fractions during different periods of growth and development, g/head (n = 50)

Age,			Groups		
weeks	Control (base diet)	Experimental group I	Experimental group II	Experimental group III	Standard
		(diet with probiotics)	(diet with mannan-rich	(diet with probiotics and	values
			fractions)	mannan-rich fractions)	
0	$52.35 \pm 0.57*$	$52.64 \pm 0.37*$	$53.22 \pm 0.67*$	$52.66 \pm 0.81*$	52
1	$205.52 \pm 1.43*$	$206.53 \pm 0.58*$	$208.42 \pm 0.37*$	$215.53 \pm 0.48*$	206
2	$640.37 \pm 2.93*$	$642.34 \pm 3.25*$	$645.38 \pm 5.34*$	$678.59 \pm 13.73*$	645
3	$1239.17 \pm 5.52*$	$1247.72 \pm 5.51*$	$1258.42 \pm 14.53*$	1305.48±34.27*	1257
4	$1814.58 \pm 7.74*$	$1874.25 \pm 22.47*$	$1883.58 \pm 11.43*$	$1933.53 \pm 31.45*$	1876
5	$2351.34 \pm 33.34*$	$2404.43 \pm 27.48*$	$2486.35 \pm 42.28*$	$2592.63 \pm 47.81*$	2503
6	$2918.42 \pm 27.56*$	$2948.27 \pm 25.58*$	$2915.37 \pm 33.59*$	$3197.37 \pm 49.56 *$	3100
7	$3319.68 \pm 26.85*$	$3419.62 \pm 24.37*$	$3528.63 \pm 25.57*$	3683.87±25.79*	3500

S. enteritidis reisolation rate decreased by 53.6% and C. jejuni – by 66.2% in ducks receiving fractions rich in mannanooligosaccharides, compared to the control group. Experiments showed that the addition of prebiotic fractions to the diet did not affect the concentration of lactobacilli, bifidobacteria, enterococci, and anaerobic bacteria.

Bifido- and lactobacteria have antagonistic activity against circulating strains of *S. enteritidis* and *C. jejuni.* 88.9% of *S. enteritidis* isolates were susceptible to bifidobacteria and 81.5% of the studied strains were susceptible to lactobacilli. 92.6% of the isolated *Campylobacter jejuni* were susceptible to

bifidobacteria, 76.9% of *Campylbacter* strains were susceptible to lactobacteria.

We developed a method of preventing bacterial infections and increasing ducks' productivity based on the combined use of bifido- and lactobacteria $(1.5 \times 10^9 \text{ CFU/mL})$ in a ratio of 1:1 with water and fractions enriched with mannan oligosaccharides (0.4 kg/t) together with feed. We recommend the preparation from the first day of birds' life till the end of growing period.

Preventive measures improved the preservation of the duck population by 8.76%, ensuring the average daily increase by 6.9% and the reduction of feed costs by 4.98% for 1 kg of growth throughout the growing period.

During the experiment, we recorded a significant decrease in *Salmonella* and *Campylobacter* colonization in the poultry intestines and improved average daily growth. The biologically active supplements provided a significant advantage in industrial duck farming.

We demonstrated the effectiveness of natural and environmentally safe methods: yeast fractions rich in mannan oligosaccharides, probiotics, and their combined use. The method was effectively implemented in Ukrainian poultry farms.

CONTRIBUTION

Co	ncept	_	O.I	. Kasjane	nko,	L.V.	Nagornaya,
S.M.	Kas	jane	nko;	Design	_	V.V.	Melnychuk,
S.M.	Kasja	anen	ko;	Observatio	n –	O.I.	Kasjanenko,
V.A.	Yevs	tafie	va;	Resources	_	S.M.	Kasjanenko;
Mater	ials	_	V.A.	Yevstafie	eva,	L.V.	Nagornaya,

S.M. Kasjanenko; Data collection and/or processing –
S.M. Kasjanenko, O.I. Kasjanenko, L.V. Nagornaya,
V.A. Yevstafieva; Analysis and/or interpretation –
O.I. Kasjanenko, L.V. Nagornaya, V.V. Melnychuk,
V.A. Yevstafieva; Search for literature –
O.I. Kasjanenko, S.M. Kasjanenko, V.V. Melnychuk,
G.A. Lukyanova, I.A. Gurenko; Writing the manuscript
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G.A. Lukyanova, I.A. Gurenko; Critical review –
O.I. Kasjanenko, G.A. Lukyanova, I.A. Gurenko.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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Assessing protopectin transformation potential of plant tissue using a zoned criterion space

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Abstract:

Introduction. The existing diversity of plant raw materials and products predetermine the prospects of studying their potential as sources of pectin substances. However all current classifications are either fragmented or inconsistent.

Study objects and methods. Our theoretical ivestigation aimed to develop an adequate classification for all taxa of plant origin, as well as their tissues and derivatives as pectin-containing materials. We developed criteria for assessing transformation potential of the protopectin complex based on the mass fractions of biologically active non-uronide components, native water-soluble pectin, the protopectin complex, and pectin substances. Individual boundary conditions were based on individual pectin potential, protopectin fragmentation potential, and pectin isolation potential.

Results and discussion. Based on the boundary conditions, we defined an universal criterion space that included a set of points M in the coordinates expressed by three main criteria. According to individual boundary conditions, the criterion space was divided, or zoned, into four domains corresponding to protopectin fragmentation potential. They were characterized by: 1) lack of pectin potential, 2) ineffective protopectin fragmentation, 3) ineffective isolation of fragmentation products, and 4) effective isolation. Finally, we developed a generalized algorithm to determine the location of points $M[\mu_1, \mu_2, \mu_3]$ in the zoned criterion space, characterizing the plant tissue.

Conclusion. Our approach can be used to assess any plant tissue for its protopectin transformation potential, which determines the technological influence on its pectin potential. This approach is universal, i.e., applicable to both plant tissue and its derivatives.

Keywords: Protopectin complex, potential, transformation, evaluation system, criterion space

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INTRODUCTION

Food technology is currently striving to maximize the potential of raw materials and use new, nontraditional sources of essential nutraceuticals and food components with biological (antioxidants, enterosorbents, etc.) and/or technological (thickeners, stabilizers, etc.) functional activity [1, 2]. The most promising way to achieve that is a biotechnological approach that makes use of both living cultures of microorganisms and isolated enzyme systems. When using isolated enzyme systems, this approach involves a multiple stage fragmentation of a native supramolecular complex of plant and/or animal cell walls into target components with a wide range of physicochemical and/ or technological properties [3–5].

One of the methods within this approach is to activate the potential of a multicomponent polymer matrix of cell walls and intercellular spaces. This method has a limited use in processing agricultural raw materials. It mainly consists in partial or complete

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degradation (depolymerization) of its individual components to change the consistency or transparency of the final product, or to clear it of degradation products and improve its sensory characteristics. Most certainly, a targeted use of this polymer matrix is complicated by its highly heterogeneous components, a system of bonds between them, and highly entangled polymer chains [6]. Moreover, the heterogeneity of individual matrix components is a serious obstacle to controlling their properties during extraction [7, 8].

Pectin substances are among major carbohydrate biopolymers that have a wide variety of functional and technological characteristics [9, 10]. In a plant cell, they are represented by two main fractions – native water-soluble pectin and a native water-insoluble protopectin complex. The last one is the most valuable for transformation due to its molecular structure and composition [9].

The structure of cell walls in almost all terrestrial plants makes them a potentially good resource for the industrial production of pectin [6, 11, 12]. However, it is difficult to implement. Since the protopectin complex is a branched supramolecular structure incorporated into the cell wall, its transformation is mainly fragmentation into water-soluble polymers (soluble pectin). In addition, mass fractions of pectin substances and the protopectin complex may depend on the type, grade, and purpose of raw materials, their structure and phase of development, soil and weather conditions for their vegetation, as well as localization, duration and storage conditions, processing intensity, etc. [10, 13]. In this regard, the choice of a plant as a pectin-containing material should be determined by the purpose of its use.

Raw materials can be classified according to the size of their pectin potential – "high", "medium," and "small" ("low", "insignificant") [9, 10, 14]. The only fundamental approach to pectin production was offered by Donchenko in [15] and supplemented by Rodionova *et al.* in [19, 20] (works [16–18] are actually based on [15]). Although this approach is rather fragmented, it can be used as a basis for developing a universal system that takes into account the native pectin potential of plant tissue.

The protopectin complex is a key object whose fragmentation enables us to use the biomass of a plant material as a source of pectin substances. Due to the presence of certain plant organisms, mainly a natively soluble fraction of pectin, biomass can be attributed to potential sources of pectin. On the other hand, the biomass of certain taxonomic elements may contain a small amount of pectin, which makes its use ineffective.

Therefore, we found it relevant to develop a clearcut classification of plant bio-resources into groups to determine the prospects of their use as pectin-containing raw materials.

In this regard, we aimed to develop a system of criteria for assessing the transformation potential of native complexes of plant carbohydrate biopolymers exemplified by pectin. To achieve this aim, we set the following objectives:

 working out criteria to assess the transformation potential of native plant biopolymers and the concept of their applicability, and

 developing a system of boundary conditions and an universal algorithm for classifying plant materials according to the transformation potential of their native pectin components.

STUDY OBJECTS AND METHODS

According to existing data, all plant materials can be classified into four main groups, namely:

 bio-resources with sufficient potential for protopectin fragmentation and subsequent isolation of its products as independent substances;

 bio-resources with sufficient potential for protopectin fragmentation, but with insufficient potential for isolation of its products;

- bio-resources with insufficient potential for protopectin fragmentation, but with sufficient potential for natively soluble pectin;

- bio-resources with no pectin potential.

On the one hand, this differentiation involves unifying plant characteristics and reducing them to certain generalized values. On the other hand, it involves dividing the domain of generalized values into four fixed zones. As we know, a universal tool for unifying an arbitrary set of source factors is a range of anonymized criteria reducible to a certain system with the use of boundary conditions [21, 22]. Thus, we can apply a criteria-based approach to fulfilling our objectives.

To be able to scale the criteria to determine clear boundary conditions, we used Harrington's individual desirability function in its canonical form [23]:

$$d_{i} = e^{-e^{-(b_{i}_{0}+b_{i}_{1},\varphi_{i})}}$$
(1)

where d_i is the dimensionless value of Harrington's individual desirability function; b_{i0} is the constant; b_{i1} is the coefficient; and φ_i is the dimensionless operator of Harrington's individual desirability function.

We introduced the first and second individual criteria for protopectin fragmentation potential among the main criteria to assess the native pectin potential.

Let us begin with the first criterion. According to [7, 8], the presence of pectin in the tissue or a certain amount of protopectin in the cell wall matrix is not sufficient for assessing the native pectin potential of plant tissue. The tissues of many plant organisms also contain a significant amount of organic and mineral components with valuable vitamins and antioxidant activity, pronounced aroma, micro- and macronutrient values, etc. [17]. They are also highly sensitive to active technological impact factors. During protopectin fragmentation, organic and mineral components can enter into uncontrolled interactions, resulting in a partial or complete loss of their biological potential. Therefore,

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when assessing the native pectin potential, we should take into account the presence of these biologically active components among other significant factors.

Thus, we decided a complex operator as an independent variable, taking into account mass fractions of protopectin and biologically active components in the tissue:

$$\varphi_1 = \frac{\omega_{pp}}{\sum_{i=1}^{\lambda} \omega_i + \omega_{pp}} \tag{2}$$

where ω_{pp} is the mass fraction of protopectin, mg in 100 g; ω_i is the mass fraction of the *i*-th biologically active component, mg/100 g; and λ is the number of biologically active components in the tissue ($\lambda \in N$).

To apply this operator in practice, we transformed it as follows:

$$\varphi_{1} = \frac{1}{\frac{\sum_{i=1}^{\lambda} \omega_{i}}{\omega_{pp}} + 1}} = \frac{1}{\mu_{1} + 1}$$
(3)

where

$$\mu_1 = \frac{\sum_{i=1}^{\lambda} \omega_i}{\omega_{pp}} \tag{4}$$

Thus μ_1 is the first dimensionless individual criterion of protopectin fragmentation potential.

As we can see, with all possible values of ω_{pp} and $\sum_{i=1}^{\lambda} \omega_i$, this criterion has the following range of definition:

$$\mu_1 \in [0; \infty) \tag{5}$$

In this case, Harrington's individual desirability function can be expressed as:

$$d_1 = e^{-e^{-(b_{10}+b_{1},\phi_1)}} = e^{-e^{-\left(b_{10}+\frac{b_{11}}{\mu_{1}+1}\right)}}$$
(6)

where d_1 is the dependent dimensionless variable; b_{10} is the empirical dimensionless constant; and b_{11} is the empirical dimensionless coefficient.

To determine the numerical values of b_{10} and b_{11} , we had to set the primary relations between the pairs $\{\mu_{11}; d_{11}\}$ and $\{\mu_{12}; d_{12}\}$, for which we proceeded from the following considerations.

If an *i*-th biologically active component has a specific measure of value p_i , the total measure of value for all biologically active components under consideration is:

$$v_{bac} = \sum_{i=1}^{\lambda} m_i \cdot p_i = \frac{m}{100} \cdot \sum_{i=1}^{\lambda} \omega_i \cdot p_i \tag{7}$$

where v_{bac} is the total measure of value for all biologically active components, units; m_i is the mass of the *i*-th component, mg/100 g of plant tissue; *m* is the tissue mass, mg; p_i is the specific measure of value of the *i*-th component, units/mg; and ω_i is the mass fraction of the *i*-th component in the plant tissue, %.

If specific measures of value for the components are expressed through some average specific measure of value

$$p_{av} = \frac{\sum_{i=1}^{\lambda} m_i \cdot p_i}{\sum_{i=1}^{\lambda} m_i}$$
(8)

then formula (7) looks as follows:

$$v_{bac} = \frac{m}{100} \cdot \sum_{i=1}^{\lambda} \omega_i \cdot p_{av} = \frac{m \cdot p_{av}}{100} \cdot \sum_{i=1}^{\lambda} \omega_i \qquad (9)$$

from which

$$\sum_{i=1}^{\lambda} \omega_i = \frac{v_{bac} \cdot 100}{p_{av} \cdot m} \tag{10}$$

If we apply similar considerations to protopectin, then:

$$v_{pp} = m_{pp} \cdot p_{pp} = \frac{m \cdot \omega_{pp}}{100} \cdot p_{pp}$$
(11)

where v_{pp} is the total measure of protopectin value, units; m_{pp} if the mass of protopectin in the tissue, mg; p_{pp} is the specific measure of protopectin value, units/mg; and ω_{pp} is the mass fraction of the *i*-th component in the plant tissue, %.

From Eq. (11), it follows that

$$\omega_{pp} = \frac{v_{pp} \cdot 100}{p_{pp} \cdot m} \tag{12}$$

Thus, formula (4) can be presented as:

$$\mu_1 = \frac{v_{bac} \cdot p_{pp}}{v_{pp} \cdot p_{av}} \tag{13}$$

Grouping similar values on its sides, formula (13) can be transformed as:

$$\frac{v_{bac}}{v_{pp}} = \frac{\mu_1 \cdot p_{av}}{p_{pp}} \tag{14}$$

Respectively, if v_{pp} , protopectin fragmentation makes no sense, even with its significant amount in the tissue. Therefore, a prerequisite for protopectin fragmentation is:

$$\mu_1 \le \frac{p_{pp}}{p_{av}} \tag{15}$$

If p_{av} is expressed as \overline{p}_{av} – in fractions of p_{pp} , – then condition (15) looks as follows:

$$\mu_1 \le \overline{p}_{av}^{-1} \tag{16}$$

When calculating \overline{p}_{av} , it is advisable to use \overline{p}_i rather than p_i , its value reduced to p_{pp} :

$$\overline{p}_{av} = \frac{p_{av}}{p_{pp}} = \frac{\sum_{i=1}^{\lambda} m_i \cdot \overline{p}_i}{\sum_{i=1}^{\lambda} m_i}$$
(17)

Theoretically, \overline{p}_i can be determined using several approaches. However, we believe that the most appropriate approach is based on a daily human need for individual nutrients. This approach is least opportunistic (compared to the financial approach) and subjective (compared to direct expert assessments). Naturally, daily

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Table 1 Specific measures of value for biologically active components and pectin in 100 g of plant tissue

Component	Recommended daily Estimated daily requirement		Specific measure of value, mg ⁻¹		
	requirement, units	mg	mg/kg	p_i	\overline{p}_i
1	2	3	4	5	6
Protein, g			800.00 ^{III}		
Amino acids, mg/kg ^{III}					
- essential amino acids:					
histidine			14	0.071428571	2.198
isoleucine			19	0.052631579	1.619
leucine			42	0.023809524	0.733
lysine			38	0.026315789	0.81
methionine			13.16 ^I	0.075987842	2.338
phenylalanine + tyrosine			27	0.037037037	1.14
threonine			16	0.0625	1.923
tryptophan			4	0.25	7.692
valine			19	0.052631579	1.619
cysteine			5.84 ^I	0.171232877	5.269
– non-essential amino acids			514.15 ^{II}	0.001944958	0.06
– other amino acids			87.85 ^{IV}	0.011383039	0.35
Lipids, g ^v	69.9	69 900	1 075.38		
- saturated fatty acids	21.2	21 200	326.15	0.003066074	0.094
- monounsaturated fatty acids	25.4	25 400	390.77	0.00255905	0.079
– polyunsaturated fatty acids	23.3	23 300	358.46	0.002789712	0.086
Digestible carbohydrates, g^{VI}	275	275 000	4 230.77	0.000236364	0.007
Pectin, g ^{VII}	2	2 000	30.77	0.0325 ^x	1
Minerals ^{VIII}	-	2000	20117	0.0020	•
– Ca mg	1 000	1.000	15 38462	0 064999981	2
– Mg mg	400	400	6 1 5 3 8 5	0 162499898	5
– K mg	2 500	2 500	38 46154	0.025999999	0.8
– Na mg	1 300	1 300	20	0.05	1 538
– P mg	800	800	12 30769	0.081250015	2 5
– Cl mg	2 300	2,300	35 38462	0.028260866	0.87
– Fe mg	14 4	14.4	0 22154	4 513857543	138 888
-7n mg	12	12	0.18462	5 416531253	166 663
– L ug	150	0.15	0.00231	432 9004329	13 320 013
$-C_{11}$ mg	1	1	0.01538	65 01950585	2 000 6
– Mn mg	2	2	0.03077	32 49918752	999 975
– Se ug	63	0.063	0.00097	1 030 927835	31 720 856
$-Cr \mu g$	50	0.05	0.00077	1 298 701299	39 960 04
	70	0.07	0.00108	925 9259259	28 490 028
$-C_0 \mu g$	10	0.01	0.00015	6 666 666667	205 128 205
-Si mg	30	30	0.46154	2 166659444	66 666
– F mg	4	4	0.06154	16 24959376	499 988
Vitamins and provitamin ^{IX}		•	0.00121	10.21909070	177.700
– water soluble					
ascorbic acid (vitamin C) mg	90	90	1 38462	0 722219815	22 222
thiamine (vitamin B) mg	15	15	0.02308	43 32755633	1 333 156
riboflavin (vitamin B_1), mg	1.5	1.8	0.02769	36 11412062	1 111 204
vitamin B mg	2	2	0.03077	32 49918752	999 975
vitamin \mathbf{B}_{6} , mg	3	0.003	0.00005	20000	615 384 615
niacin mg	20	20	0.30769	3 250024375	100.001
nantothenic acid mg	5	5	0.07692	13 00052002	400.016
hiotin ug	50	0.05	0.00077	1298 7012002	39 960 04
folic acid and folates up	400	0.03	0.00615	162 601626	5 003 127
_ fat soluble	ŕVV	т.ч	0.00015	102.001020	5 005.127
carotenoids mg	5	5	0 07692	13 00052002	400.016
vitamin D µg	10	0.01	0.00015	6 666 666667	205 128 205
ν mainin 12, μg	1 V	0.01	0.00015	0.000.000007	200 120.200

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				Continua	tion of the table 1
1	2	3	4	5	6
vitamin E, mg	15	15	0.23077	4.333318889	133.333
vitamin K, µg	120	0.12	0.00185	540.5405405	16 632.017
- pseudo-vitamins					
inositol, mg	500	500	7.69231	0.129999961	4
L-carnitine, mg	300	300	4.61538	0.216666883	6.667
coenzyme Q10 (ubiquinone), mg	30	30	0.46154	2.166659444	66.666
lipoic acid, mg	30	30	0.46154	2.166659444	66.666
vitamin U, mg	20	20	0.30769	3.250024375	100.001
orotic acid (B13), mg	30	30	0.46154	2.166659444	66.666
paraminobenzoic acid, mg	100	100	1.53846	0.65000065	20
choline, mg	500	500	7.69231	0.129999961	4
Flavonoids, mg ^{vIII}	250	250	3.84615	0.26000026	8

I – according to [24] and the ratio in [25]

II - according to the ratio between essential and non-essential amino acids in [25]

III – according to the recommended dietary allowance in [24]

IV - the value is a difference between the daily requirement for protein and the sum of essential and non-essential amino acids

V - according to [24] and [26], based on a daily energy requirement of 2000 kcal

VI - according to [27] and [28]

VII – according to [18]

VIII – according to [28]

IX – according to [28] and [29, 30]

X – the value corresponds to p_{ps}

requirements for certain components depend on our knowledge of biochemical processes in the human body, as well as on the constantly changing environmental situation in the world [24]. However, these factors should not significantly affect \overline{p}_{av} .

The value of \overline{p}_{av} was calculated in several stages.

At the *first* stage, we determined daily requirements for each of the biologically active components (u_i) and pectin (u_{ps}) based on a daily energy requirement of 2000 kcal and an average body weight of 65 kg. The differences in daily requirements for men and women were averaged. For comparability, all the values were presented in mg/kg of body weight.

At the *second* stage, we calculated specific measures of value for biologically active components (p_i) and pectin (p_{ps}) :

$$p_i = u_i^{-1}$$
 (18)

$$p_{ps} = u_{ps}^{-1}$$
 (19)

The specific measures of value for pectin p_{ps} and protopectin p_{pp} were numerically identical since protopectin is only valuable for the human body in the form of its fragmentation products. To simplify, we assumed that processing resulted in all protopectin fragmented in a targeted manner (i.e., into fragments that could be identified as pectin).

At the *third* stage, we determined specific measures of value in the fractions of the specific measure of pectin values \overline{p}_i .

The calculation results are shown in Table 1.

At the *fourth* stage, we calculated the value of \overline{p}_{av}^{-1} (Table 2). Based on the data in [31], we determined the content of biologically active components in 100 g of tissue for 21 types of plant materials from the classification presented in [16]. For each type of raw

material, formula (17) was used to calculate the values of $\overline{p}_{av(j)}$ and $\overline{p}_{av(j)}^{-1}$, where $j \in \mathbb{N}$.

Some assumptions were made in the calculations. For example, the mass fractions of the components which were not available in the database were assumed as equal to zero [31]. The amount of carotenoids was calculated based on the biological potential of each type of raw material as $m_{car} = m_{\beta-car} + \frac{1}{2} \cdot \sum_{i=1}^{n} m_{i(o,c)}$, where $m_{\beta-car}$ is the mass fraction of β -carotene, mg/100 g; $\sum_{i=1}^{n} m_{i(o,c)}$ is the sum of mass fractions of other carotenoids, mg/100 g [24]. The amount of tocopherols was also calculated taking into account the biological potential of each type of raw material as $m_{tak} = m_{\alpha-toc} + \frac{1}{10} \cdot m_{\gamma-toc}$, where $m_{\alpha-toc}$ and $m_{\gamma-toc}$ are the mass fractions of α - and γ -tocopherols, respectively; mg/100 g [24]. To determine the sum of the remaining amino acids, we subtracted the mass fractions of essential and non-essential amino acids from the mass fraction of protein.

The calculation results are shown in Table 2.

Since $\overline{p}_{av(j)}^{-1}$ values were significantly different for different types of raw materials, we calculated the average $\overline{p}_{av(av)}^{-1}$ and the margin of error Δ to determine boundary values (μ_{11} and μ_{12}):

$$\overline{p}_{av(av)}^{-1} = \frac{\sum_{j=1}^{\zeta} \overline{p}_{av(j)}^{-1}}{\zeta}$$
(20)

$$\Delta = t_{(\alpha; \zeta - 1)} \cdot \sqrt{\frac{\sum_{j=1}^{\zeta} \left(\overline{p}_{av(j)}^{-1} - \overline{p}_{av(av)}^{-1}\right)^2}{\zeta \cdot (\zeta - 1)}}$$
(21)

where ζ is the number of raw material types; $t_{(\alpha; \zeta-1)}$ is Student's t-test; and α is the probability of error (0.05).

Based on the above, the value of μ_{11} for the first pair $\{\mu_{11}; d_{11}\}$ was calculated as:

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Raw materials	$\overline{P}_{av(j)}$	$\overline{p}_{av(j)}^{-1}$	Raw materials		$\overline{p}_{av(j)}$	$\overline{p}_{av(j)}^{-1}$
Carrot	0.8282	1.207	Persimmon		0.0887	11.274
Beetroot	0.4156	2.406	Grapefruit		0.2355	4.246
Watermelon	0.2860	3.497	Lemon	with skin	0.6618	1,511
Pumpkin	0.6578	1.520		without skin	0.3057	3,271
Melon	0.1749	5.718	Orange		0.2729	3.664
Apples	0.0783	12.771	Tangerine		0.1691	5.914
Quince	0.0993	10.070	Currants	red	0.2654	3,768
Pears	0.0889	11.249		black	0.4389	2,278
Figs	0.1137	8.795	Cranberry		0.3147	3.178
Pomegranate	0.1671	5.984	Gooseberry		0.2935	3.407
Grapes	0.1057	9.461	Feijoa		0.2074	4.822

Table 2 Weighted average reduced measures of raw materials value in non-uronide biologically active components

$$\mu_{11} = \overline{p}_{av(av)}^{-1} - \Delta \tag{22}$$

The value of μ_{12} for the second pair $\{\mu_{12}; d_{12}\}$ was calculated as the second order of μ_{11} :

$$\mu_{12} = \left(\overline{p}_{av(av)}^{-1} - \Delta\right)^2 \tag{23}$$

The critical (boundary) values of μ_1 were based on the analysis of Harrington's desirability function, using μ_{11} and μ_{12} as reference values. Since they are preset, the calculated values were rounded to the nearest whole number.

Despite the rigor of expression (16), its righthand side is an empirical value based on the chemical composition of a finite number of plant raw materials and, therefore, it cannot be considered *a priori*. To make up for this feature, we further determined the critical values of μ_1 on the basis of Harrington's desirability function, using μ_{11} and μ_{12} as reference values. Since a smaller reference value corresponded to a larger value of Harrington's individual desirability function, we defined a condition $Cond_{d_1}$ that determined the individual form of the function as:

$$Cond_{d_1} = \left[\frac{d_{11}}{\mu_{11}} :\Leftrightarrow \frac{0.60}{3}; \frac{d_{12}}{\mu_{12}} :\Leftrightarrow \frac{0.40}{10}\right]$$
 (24)

Based on $Cond_{d_1}$, we calculated the values of the constant and the coefficient: $b_{10} = -0.246$; $b_{11} = 3.673$.

The critical values of the first criterion for the protopectin fragmentation potential at the points with standard critical values of the desirability function can be calculated using Eq. (6) with the variable μ_1 :

$$\mu_{l}[D_{i}] = -1 - \frac{b_{l1}}{b_{l0} + \ln[-\ln(d_{li})]}$$
(25)

where $\mu_1[D_i]$ is the value of the criterion μ_1 at the critical



Figure 1 Graphic interpretation of Harrington's individual desirability function given condition $Cond_d$, and variable μ_1

point D_i of Harrington's individual desirability function determined by Eq. (6) and corresponding to d_{1i} ; and d_{1i} is the standard *i*-th critical (canonical) value d_1 of Harrington's individual desirability function.

The graphic interpretation of Harrington's individual desirability function corresponding to the condition $Cond_{d_1}$ is given in Fig. 1. For each value of d_{1i} , we determined the corresponding values of $\mu_1[D_i]$.

As we can see, the μ_1 range of definition includes four domains separated by the critical values of $\mu_1[D_i]$, where i = 1, 2, 3. By definition, domain IV includes those μ_1 values at which the fragmentation of the protopectin complex makes no sense due to a low value of the individual function of desirability.

Domain III covers those μ_1 values at which the individual desirability function is large enough for protopectin fragmentation to make sense, but insufficiently large to neglect non-uronide bioactive components and isolate the products of fragmentation.

In domains I and II, the individual desirability function is so large that the content of non-uronide bioactive components in plant tissue can be completely ignored.

Based on the physical meaning of the boundary conditions for μ_1 , we established two individual boundary conditions that partially determined the native pectin potential of plant tissue.

Boundary condition I:

 $-\mu_1 > \mu_1[D_3]$ means the absence of the first individual potential for protopectin fragmentation;

 $-\mu_1 \le \mu_1[D_3]$ means the presence of the first individual potential for protopectin fragmentation.

Boundary condition II:

 $- \mu_1[D_3] \ge \mu_1 > \mu_1[D_2]$ means the absence of the first individual potential for isolation of protopectin fragmentation products;

 $-\mu_1 \le \mu_1[D_2]$ means the presence of the first individual potential for isolation of protopectin fragmentation products.

Next, we determined the structure and properties of the second dimensionless individual criterion for the protopectin fragmentation potential.

The second independent variable was a complex operator based on the mass fraction of protopectin in the tissue:

$$\varphi_2 = \frac{\omega_{pp}}{100} = \mu_2 \tag{26}$$

where φ_2 is the dimensionless operator of Harrington's individual desirability function; and μ_2 is the second dimensionless individual criterion for the protopectin fragmentation potential.

Harrington's individual desirability function was expressed as:

$$d_2 = e^{-e^{-(b_{20}+b_{21}\varphi_2)}} = e^{-e^{-(b_{20}+b_{21}\varphi_2)}}$$
(27)

Thus, the condition $Cond_{d_2}$ that determined the individual function was set as:

$$Cond_{d_2} = \left[\frac{d_{21}}{\mu_{21}} :\Leftrightarrow \frac{0.35}{0.001}; \frac{d_{22}}{\mu_{22}} :\Leftrightarrow \frac{0.65}{0.05}\right]$$
 (28)

Based on $Cond_{d_2}$, we calculated the values of the constant and the coefficient: $b_{20} = -6.68 \times 10^{-2}$ and $b_{21} = 18.179$. The critical values of the μ_2 criterion were calculated as:

$$\mu_2[D_i] = -\frac{b_{20} + \ln\left[-\ln\left(d_{2i}\right)\right]}{b_{21}} \tag{29}$$

where $\mu_2[D_i]$ is the value of μ_2 at the critical point D_i of Harrington's individual desirability function calculated by Eq. (6) and corresponding to d_{2i} ; d_{2i} is the standard *i*-th critical (canonical) value d_2 of Harrington's individual desirability function.



Figure 2 Graphic interpretation of Harrington's individual desirability function given condition $Cond_{d_1}$ and variable μ_2

The graphic interpretation of Harrington's individual desirability function corresponding to the condition $Cond_{d_2}$ is presented in Fig. 2. For each value of d_{2i} , we calculated the corresponding values of $\mu_2[D_i]$.

Just like with μ_1 , the μ_2 range of definition includes four domains separated by the critical values of $\mu_2[D_i]$, where i = 1, 2, 3.

By definition, domain IV covers those values of μ_2 at which the fragmentation of the protopectin complex makes no sense. This led us to formulate the third individual boundary condition:

 $-\mu_2 < \mu_2[D_3]$ means the absence of the second individual potential for protopectin fragmentation;

 $-\mu_2 \ge \mu_2[D_3]$ means the presence of the second individual potential for protopectin fragmentation.

We should note that fragmentation potentials I and II are categorical, i.e., if one of them is absent, the total fragmentation potential is absent as well.

Domains I, II, and III include such values of μ_2 that ensure not only protopectin fragmentation, but also the isolation of fragmentation products. Based on the canonical reference values of the individual desirability function, we formulated the fourth boundary condition:

- $\mu_2[D_3] \le \mu_2 < \mu_2[D_1]$ means the absence of the second individual potential for isolation of protopectin fragmentation products;

 $-\mu_2 \ge \mu_2[D_1]$ means the presence of the second individual potential for isolation of protopectin fragmentation products.

Similar to the first and the second fragmentation potentials, the individual isolation potentials are categorical.

The third independent variable was a complex operator based on the mass fraction of pectin substances in the tissue:

$$\varphi_3 = \frac{\omega_{ps}}{100} = \mu_3 \tag{30}$$

where φ_3 is the dimensionless operator of Harrington's individual desirability function; ω_{ps} is the total amount of pectin substances, %; and μ_3 is the third dimensionless individual criterion for the protopectin fragmentation potential.

In this case, the condition $Cond_{d_3}$ that determined the individual function was calculated as:

$$Cond_{d_3} = \left[\frac{d_{31}}{\mu_{31}} :\Leftrightarrow \frac{0.40}{0.01}; \frac{d_{32}}{\mu_{32}} :\Leftrightarrow \frac{0.65}{0.07}\right]$$
 (31)

Based on expression (31), we calculated the constant and the coefficient as $b_{30} = -3.8367 \times 10^{-2}$ and $b_{31} = 12.5788$, respectively, and the critical boundaries of μ_3 , as:

$$\mu_{3}[D_{i}] = -\frac{b_{30} + \ln\left[-\ln\left(d_{3i}\right)\right]}{b_{31}}$$
(32)

where $\mu_3[D_i]$ is the value of μ_3 at the critical point D_i of Harrington's individual desirability function calculated by (6) and corresponding to d_{3i} ; and d_{3i} is the standard *i*-th critical (canonical) value d_3 of Harrington's individual desirability function.

Figure 3 shows the graphic interpretation of Harrington's individual desirability function given $Cond_{d_3}$. For each value of d_{3i} , we calculated the corresponding values of $\mu_3[D_i]$.

Here, we can clearly see domain IV with no pectin potential in the plant tissue.

As a result, we formulated the fifth individual boundary condition:

 $-\mu_3 < \mu_3[D_3]$ means the absence of pectin potential;

 $-\mu_3 \ge \mu_3[D_3]$ means the presence of pectin potential.

Thus, the pectin potential is categorical.

The fourth independent variable was a complex operator based on the ratio of the mass fractions of protopectin and pectin substances in the tissue:

$$\varphi_4 = \frac{\omega_{pp}}{\omega_{ps}} = \frac{1}{\mu_4 + 1} \tag{33}$$

where φ_4 is the dimensionless operator of Harrington's individual desirability function; ω_{sp} is the mass fraction of natively soluble pectin substances, %; and μ_4 is the third dimensionless individual criterion for the protopectin fragmentation potential calculated as:

$$\mu_4 = \frac{\omega_{sp}}{\omega_{pp}} \tag{34}$$

Then, the condition $Cond_{d_4}$, which determined the individual function, was calculated as:

$$Cond_{d_4} = \left\lfloor \frac{d_{41}}{\mu_{41}} :\Leftrightarrow \frac{0.65}{2.50}; \frac{d_{42}}{\mu_{42}} :\Leftrightarrow \frac{0.80}{1.25} \right\rfloor$$
(35)

Based on expression (35), we calculated the constant and the coefficient ($b_{40} = -0.3419$, $b_{41} = 4.1441$).

Based on $Cond_{d_4}$, the critical boundaries of μ_4 were calculated as:

$$\mu_4[D_i] = -1 - \frac{b_{41}}{b_{40} + \ln\left[-\ln\left(d_{4i}\right)\right]}$$
(36)

where $\mu_4[D_i]$ is the value of μ_4 at the critical point D_i of Harrington's individual desirability function calculated by (6) and corresponding to d_{4i} ; and d_{4i} is the standard *i*-th critical (canonical) value d_4 of Harrington's individual desirability function.

Figure 4 shows the graphic interpretation of Harrington's individual desirability function given $Cond_{d,2}$, with d_{4i} values corresponding to $\mu_4[D_i]$ values.

Based on the logical content of d_{4i} and the numerical values of $\mu_4[D_i]$, the range of definition can be divided into four domains that determine the fragmentation potential of the protopectin complex and the isolation potential of fragmentation products.

According to Fig. 4, domain IV covers those values μ_4 at which the mass fraction of water-soluble pectin exceeds that of the protopectin complex so much that there is practically no reason for its individual fragmentation. Thus, we determined the sixth boundary condition as follows:

 $-\mu_4 > \mu_4[D_3]$ means the absence of the third individual potential for protopectin fragmentation;

 $-\mu_4 \le \mu_4[D_3]$ means the presence of the third individual potential for protopectin fragmentation.

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Figure 3 Graphic interpretation of Harrington's individual desirability function given condition $Cond_{d_{a}}$ and variable μ_{3}



Figure 4 Graphic interpretation of Harrington's individual desirability function given condition $Cond_{d_1}$ and variable μ_4

Following the same pattern, we determined the seventh boundary condition (VII), namely:

 $-\mu_4[D_2] < \mu_4 \le \mu_4[D_3]$ means the absence of the third individual potential for isolation of protopectin fragmentation products;

 $-\mu_4 \le \mu_4[D_1]$ means the presence of the third individual potential for isolation of protopectin fragmentation products.

In addition, boundary conditions VI and VII are based on:

$$\mu_4 \le \mu_4[D_i] \tag{37}$$

where i = 3 for condition VI and, i = 2 for condition VII. However, μ_4 can be expressed as:

$$\mu_4 = \frac{\omega_{sp}}{\omega_{pp}} = \frac{\omega_{ps} - \omega_{pp}}{\omega_{pp}} = \frac{\mu_3 - \mu_2}{\mu_2} \tag{38}$$

Then, given the presence of the third individual fragmentation potential:

$$\mu_3 \le \mu_2 \cdot (\mu_4[D_i] + 1) \tag{39}$$

Thus, the third individual potentials of fragmentation and isolation are relative since they are involved in the formation of respective total potentials indirectly, through expressions in which they act as one of the variables.

If we assume that there is a certain criterion space with coordinates μ_1 , μ_2 and μ_3 , the pectin potential of any plant material can be clearly determined as a geometrical location of the point $M[\mu_1, \mu_2, \mu_3]$ corresponding to the material under analysis.

Based on the *a priori* assumption that

$$\omega_{ps} + \sum_{i=1}^{\lambda} \omega_i \le 100 \tag{40}$$

we can establish the eighth boundary condition (VIII): the top boundary of the range of definition for all possible values of $M[\mu_1, \mu_2, \mu_3]$ is determined by the following basic proposition:

$$\mu_{3(top)} = 1 - \mu_1 \cdot \mu_2 \tag{41}$$

In addition, since a part cannot be larger than a whole, it is also true that:

$$\omega_{pp} \le \omega_{ps}$$
 (42)

which leads to the following condition:

$$\mu_3 \ge \mu_2 \tag{43}$$

i.e., the bottom boundary of the range of definition for all possible values of $M[\mu_1, \mu_2, \mu_3]$ is determined by the second basic proposition:

$$\mu_{3(bot)} = \mu_2 \tag{44}$$

The last formula is an expression of boundary condition IX.

RESULTS AND DISCUSSION

Thus, according to boundary conditions VIII and IX, a set (A) of all points $M[\mu_1, \mu_2, \mu_3]$ can be defined as

$$M[\mu_1, \mu_2, \mu_3] \in [\mu_{3(bot)}, \mu_{3(top)}]|_{\mu_1 \ge 0; \, \mu_2 \ge 0; \mu_3 \ge 0}, \quad (45)$$

graphically presented in Fig. 5.

The logic of assessing plant bioresources for the presence of pectin substances determines general boundary conditions for defining a set of points $M[\mu_1, \mu_2, \mu_3]$ as the following hierarchy: "individual pectin potential \rightarrow individual fragmentation potential of the protopectin complex \rightarrow individual isolation potential of protopectin fragmentation products". Thus, the entire set of points $M[\mu_1, \mu_2, \mu_3]$ can be divided into four subsets:

- subset A_1 characterized by the absence of a common pectin potential in all the elements;

- subset A_2 where $A_2 \cap A_1 = \emptyset$ and all the elements have a common pectin potential, but lack a common potential for protopectin fragmentation;

- subset A_3 where $A_3 \cap A_2 = \emptyset$ and all the elements have common pectin and protopectin fragmentation potentials, but lack a common isolation potential for fragmentation products; and

- subset A_4 where $A_4 \cap A_3 = \emptyset$ and all elements have common pectin and protopectin fragmentation

potentials, as well as isolation potential for fragmentation products.

By definition, the following is true for all the subsets:

$$A_1 \cap A_2 \cap A_3 \cap A_4 = \emptyset \tag{46}$$

Based on the above, the existence of A_1 corresponds to:

$$\mu_2 \le \mu_3 < \mu_3[D_3] \tag{47}$$

The area of definition for all A_1 elements is partially presented in Fig. 6.

The existence of subset A_2 corresponds to:

$$1-\mu_{1}\cdot\mu_{2} \geq \mu_{3} \geq \left\{ \begin{cases} \mu_{2}\cdot(\mu_{4}[D_{3}]+1), \mu_{2} \geq \mu_{2}[D_{3}] \\ \mu_{3}[D_{3}], \mu_{2} < \mu_{2}[D_{3}] \end{cases} \right. \\ \left\{ \begin{array}{l} \mu_{2}, \mu_{2} \geq \mu_{3}[D_{3}] \\ \mu_{3}[D_{3}], \mu_{2} < \mu_{3}[D_{3}] \\ \mu_{3}[D_{3}], \mu_{2} < \mu_{3}[D_{3}] \end{array} \right| \mu_{1} > \mu_{1}[D_{3}] \end{cases}$$

$$(48)$$

Figure 7 shows a partial area of definition for all A_2 elements.

The existence of A_1 corresponds to:

$$\begin{cases} 1-\mu_{1}\cdot\mu_{2} & \mu_{1} \leq \mu_{1}[D_{3}] \\ \mu_{2}\cdot(\mu_{4}[D_{3}]+1) & \mu_{2} \geq \mu_{2}[D_{3}] \\ \end{pmatrix} = \\ \geq \begin{cases} \mu_{2}\cdot(\mu_{4}[D_{2}]+1) & \mu_{1} \leq \mu_{1}[D_{2}] \\ \mu_{2} \geq \mu_{2}[D_{1}] \\ \mu_{2} & \mu_{1}[D_{3}] \geq \mu_{1} > \mu_{1}[D_{2}] \\ \mu_{2}[D_{3}] \leq \mu_{2} < \mu_{2}[D_{1}] \end{cases}$$

$$(49)$$

Figure 8 presents the area of definition for all A_3 elements.

The existence of subset A_4 corresponds to:

$$\begin{cases} \left\{ 1 - \mu_{1} \cdot \mu_{2} \\ \mu_{2} \cdot (\mu_{4}[D_{2}] + 1) \right\} > \mu_{3} \ge \mu_{2} \end{cases} \quad \begin{array}{l} \mu_{1} \le \mu_{1}[D_{2}] \\ \mu_{2} \ge \mu_{2}[D_{1}] \end{cases} \tag{50}$$

The area of definition for all A_4 elements is presented in Fig. 9.

Thus, the specific value $M[\mu_1, \mu_2, \mu_3]$ that shows its belonging to one of the subsets A_i (where i = 1, 2, 3, 4) in the zoned criterion space clearly determines the plant tissue's overall potential for protopectin fragmentation.

Our approach to classifying plants as pectincontaining materials, which is based on a system of criteria and a zoned criterion space, has clear advantages over existing methods due to its objectivity determined by the boundary conditions.

However, when analyzing this approach, we can easily see that the μ_{j1} and μ_{j2} values corresponding to d_{j1} and d_{j2} in the conditions $Cond_{d_j}|_{j=2,3,4}$ were set *a priori*, based on general assumptions regarding the degree of acceptability of certain μ_j values within Harrington's individual desirability functions in accordance with the boundary (canonical) values of *d*. Yet, the conditions $Cond_{d_j}|_{j=2,3,4}$ determine the coefficients and constants, and, consequently, individual desirability

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Figure 5 Definition area of the criterion space



Figure 7 Partial definition area for subset A,

functions, as well as numerical values of $\mu_j [D_i]$. Therefore, at this stage, our approach has a general, conceptual form requiring further research.

Based on the results, we developed a generalized algorithm to determine the geometric location of plant tissue in the zoned criterion space, or $M[\mu_1, \mu_2, \mu_3]$ belonging to one of the subsets (Fig. 10). We can use this algorithm to assess any plant tissue's potential for transformation of the protopectin complex, which determines the influence of any technological impact on its pectin potential.

The approach that we used to determine the criterion space and boundary conditions for its zoning explicitly suggests that this algorithm is universal for classifying plant tissue or its derivatives as pectin-containing materials. Thus, the algorithm is applicable to any type of plant material for which the μ_1 , μ_2 and μ_3 criteria can be numerically expressed.

CONCLUSION

To sum up, our investigation showed the following results.



Figure 6 Partial definition area for subset A₁



Figure 8 Partial definition area for subset A₃



Figure 9 Partial definition area for subset A_{4}

1. We developed a system of criteria to assess the transformation potential of the protopectin complex in plant tissue. This system is based on the geometrical Kondratenko V.V. et al. Foods and Raw Materials, 2020, vol. 8, no. 2, pp. 348-361



Figure 10 Algorithm for plant tissue classification according to protopectin fragmentation potential based on the geometric location in the zoned criterion space

location of $M[\mu_1, \mu_2, \mu_3]$ – the point that corresponds to the material under analysis – in a zoned criterion space with coordinates in the form of dimensionless individual criteria for protopectin fragmentation potential.

2. The dimensionless individual criteria for protopectin fragmentation potential included the ratio between the mass fractions of biologically active components and protopectin in plant tissue, the mass fraction of the protopectin complex expressed in unit fractions, and the mass fraction of total pectin substances expressed in unit fractions.

3. We established nine individual boundary conditions, individual pectin potential, two individual fragmentation potentials, and three individual isolation potentials for pectin substances, which altogether determine a system of zoning the criterion space.

4. The boundary conditions in the definition area for a set of points $M[\mu_1, \mu_2, \mu_3]$ had the following hierarchy: individual pectin potential \rightarrow individual fragmentation potential of the protopectin complex \rightarrow individual isolation potential of protopectin fragmentation products.

5. We developed an algorithm to classify plant tissues according to protopectin fragmentation potential based on the geometric location in the zoned criterion space.

CONTRIBUTION

All the authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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Identification of total aromas of plant protein sources

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Abstract:

Introduction. Due to the deficit and high cost of complete animal protein, the search and analysis of alternative sources is an actual scientific trend. Lentils is a good alternative to animal protein, but the pronounced bean smell and taste limit its full or partial use in food production. The aim of the work was to determine the total aromas of lentils when germinated to eliminate the bean taste and smell.

Study objects and methods. The object of the study was brown lentil beans germinated under laboratory conditions. Samples of the equilibrium gas phase formed over samples of wet and sprouted beans were investigated. The analysis of total aromas was carried out on a laboratory odor analyzer MAG-8 ("electronic nose") by the method of piezoelectric quartz micro-weighing with an array of sensors.

Results and discussion. The study results showed qualitative and quantitative differences in the equilibrium gas phase over samples of wet and germinated grain. The quantitative analysis showed that the content of volatile compounds over sprouted grain is 12% less than over wet. The qualitative composition of the samples of wet and sprouted grain differed by 60%, which confirmed the influence of germination on the composition of the equilibrium gas phase and the possibility of eliminating bean odor. Testing showed that the use of pre-processed lentil grains allows to replace up to 50% of raw meat in minced products (minced food, chopped food) without changing the smell of the products.

Conclusion. According to the results obtained, preliminary processing of lentils by germination will allow using this bean culture as an alternative source of animal protein to expand the range, and improve the quality of meat and dairy products.

Keywords: Lentils, germination, amino acid composition, biological value, total flavors, total analytic signal, the equilibrium gas phase

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INTRODUCTION

The lack of animal protein resources on the planet arose a long time ago, and a global protein deficit in human nutrition continues to grow. According to some experts, for example, over next 20 years, the shortage of meat will remain one of the global problems of mankind.

At the same time, in foreign and domestic technologies of obtaining food products, there is a positive experience in the complete or partial replacement of expensive and difficultly reproducible animal protein resources with vegetable ones, including meat and dairy products [1–3].

The use of alternative sources of protein allows manufacturers to simultaneously solve economic and

technological problems, such as reducing production costs, stabilizing and improving the quality of meat systems, increasing product yield, etc.

Currently, a consumer begins to find benefits of consuming products with alternative sources of protein, namely a lower cost and the ability to provide themselves with healthy food in the required amount.

The experience of using vegetable proteins on an industrial scale is mainly associated with imported soy [4]. However, at the moment, during the unfolding of measures to implement the provisions of the Food Security Doctrine, either ensuring healthy nutrition, or the development of domestic technologies, and rejection of imports become relevant. Russian scientists have

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Table 1	Digestive	enzvme	inhibitors	in legumes
I HOIC I	Digestive	enzyme	minoncorb	in regames

	Soybean		Lentils
Inhibitors	Their action	Inhibitors	Their action
1. Protease inhibitors	Binding trypsin and chymotrypsin, which are secreted by the animal's pancreas, thereby reducing the efficiency of digestion of feed.	1. Trypsin inhibitor	Inhibiting the activity of trypsin, which is synthesized by the pancreas in the form of zymogen, activated in the duodenum 12, and catalyzes the hydrolysis of proteins and peptides. Trypsin is involved in the activation of many digestive enzymes through a limited proteolysis mechanism.
2. Urease Inhibitors	Suppressing urease activity, thereby reducing urea hydrolysis with ammonia formation, and neutralizing its toxic effect on the body.		
3. Lipoxy- genase inhibitors	Inhibiting the activity of lipoxeginase involved in the enzymatic rancidity of fats during storage with the formation of aldehydes and ketones, which give an unpleasant smell and taste.	_	

proved the benefits of legume proteins traditionally grown in Russia (Saratov, Samara, Orenburg and Penza regions and Altai Krai), the potential of which can be significantly increased by pre-germination [1].

Among alternative sources of protein, legumes occupy priority positions in terms of protein quantity and quality. That is why they are preferred when creating, for example, meat food systems [5, 6]. Among them, lentils, known for its healing properties, should be distinguished. By the quantity and quality of amino acids, the lentil protein is closest to the first grade beef protein. Compared to other legumes, lentils have a more balanced amino acid composition, containing an increased amount of valuable vitamins, macro-, microelements (including iodine), and less oligosaccharides that cause intestinal flatulence.

Lentils is one of few cultures containing only one digestive enzyme inhibitor that acts on trypsin (Table 1).

Lentils have good botanical properties, develop and fruit well in Central Russia (Chernozemye, Volga region).

The choice of lentils as an object of the study is justified by the results of chemical analysis obtained by Howell, according to which this culture has a number of obvious advantages compared to other legumes [9]. However, the test results achieved in the conditions of the "Kalacheevsky" meat factory showed the limited use of lentils even in small quantities (5–8%) due to its bean taste and smell. In this regard, germination is of practical interest, not only to improve the balance of the amino acid composition, and to increase the content of micronutrients, but also to assess the possibility of eliminating undesirable sensory properties.

Many researchers confirm that, as a result of germination, a decrease in the oligosaccharide fraction is observed (Fig. 1), additional vitamins are synthesized, increasing the total nutritional and biological value of the product [10–17]. However, the information concerning change in organoleptic properties is

extremely insufficient, and ungeneralized [13–16]. Lentil products (flour, concentrate, isolate) combine well and replace food systems of animal origin (meat, dairy).

Our previous results proved that germinating significantly improved the biological value of raw materials, namely the content of proteins, vitamins, and minerals. The amount of amino acids increased by a factor of 1.5-2 (Fig. 1).

The amino acid composition of the protein becomes more balanced, with the score close to the score of the reference protein (Fig. 2). A significant increase in lysine and tryptophan, the most valuable amino acids, can be mentioned. Lysine is a deficient amino acid, which, combined with vitamins, strengthens the immune system, promotes calcium absorption from the intestine, as well as contributes to cellular protein and bone tissue formation. Tryptophan is involved in the serotonin (the hormone of happiness) formation; mood, sleep quality, level of pain threshold and susceptibility to various irritants and inflammations depend on its concentration.

An increase in the content of minerals and vitamins, as well as a decrease in the number of oligosaccharides,



Figure 1 Amino acid composition of lentil grains, beef and poultry meat



Figure 2 Effect of germination on lentil amino acid score

an anti-nutritional factor of legumes, were also noted (Table 2).

Identification of total aromas by sensory methods, taking into account the nutritional value of lentils, is especially important. All these indicators belong to food chemistry, the most important section in the study of food products, providing important information in the selection of raw materials, and it also means for a consumer a better applicability of the product in a food system [17, 18]. The information obtained will allow us to construct a product with properties (chemical composition, appearance, taste, and smell) close to those of meat.

The aim of the study was to assess the total aromas of lentils during germination, to eliminate extraneous odor by partial or complete replacement of animal proteins in food systems of animal origin.

STUDY OBJECTS AND METHODS

The object of study was brown lentil beans (State Standard 7066-77¹) germinated in laboratory conditions at Voronezh State University of Engineering Technologies.

Grains of untreated lentils were germinated at 21–23°C for 3–4 days, preventing their complete drying out.

The general chemical composition of sprouted lentils is presented in Table 2, and the amino acid composition of proteins in Fig. 1. It can be seen from the data that germinated lentil grains have significant advantages in the content of the most important nutrients.

The study of total aromas was carried out on a MAG-8 laboratory (experimental) odor analyzer (Fig. 3) with the electronic nose methodology, by the method of piezoelectric quartz weighing with an array of sensors [20–22]. We analyzed three samples: wet grain, germinated grain and water.

The sensor array consisted of eight sensors based on BAW-type piezoelectric quartz resonators with an oscillation frequency of 10.0 MHz and with diverse film sorbents on electrodes.

Coatings are selected in accordance with the test objective (possible emission from samples of various organic compounds):

Sensor 1 – Multilayer Carbon Nanotubes, MCNT;

Sensor 2 – Polyethylene glycol succinate, PEGS;

Sensor 3 – Polyethylene glycolsebacinate, PEGSb;

Sensor 4 – Polyethylene Glycol Adipate, PEGA;

Sensor 5 – Polyethylene glycol-2000, PEG-2000;

Sensor 6 - Dicyclohexane-18-Crown-6, DCH18C6;

Sensor 7 – Twin-40, Tween;

Sensor 8 - Polyethylene Glycolphthalate, PEGP.

Grain samples were placed in glass tubes (10 g in each), tightly closed, kept at room temperature $(20 \pm 1^{\circ}C)$ for at least 20 min to saturate the equilibrium gas phase over the samples. Then, we determined moisture content. which amounted to 51.2%. 3 cm³ of the equilibrium gas phase was taken through a membrane with individual syringes and introduced into the detection cell. The background of the array of sensors was from 15 to 30 Hz·s. The measurement time was 60 s, the mode for recording sensor responses was uniform with a step of 1 s, the optimal algorithm for presenting

 Table 2 Effect of germination on chemical composition of lentil seeds

Indicators	Content in 100 g of product					
	Before germination	After germination				
Proteins, g	26.15	29.56				
Fat, g	1.2	1.1				
Carbohydrates, g	53.7	41.06				
including glucose	8.45	13.64				
Oligosaccharides:						
raffinose	0.9	0.5				
stachyose	2.7	2.1				
verbascose	1.4	0.8				
Starch	33.8	24.12				
Cellulose	3.65	3.04				
Ash	3.65	3.31				
Moisture	12.33	18.1				
Minerals, mg						
calcium	84.23	84.62				
phosphorus	401.16	400.3				
magnesium	78.9	76.3				
iron	12.06	12.32				
sodium	56.12	55.91				
potassium	659.18	659.51				
Vitamins, mg						
B1	0.5	0.78				
B2	0.21	0.48				
PP	1.8	2.21				
С	-	0.04				
β-carotene	0.03	0.08				

¹ State Standard 7066-77. Lentil for human consumption (plateshaped). Requirements for state purchases and deliveries. Moscow: Izdatel'stvo standartov; 2003. 8 p.



Figure 3 A general view of the workplace with the MAG-8 analyzer

responses was based on the maximum responses of individual sensors. The measurement error was 10%.

The total analytical signal is generated by using the integrated signal processing algorithm of eight sensors in the form of a "visual imprint". To determine the total composition of sample smell, we used the full "visual imprints" of the peaks (the largest responses of eight sensors), constructed from the maximum responses of the sensors in the equilibrium gas phase of the samples during the measurement time (no more than 1 min). The similarity and difference in the composition of the volatile odor fraction over the analyzed samples was established [20]. Slight differences in the composition of the gas mixture were established by comparing the kinetic "visual imprints" constructed from the responses of all the sensors recorded at different times over the entire measurement interval. The nature of the components mixture is more apparent in such analytical signals. Both types of signals, as well as the area of the figures are calculated automatically in the instrument software.

The following criteria for assessing differences in the smell of the analyzed samples are selected:

A qualitative characteristic – the form of a "visual imprint" with characteristic distributions along the

response axes, was determined by the set of compounds in the equilibrium gas phase.

Quantitative characteristics:

1) The total area of the full "visual imprint" $(S\Sigma, Hz \cdot s)$ was used to estimate the total intensity of the aroma proportional to the concentration of volatile substances, including water. This parameter was constructed from all signals of all sensors for the full measurement time;

2) The maximum signal of sensors with the most active or specific sorbent films (Δ Fmax, Hz) was applied to assess the content of individual classes of organic compounds in the EGP by the normalization method [21, 22];

3) The identification parameter (Aij) was used to identify individual classes of compounds in a mixture. This parameter was calculated from the signals of the sensors in the analyzed samples and for standard compounds.

Sensor responses were recorded, processed and compared in the software of the MAG Soft analyzer.

RESULTS AND DISCUSSION

In the course of experimental studies (Table 3), the total content of volatile compounds in the EGP was found to correlate with the total analytical signal of the "electronic nose" – the area of the "visual imprint" of the response peaks.

Insignificant differences were found in the total odor intensity over samples of wet and germinated grains, however, the contribution to the total sorption response of different classes of compounds is not equal. To establish differences in the composition (qualitative and quantitative) of the volatile odor fraction, we analyzed the total content of readily volatile components in the equilibrium gas phase over the samples (Fig. 4).

The shape of the "visual imprint" of the sensor responses in the array showed insignificant differences in the chemical composition of the equilibrium gas phase over samples of wet and germinated grain. The content of volatile compounds in the equilibrium gas phase over germinated grain was less by 12% than over wet grain.

Additionally, we noted the change in the quantitative composition of the odor above the samples according to the relative content of the main classes of volatile compounds, evaluated by the normalization method (Table 4).

Table 3 "Visual imprint" area of the sensor signals (S1-S8) in the equilibrium gas medium above the samples

Samples	S1-MCNT	S2-PEGS	S3-PEGSb	S4-PEGA	S5-PEG-2000	S6-DCH18 C6	S7-Tween	S8-PEGP	S_{sum} , $Hz \cdot s$
Wet grain	9	13	9	14	14	9	11	11	353
Germinated	8	13	8	12	13	9	11	11	312
grain									
Water	18	21	15	24	23	17	21	22	1135

MCNTs - multilayer carbon nanotubes, PEGS - polyethylene glycol succinate, PEGSb - polyethylene glycol sebacinate, PEGA - polyethylene glycol adipate, PEG-2000 - polyethylene glycol, DCH18C6 - dicyclohexane-18-Crown-6, Tween - Twin 40, PEGP - polyethylene glycolphthalate

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Figure 4 "Visual prints" of the maximum sensor signals in the equilibrium gas phase above the samples. The rotary axis indicates the numbers of the sensors in the array (experimental part), and the vertical – the responses of the sensors at a particular point in time (Δ Fmax, Hz)

After germination, a decrease in the intensity of aroma was noted in the universal indicator, as well as in the "O-containing", "alcohols, ketones, amines" and "alcohols, ketones, water" indicators.

Alcohols are the most commonly found compounds in natural essential oils. As part of essential oils, they do not only add a peculiar aroma, but also contribute to the manifestation of antiseptic activity against bacterial and viral infections, have analgesic, anesthetic and tonic effects, as well as regulate hormonal activity. The absence of toxicity is very important, therefore essential oils with a predominant alcohol content are relatively safe.



Figure 5 Spectra of identification parameters for grain and water. The X-axis indicates the numbers of identification parameters, the Y-axis indicates their values

Natural essential oils with a high content of ketones can cause side effects: neurotoxicity (negatively affect the functions of the nervous system), embryotropic effect (dangerous during pregnancy), and hepatotropic effect (disrupt liver function).

Some amines are very toxic substances. Inhalation of their fumes and contact with skin are dangerous. Aliphatic amines affect the nervous system, as well as cause violations of the permeability of the walls of blood vessels and cell membranes, liver functions and the development of dystrophy.

We found that grain samples differed in quality composition. For a more visual presentation of the results, a spectrum of identification parameters was constructed (Fig. 5). The compositions of the equilibrium gas phase can be considered identical if the spectra coincide within the error (equal to or greater than 0.1).

Germinated grain contained fewer components in quality composition than wet grain. To establish such differences, the distribution by identification parameters of their absolute differences from water was traced (Fig. 6).

Differences by more than \pm 0.1 units are significant and indicate a different composition of compounds in the equilibrium gas phase above the samples. In Fig. 5 the allowable difference interval is marked with black lines.

A significant change in the qualitative composition of the sprouted grain compared to wet grain was

Table 4 Signal ratio of several sensors in the matrix for test samples

Samples	S1-MCNT	S2-PEGS	S3-PEGSb	S4-PEGA	S5-PEG-2000	S6-DCH18 C6	S7-Tween	S8 -PEGP
	Universal	N-containing	Alcohols,	Alcohols,	O-containing	Alcohols, acids	Aliphatic	Ether
	sensor		ketones, amines	ketones, water			acids	
Wet grain	10	14.4	10	15.6	15.6	10	12.2	12.2
Germinated grain	9.4	15.3	9.4	14.1	15.3	10.6	12.9	12.9
water	11.2	13	9.3	14.9	14.3	10.6	13	13.7

 $MCNT - multilayer \ carbon \ nanotubes, \ PEGS - polyethylene \ glycol \ succinate, \ PEGSb - polyethylene \ glycol \ sebacinate, \ PEGA - polyethylene \ glycol, \ DCH18C6 - \ dicyclohexane-18-Crown-6, \ Tween - Twin, \ PEGP - polyethylene \ glycol \ philate \ sebacinate \ philate \ sebacinate \ philate \ sebacinate \ philate \ sebacinate \ philate \ p$



Figure 6 Absolute differences in identification parameters for grain samples compared to water. The X axis is the number of identification parameters

established. Water was also very different from both grain samples, which suggests that not only water vapor, but also other organic volatile compounds were present in the equilibrium gas phase. The qualitative composition of the EGP above wet and germinated samples differed significantly (for selected points – by 60%).

In parallel, an analysis of the sensory characteristics of the grain was carried out. A significant decrease in the sharp smell of legumes after germination was found. This makes it possible to reduce the legume smell, which is a drawback when added to food products, or when traditional raw materials are completely replaced.

CONCLUSION

Testing in laboratory and pilot production conditions showed that the use of pre-processed lentil grains would allow replacing up to 50% of raw meat in minced products (ready-to-cook food, cupats) without changing the smell of the products. Smell is easily masked by spices and food additives. The products possess juiciness and attractive appearance.

The conducted studies opened up new prospects for the creation of meat and vegetable products enriched with biologically active substances, that have the possibility of wider use of domestic raw materials and the development of import-substituting technologies for healthy nutrition products.

Germinated lentils are supposed to be used both as part of meat systems and as an independent ingredient for salads, as well as when creating products that simulate meat for fasting, or when creating enriched extruded products for people who lose weight (bread, bran, etc.).

CONTRIBUTION

L.V. Antipova – 40%, T.A. Kuchmenko – 30%, A.A. Osmachkina – 20%, N.A. Osipova – 10 %.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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Panax ginseng callus, suspension, and root cultures: extraction and qualitative analysis

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Abstract:

Introduction. In recent years, scientists have been actively searching for medicinal plants containing biologically active substances with geroprotective properties to treat diseases of old age, in particular cancer, diabetes, cardiovascular diseases, and others. Ginseng (*Panax ginseng* L.) is a promising source of geroprotective compounds. We aimed to select optimal parameters for extracting organic compounds from ginseng callus, suspension, and root cultures and analyze their qualitative composition.

Study objects and methods. We studied ginseng callus, suspension, and root cultures, as well as their extracts. Biologically active substances were extracted with 30 to 70% ethanol. Organic compounds were determined by thin-layer chromatography. The results for each plant were archived and analyzed for the presence of quercetin, mangiferin, luteolin, rutin, quercetin-2-D-glucoside, malvidin, as well as caffeic, cinnamic, ferulic, and sinapinic acids.

Results and discussion. We developed a procedure for screening solvents and performed a fractional qualitative analysis of biologically active substances extracted from ginseng. As a result, we established the optimal parameters for extracting biologically active substances from the dried biomass of ginseng cultures. In all cases, temperature and the ratio of solvent to biomass were the same (50° C, 1:5). However, the extraction time and ethanol concentration differed, amounting to 60 min and 50% for callus cultures, 30 min and 60% for suspension cultures, and 60 min and 70% for root cultures. The qualitative analysis of organic compounds showed the presence of rutin (0.25), quercetin (0.75), and mangiferin (0.57), as well as caffeic and sinapinic acids in the extracts. *Conclusion*. Our set of experiments to isolate biologically active substances from ginseng callus, suspension, and root cultures resulted in selecting the optimal extraction parameters and analyzing the extracts for the presence of organic compounds.

Keywords: Plant cultures, Panax ginseng, ginseng, plant extracts, geroprotective properties, gerontology

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INTRODUCTION

Modern medicine and biology are actively searching for new drugs with geroprotective effects [1–8]. Highly useful in this regard are extracts of medicinal plants [8, 9].

A common extraction method involves using a reflux condenser, a Soxhlet extractor, mechanical stirring, and ultrasound. Soxhlet extraction takes place at 80–90°C and lasts from 20 to 24 h. Such parameters make it possible to efficiently extract biologically active compounds, such as saponins [10–13].

Modern extraction methods include ultra-high pressure extraction (UHPE), ultra-temperature extraction (UTE), pressurized liquid extraction (PLE), microwave-assisted extraction (MAE), pressurized hot-water extraction (PHWE), and supercritical fluid extraction (SFE) [9–12, 14–17].

Compared to traditional techniques, modern methods use smaller amounts of solvents, are easily automated, and take little time. However, they are hardly more effective than, for example, Soxhlet extraction or

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mechanical mixing [13, 18]. Moreover, pressurized hotwater extraction and supercritical fluid extraction are technically quite difficult to perform [10, 14, 19, 20].

Among medicinal plants with geroprotective properties are Schisandra chinensis L., Scutellaria baicalensis L., Rhodíola rósea L., Ginkgo biloba L., and others [21–24]. The most highly valued geroprotective medicinal plants include Panax ginseng L., Aralia mandshurica L., and Eleutherococcus senticosus L. [25]. Since the 1980s, scientists have known of their antitumor effects [25–27].

Ginseng (*Panax ginseng* L.) is a slowly growing perennial plant that is often used as a functional component and a phytotherapeutic agent to prevent and treat various diseases, such as cancer, allergies, inflammatory diseases, and diabetes mellitus [26–31].

According to scientific literature, ginseng extract is used as an adaptogen to increase physical performance, vitality, immunity, as well as resistance to stress and aging [26, 32–34]. It also lowers total cholesterol and low-density lipoproteins, thereby improving a blood lipid profile [31, 32].

However, this plant is included in the Red Book of the Russian Federation and the collection of young roots is prohibited due to its depletion. In addition to low seed productivity and relatively slow growth, ginseng population is irreparably damaged by forest fires and human activities in its endemic areas [35, 36].

Therefore, a justified solution would be to use the plant's cell and organ cultures as an alternative source of renewable medicinal material [30, 32, 35-37]. In our study, we used ginseng callus, suspension, and root cultures – obtained in the early stages of research – as a source of biologically active substances.

We aimed to select optimal extraction parameters and perform a qualitative analysis of organic compounds isolated from ginseng callus, suspension, and root cultures.

STUDY OBJECTS AND METHODS

Our study objects included callus, suspension, and root cultures of ginseng (*Panax ginseng* L.) obtained *in vitro*, as well as their extracts.

To determine the optimal parameters for extracting biologically active substances from ginseng by reflux extraction, we analyzed several extraction systems for their effectiveness. A water-ethanol mixture was selected as an extractant due to its safety (GRAS), economic efficiency, and the ability to extract a wide range of biologically active substances from plant materials [38, 39]. We screened the solvents and performed a qualitative analysis of organic compounds (Fig. 1). The percentage of ethanol in the solvents is indicated in mass fractions. The yield of extracts (%) is expressed in terms of 100 g of dry raw material.

To extract biologically active substances from ginseng callus cultures, we placed 3 ± 0.001 g of dry powdered callus culture in a 50 mL plastic tube and added 40 mL of 30, 40, 50, 60, or 70% solvent according to the screening scheme (Fig. 1). The tube was connected to a reflux condenser. After 60 min of extraction, we separated the dry mass from the solution by filtration. To remove suspended particles, we centrifuged the filtrate at 3900 rpm. Ethanol was evaporated from a 100 mL pre-weighed flask under reduced pressure. After evaporation, we weighed the flask and measured the extract yield.

Then, we dissolved the residue in a minimum amount of the solvent and determined the qualitative composition of organic compounds in the extract by thin-layer chromatography.

The chromatograms for each plant were archived and analyzed for the presence of quercetin (Sigma-Aldrich, USA, \geq 95%), mangiferin (Sigma-Aldrich, USA, \geq 98%), luteolin (Sigma-Aldrich, USA, \geq 98%), rutin (Sigma-Aldrich, USA, \geq 94%), quercetin-2-D-glucoside (Sigma-Aldrich, USA, \geq 95%), caffeic acid (Sigma-Aldrich, USA, \geq 98%), cinnamic acid (Acros Organics, Belgium,



Figure 1 Solvents efficiency in extracting biologically active substances from ginseng



Figure 2 Solvents efficiency in extracting biologically active substances from ginseng callus cultures

 \geq 98%), ferulic acid (Sigma-Aldrich, USA, \geq 99%), sinapinic acid (Honeywell, USA, \geq 95%), and malvidin (Sigma-Aldrich, USA, \geq 90%).

To prepare ginseng suspension and root cultures for the experiment, they were pre-dried to constant weight. Then, 0.5–2.0 g samples of dried cultures were extracted with solvents for callus cultures.

Thin-layer chromatography was performed as described in Pharmacopeia Article 1.2.1.2.0003.15. After evaporation of the solvent from the total extract, we dissolved the dry residue in 1 mL of a suitable extractant (methanol, methylene chloride or acetone) and applied it to the plate with a glass capillary for thin-layer chromatography.

Then, we placed the plate in a chamber and added a suitable eluent. When we used silica gel without modification, chromatography was performed in the CH_2Cl_2 :MeOH system with a 0–10% methanol gradient, in increments of 1%. For reversed-phase chromatography, we used the H₂O:MeCN eluent system with a 0-20% acetonitrile gradient, in increments of 2%, and 0.1% trifluoroacetic acid as a modifier.

We separated the fractions with high-performance liquid chromatography (HPLC), using a Prominence LC-20 chromatograph with diode-array detection (Shimadzu, Japan) and a 250×4.6 mm Kromasil C18 chromatographic column with 5 µm sorbent particles. A mixture of water with o-phosphoric acid, pH = 4.6 (A) and acetonitrile (B) were used as a mobile phase. The gradient elution modes (% B) were 0–20 and 20–60 min with a gradient change of 10–20% and 20–50%, respectively. The eluent flow rate was 1.0 mL/min; the temperature of the column thermostat was 35°C. In the preparative accumulation mode, the eluent was used without the acid.

The instrument was calibrated with caffeine (Sigma-Aldrich, USA, \geq 90%).

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian NMR System 400 spectrometer with a silent Ceccato OFCS 5/8 SD compressor (Varian, USA), with DMSO-D6 used as a solvent and tetramethylsipane as the internal standard.

RESULTS AND DISCUSSION

To analyze the efficiency of various extraction systems, we obtained average yields of solids in total extracts. Total extract yields depending on the solvent's concentration are presented in Fig. 2.

Based on the results, we selected 50% ethanol as a solvent to extract biologically active substances from the dried biomass of ginseng callus cultures by reflux extraction. Further selection parameters are shown in Tables 1, 2.

According to Table 1, the maximum yield of biologically active substances extracted from dried ginseng callus cultures ($5.88 \pm 0.59\%$) at 45° C was provided by a 1:5 ratio of solvent to biomass and the

Table 1 Dry extract yield of biologically active substances from dried ginseng callus culture biomass depending on extraction time (at 45° C)

Solvent:culture	Extract yield depending on duration, %						
ratio	10 min	30 min	60 min	120 min	180 min	360 min	
1:1	0.50 ± 0.05	0.81 ± 0.08	1.22 ± 0.12	1.29 ± 0.13	1.38 ± 0.14	1.38 ± 0.14	
1:2	0.80 ± 0.08	0.94 ± 0.09	1.35 ± 0.14	1.58 ± 0.16	1.67 ± 0.17	1.71 ± 0.17	
1:5	1.20 ± 0.12	1.80 ± 0.18	2.78 ± 0.28	5.88 ± 0.59	5.95 ± 0.60	5.81 ± 0.58	
1:10	1.40 ± 0.14	1.98 ± 0.20	2.98 ± 0.30	5.94 ± 0.59	5.97 ± 0.60	6.04 ± 0.60	
1:20	1.40 ± 0.14	2.01 ± 0.20	3.01 ± 0.30	5.95 ± 0.60	6.01 ± 0.60	6.07 ± 0.61	

Table 2 Temperature selection for extracting biologically active substances from ginseng callus cultures

Temperature, °C	Extract yield depending on duration, %							
	10 min	30 min 60 min 120 min 180 min 360 min						
25	1.20 ± 0.12	1.80 ± 0.18	2.78 ± 0.28	5.88 ± 0.59	5.95 ± 0.60	5.81 ± 0.58		
40	1.55 ± 0.16	1.98 ± 0.20	3.92 ± 0.39	6.21 ± 0.62	6.18 ± 0.62	6.24 ± 0.62		
50	1.79 ± 0.18	2.35 ± 0.24	6.98 ± 0.70	7.05 ± 0.71	7.01 ± 0.70	7.12 ± 0.71		
80	1.62 ± 0.16	2.14 ± 0.21	6.04 ± 0.60	6.12 ± 0.61	6.14 ± 0.61	6.17 ± 0.62		

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Figure 3 Solvents efficiency in extracting biologically active substances from ginseng suspension cultures

extraction time of at least 120 min. Noteworthily, a further increase in duration had no effect on the yield of biologically active substances.

Next, we optimized the temperature and time of extraction (Table 2).

We found that the duration of 60 min and a temperature of 50°C produced the optimal yield of biologically active substances from the dried biomass of ginseng callus cultures ($6.98 \pm 0.70\%$).

Next, we determined the optimal parameters to obtain total extracts from the dried biomass of ginseng suspension cultures with various solvent concentrations (Fig. 3).

Based on the results, we selected 60% ethanol as the most optimal solvent to obtain total extracts of biologically active substances from the dried biomass of ginseng suspension cultures using the reflux extraction method. Further selection parameters are shown in Tables 3–4.

We found that the maximum yield of biologically active substances extracted from dried ginseng suspension cultures ($8.78 \pm 0.88\%$) at 45°C was provided



Figure 4 Solvents efficiency in extracting biologically active substances from ginseng root cultures

by a 1:5 ratio of solvent to biomass and the extraction time of at least 120 min.

According to the results, the optimal parameters for extracting biologically active substances from ginseng with 60% ethanol (extract yield of $8.95 \pm 0.90\%$) were 50°C, 30 min extraction, and a 1:5 solvent-to-biomass ratio.

At the next stage, we optimized the parameters for obtaining total extracts from *in vitro* ginseng root cultures. The total extract yield depending on the solvent is shown in Fig. 4.

According to Fig. 4, 70% ethanol produced the highest yield of biologically active substances from the dried biomass of ginseng root cultures by reflux extraction. Further selection parameters are shown in Tables 5, 6.

According to the results, the duration of 30 to 180 min and the solvent-to-biomass ratio of 1:5 and 1:10 provided the maximum yield of biologically active substances from the dried biomass of ginseng root cultures. In particular, the yield of 11.98% was produced at a ratio of 1:10 at 45°C during 30–60 min.

Table 3 Dry extract yield of biologically active substances from dried ginseng suspension culture biomass depending on extraction time (at 45°C)

Solvent:culture		Extract yield depending on duration, %						
ratio	10 min	30 min	60 min	120 min	180 min	360 min		
1:1	2.50 ± 0.25	2.51 ± 0.25	2.62 ± 0.26	2.92 ± 0.29	2.98 ± 0.30	2.83 ± 0.28		
1:2	2.80 ± 0.28	2.94 ± 0.29	2.75 ± 0.28	2.85 ± 0.29	2.76 ± 0.28	2.91 ± 0.29		
1:5	2.93 ± 0.29	8.78 ± 0.88	8.80 ± 0.88	8.68 ± 0.87	8.95 ± 0.90	8.21 ± 0.82		
1:10	5.40 ± 0.54	8.98 ± 0.90	8.98 ± 0.90	8.94 ± 0.89	8.97 ± 0.90	8.84 ± 0.88		
1:20	6.34 ± 0.63	8.61 ± 0.86	8.51 ± 0.85	8.95 ± 0.90	8.71 ± 0.87	8.77 ± 0.88		

Table 4 Temperature selection for extracting biologically active substances from ginseng suspension cultures

Temperature, °C		Extract yield depending on duration, %						
	10 min	10 min 30 min 60 min 120 min 180 min 360 min						
25	2.20 ± 0.22	1.80 ± 0.18	2.78 ± 0.28	5.88 ± 0.59	5.95 ± 0.60	5.81 ± 0.58		
40	2.55 ± 0.26	1.98 ± 0.20	3.92 ± 0.39	6.21 ± 0.62	6.18 ± 0.62	6.24 ± 0.62		
50	2.79 ± 0.28	8.95 ± 0.90	8.40 ± 0.84	8.75 ± 0.88	8.21 ± 0.82	8.32 ± 0.83		
80	3.62 ± 0.36	7.56 ± 0.76	7.34 ± 0.73	7.12 ± 0.71	7.14 ± 0.71	7.17 ± 0.72		

Table 5 Dry extract yield of biologically active substances from dried ginseng root culture biomass depending on extraction time (at 45°C)

Solvent:culture		Extract yield depending on duration, %						
ratio	10 min	30 min	60 min	120 min	180 min	360 min		
1:1	2.35 ± 0.24	2.51 ± 0.25	2.62 ± 0.26	2.92 ± 0.29	2.98 ± 0.30	2.83 ± 0.28		
1:2	2.38 ± 0.24	2.94 ± 0.29	2.75 ± 0.28	2.85 ± 0.29	2.76 ± 0.28	2.91 ± 0.29		
1:5	2.30 ± 0.23	11.78 ± 1.18	11.80 ± 1.18	11.68 ± 1.17	11.95 ± 1.20	11.21 ± 1.12		
1:10	5.34 ± 0.53	11.98 ± 1.200	11.98 ± 1.20	11.94 ± 1.19	11.97 ± 1.20	11.84 ± 1.18		
1:20	6.54 ± 0.65	11.61 ± 1.16	11.51 ± 1.15	11.95 ± 1.20	11.71 ± 1.17	11.77 ± 1.18		

Table 6 Temperature selection for extracting biologically active substances from ginseng root cultures

Temperature, °C	Extract yield depending on duration, %						
	10 min	30 min	60 min	120 min	180 min	360 min	
25	3.20 ± 0.32	1.80 ± 0.18	2.78 ± 0.28	5.88 ± 0.59	5.95 ± 0.60	5.81 ± 0.58	
40	8.55 ± 0.86	1.98 ± 0.20	3.92 ± 0.39	6.21 ± 0.62	6.18 ± 0.62	6.24 ± 0.62	
50	7.79 ± 0.78	11.95 ± 1.20	12.40 ± 1.24	11.75 ± 1.18	11.21 ± 1.12	11.32 ± 1.13	
80	6.62 ± 0.66	11.56 ± 1.16	11.34 ± 1.13	11.12 ± 1.11	11.14 ± 1.11	10.17 ± 1.02	

 Table 7 Optimal parameters for extracting biologically active substances from the dried biomass of ginseng callus, suspension, and root cultures

Type of	Organic	Ratio of	Time,	Tempe-
ginseng	solvent	solvent to	min	rature,
culture		biomass		°C
Callus	50% ethanol	1:5	60	50
Suspension	60% ethanol	1:5	30	50
Root	70% ethanol	1:5	60	50



Figure 5 Qualitative analysis of ginseng flavonoids

Based on the results in Table 6, we selected the following parameters for extracting biologically active substances from the dried biomass of *in vitro* ginseng root cultures: extraction at 50°C during 30 min at a 1:5 ratio of solvent to dried biomass. These parameters produced a yield of $11.95 \pm 1.20\%$. We recommend to use 70% ethanol as a solvent.

Thus, we determined the optimal parameters (time, temperature, organic solvent, ratio of solvent to biomass) for extracting biologically active substances from ginseng callus, suspension, and root cultures (Table 7).

The qualitative analysis of standard compounds and biologically active substances extracted from dried ginseng callus, suspension, and root cultures showed the presence of rutin, quercetin, quercetin-glycoside, mangiferin, luteolin, apigenin, and caffeic acid.



Figure 6 NMR spectrum of mangiferin isolated from ginseng extracts

The fractions were separated by preparative HPLC (Fig. 5).

As a result, we isolated a basic substance with a retention time of 24 min, which was identified as mangiferin (Fig. 6).

Thus, rutin (0.25), quercetin (0.75), and mangiferin (0.57) were major biologically active substances found in the extracts of *in vitro* ginseng callus, suspension, and root cultures. We also identified caffeic and sinapinic acids in the extracts.

CONCLUSION

We developed a solvent screening procedure and performed a qualitative analysis of biologically active substances extracted from ginseng (*Panax ginseng* L.).

The optimal parameters for extracting biologically active substances (organic solvent, ratio of solvent to biomass, time, and temperature) were 50% ethanol, 1:5 ratio, 60 min, 50°C for ginseng callus cultures; 60% ethanol, 1:5 ratio, 30 min, 50°C for suspension cultures; and 70% ethanol, 1:5 ratio, 60 min, 50°C for root cultures, respectively.

The qualitative analysis of the extracts of ginseng callus, suspension, and root cultures showed the presence of rutin (0.25), quercetin (0.75), and mangiferin (0.57) as predominant components. The extracts also contained caffeic and sinapinic acids.

Thus, the extracts obtained by water-ethanol extraction from ginseng callus, suspension, and root cultures can be used as biologically active ingredients in the production of functional geroprotective foods.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Antibiotic activity and resistance of lactic acid bacteria and other antagonistic bacteriocin-producing microorganisms

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Abstract:

Introduction. Increased resistance of microorganisms to traditional antibiotics has created a practical need for isolating and synthesizing new antibiotics. We aimed to study the antibiotic activity and resistance of bacteriocins produced by lactic acid bacteria and other microorganisms.

Study objects and methods. We studied the isolates of the following microorganism strains: Bacillus subtilis, Penicillium glabrum, Penicillium lagena, Pseudomonas koreenis, Penicillium ochrochloron, Leuconostoc lactis, Lactobacillus plantarum, Leuconostoc mesenteroides, Pediococcus acidilactici, Leuconostoc mesenteroides, Pediococcus pentosaceus, Lactobacillus casei, Lactobacillus fermentum, Bacteroides hypermegas, Bacteroides ruminicola, Pediococcus damnosus, Bacteroides paurosaccharolyticus, Halobacillus profundi, Geobacillus stearothermophilus, and Bacillus caldotenax. Pathogenic test strains included Escherichia coli, Salmonella enterica, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus mycoides, Alcaligenes faecalis, and Proteus vulgaris. The titer of microorganisms was determined by optical density measurements at 595 nm.

Results and discussion. We found that eleven microorganisms out of twenty showed high antimicrobial activity against all test strains of pathogenic and opportunistic microorganisms. All the Bacteroides strains exhibited little antimicrobial activity against Gramnegative test strains, while *Halobacillus profundi* had an inhibitory effect on Gram-positive species only. The Penicillium strains also displayed a slight antimicrobial effect on pathogenic test strains.

Conclusion. The antibiotic resistance of the studied lactic acid bacteria and other bacteriocin-producing microorganisms allows for their use in the production of pharmaceutical antibiotic drugs.

Keywords: Lactic acid bacteria, bacteriocins, antibiotic properties, antibiotic resistance, natural sources, isolates

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INTRODUCTION

New microorganisms that are resistant to traditional antibiotics have recently become known to medicine [1]. Today, large numbers of people worldwide are dying from various infections caused by antibiotic-resistant strains of microorganisms [2]. Therefore, there is an increasingly important scientific and practical need for new antimicrobial drugs with a wide spectrum of action. Modern researchers are actively studying bacteriocins produced by Gram-positive bacteria, which are antibiotic proteins [3]. Due to their complex structure, bacteriocins can be classified as peptides with different activity, gene control, and biochemical processes [4, 5]. They do not develop antimicrobial resistance and therefore are widely used in medicine and pharmacology [4, 6]. These substances are known

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for high antibiotic activity against closely related strains of microorganisms. Lactic acid microorganisms are among the most effective producers of bacteriocins and bacteriocin-like agents [3].

Bacteriocinogenesis has apparently evolved as a result of adaptation and survival in a harmful environment, having occupied a certain niche in microbiology [7]. Bacteriocins are produced by lactic acid bacteria – natural microbiota in the digestive system of humans and animals, as well as in food raw materials, products, or animal feed. Bacteriocins colonize natural and industrial substrates [8–10]. Most often, they do not dominate over saprophytic microorganisms of spore and non-spore forms, over cocci, yeasts, molds, and Gram-negative bacteria, which inhibit antibiotics [11, 12].

Bacteriocin production is a complex process that requires optimal parameters to affect the system. Not all bacteria can synthesize bacteriocins. It has been proved that the ability to synthesize a small amount of bacteriocinogenic substances by individual strains is hereditary [13, 14]. However, the synthesis can be improved by genetic engineering, DNA-tropic substances ultraviolet rays, peroxides, chemical mutagens, and other agents [15, 16]. Since mid-20th century, extensive experiments have been in operation to create new bacteriocin-producing bacteria.

A number of Gram-positive strains, such as *Lactobacillus, Streptococcus, Bacillus, Mycobacterium, Staphylococcus, Corynebacterium, Leuconostoc, Sarcina, Micrococcus, Clostridium* and *Streptomyces*, have been reported to synthesize bacteriocins [2, 3, 17, 18].

A lot of current research is focused on bacteriocins produced by lactic acid microorganisms. For example, diacetin B-1, a bacteriocin isolated from *Lactococcus lactis*, consists of 37 amino acid residues and has a molecular weight of 4300 Da [19–21]. Scientists know of 14 strains of *Lactococcus lactis* capable of synthesizing bacteriocins. All bacteriocins inhibit the growth of *S. aureus*, *P. acidilactici*, *L. Plantarum*, and many *Listeria* species [14, 22, 23].

Amylovorin 471, a bacteriocin produced by *Lactobacillus amylovorus* DCE 471, is used as a bio-preservative in food and feed [24].

A purified form of enterocin A obtained from *Enterococcus faecium* contains 47 amino acid residues, including 4 cystine residues, and has a molecular weight of 4289 Da. Enterocin A has a similar amino acid sequence to that of nisin, a bacteriocin produced by lactic acid bacteria [25].

Bacteriocins are also formed by other types of enterococci. For example, *E. faecalis* S-48 produces a 80 kDa bacteriocin that is sensitive to proteases and has an inhibitory effect on *E. faecalis* [26].

Thus, many infectious diseases can be prevented and treated by isolating new strains of lactic acid microorganisms that produce bacteriocins with antibacterial action [27, 28]. Unlike *Lactobacillus* strains, the antimicrobial activity of *Lactococcus* strains has not been well studied [2, 14].

Therefore, there is an urgent need for isolating new antimicrobial and antibiotic-resistant bacteriocins formed by lactic acid bacteria and other antagonist microorganisms, as well as studying their properties and prospects for the pharmaceutical industry [29, 30].

We aimed to study the antibiotic activity and resistance of bacteriocins produced by lactic acid bacteria and other antagonist microorganisms isolated from natural systems in the Kemerovo region.

In particular, we aimed to:

- study the antimicrobial effect of lactic acid bacteria and other antagonist bacteriocin-producing microorganisms on pathogenic and opportunistic microflora that can cause severe infectious diseases in humans;

- select the isolates of microorganisms with bacteriocin properties (antimicrobial activity) to determine their antibiotic resistance; and

- examine the resistance of lactic acid bacteria and other antagonist microorganisms to the main antibiotics of various series.

STUDY OBJECTS AND METHODS

Microbial communities in various habitats (soil, water, animal gastrointestinal tract, animal products, etc.) were used as natural systems from which we isolated strains of bacteriocin-producing microorganisms. The sampling took place in the Kemerovo region.

Our objects of study included the isolates of bacteriocin-producing microorganism strains, such as Bacillus subtilis, Penicillium glabrum, Penicillium lagena, Pseudomonas koreenis, Penicillium ochrochloron, Leuconostoc lactis. Lactobacillus plantarum, Leuconostoc mesenteroides, Pediococcus acidilactici, Leuconostoc mesenteroides, Pediococcus Lactobacillus Lactobacillus pentosaceus. casei. **Bacteroides** fermentum, *Bacteroides* hypermegas, ruminicola, Pediococcus damnosus. Bacteroides paurosaccharolyticus, Halobacillus profundi, Geobacillus **Bacillus** stearothermophilus, and caldotenax.

Prior to isolation, we incubated microorganisms on an agar medium melted and poured into Petri dishes (covering a third or a quarter of the area), then sterilized and cooled. The incubation lasted 4–5 days at 30°C (until complete or almost complete sporulation by vegetative cells). Then, the grown colonies were suspended in 30 mL of a sterile liquid T3 medium. The flasks with the inoculated medium were placed on an orbital shaker (220 rpm, 72–80 h, 30°C). The stage of sporulation was determined by phase contrast microscopy. At the end of incubation, we found 98–100% of spores and crystals in the liquid medium in relation to the vegetative cells. The number of colony-forming units per mL of culture fluid (CFU/mL) was determined with a series of dilutions followed by incubation in Petri dishes with a T3 medium (five replicates) for 24 h at 30°C. After incubation, we counted the grown colonies and expressed the results in CFU/ml, or spores/ml in our case.

We used the following pathogenic test strains:

- *Escherichia coli* ATCC 25922 - opportunistic bacteria causing gastroenteritis in humans;

- Salmonella enterica ATCC 14028 - pathogenic bacteria causing gastroenteritis in humans;

- *Staphylococcus aureus* ATCC 25923 - pathogenic bacteria causing pneumonia, meningitis, osteomyelitis, endocarditis, infectious toxic shock and sepsis in humans;

- *Pseudomonas aeruginosa* B6643 - opportunistic bacteria causing nosocomial infections in humans;

- *Bacillus mycoides* EMTC 9 (Russian collection of extremophilic microorganisms and type cultures) - opportunistic bacteria causing foodborne toxic infections in humans;

- Alcaligenes faecalis EMTC 1882 - opportunistic bacteria causing septicemia and meningitis in newborns and intra-abdominal infections in adults;

- *Proteus vulgaris* ATCC 63 - opportunistic bacteria causing acute intestinal infections in humans.

Cultivation of microorganism test strains. Escherichia coli ATCC 25922 was cultivated on a medium composed of 10 g tryptone, 5 g yeast extract, 10 g sodium chloride, and 1 L water (pH 7.5–8.0, 37°C).

Salmonella enterica ATCC 14028 was cultivated on a medium composed of 10 g peptic digest of animal tissue, 5 g meat extract, 5 g glucose, 4 g sodium hydrogen phosphate, 0.3 g iron sulfate, 8 g bismuth sulfite, 0.025 g brilliant green, 20 g agar-agar, and 1 L water (pH 7.5–7.9, 35°C).

Staphylococcus aureus ATCC 25923 was cultivated on a medium composed of 10 g casein hydrolysate, 2.5 g yeast extract, 30 g gelatin, 10 g D-mannitol, 55 g sodium chloride, 75 g ammonium sulfate, 5 g potassium hydrogen phosphate, 15 g agar-agar, and 1 L water (pH 6.8–7.2, 30°C).

Pseudomonas aeruginosa B6643 was cultivated on a medium composed of 1 L meat water, 5 g NaCl, and 10 g peptone (pH 6.8–7.0, 37°C).

Bacillus mycoides EMTC 9 was cultivated on a medium composed of 10 g casein hydrolysate, 2.5 g yeast extract, 5 g glucose, 2.5 g potassium hydrogen phosphate, 3 g agar-agar, and 1 L water (pH 7.2–7.6, 30°C).

Alcaligenes faecalis EMTC 1882 was cultivated on a medium composed of 10 g special peptone, 5 g sodium chloride, 0.3 g sodium azide, 0.06 g chromogenic mixture, 2 g Tween-80, 1.25 g sodium hydrogen phosphate, 15 g agar-agar, and 1 L water (pH 7.3–7.5, 37°C).

Proteus vulgaris ATCC 63 was cultivated on a medium composed of 8 g peptone, 5 g sodium chloride, 1 g sodium deoxycholate, 1.5 g chromogenic mixture, 10.5 g propylene glycol, 15 g agar-agar, and 1 L water (pH 7.1-7.5, 37° C).

The quantity of microorganisms (titer) in the suspensions of overnight broth cultures grown on standard media was determined by optical density measurements at 595 nm.

Lactic acid bacteria and other antagonist microorganisms isolated from natural sources in the Kemerovo region were assessed for their antimicrobial action in two ways, using the diffusion method and measuring optical density.

Diffusion method. Test strain bacteria inoculated onto an agar medium using the spread plate technique were immediately covered with paper disks impregnated with the metabolites of microorganisms under study (10 μ L/disk). A disc with a nutrient medium was used as a control, and a disc with ciprofloxacin (a standard antibiotic) was used as a reference drug. The plates were incubated for 24 h at a temperature optimal for each test strain. The quantity of microorganisms was determined by measuring the size (mm) of a transparent zone around the disc, indicating the absence of microbial growth [31].

Optical density measurement. Test strain bacteria were incubated with the metabolites in 96-well culture plates [32]. We resuspended broth cultures aged for 12 h in a medium corresponding to the species of microorganisms to inoculate, bringing their amount to \sim 105 CFU/mL. At the same time, we added the cell suspension and the metabolites under study to the wells in an amount of 1/10 of the total volume. A liquid nutrient medium was used as a control and ciprofloxacin was used as a reference drug (10 µg/mL). The total volume of the suspension in the well was 200 µL. The experiments were performed in duplicate. Incubation was carried out on a shaker at 580 rpm at a temperature optimal for each test strain. After 24 h, we measured the optical density on a PICO01 spectrophotometer (Picodrop Limited, UK) at 595 nm. The bactericidal activity was determined by changes in the optical density compared to the control. In the wells where cell growth stopped or slowed down, the optical density was lower than in those with normal growth.

Microbial spores were stained according to the Schaeffer-Fulton method. The method uses a combined effect of a concentrated brilliant green solution and temperature on the impermeable spore membrane with further decolorization of the cytoplasm of a vegetative cell and its contrast staining with safranin. Microscopic examination showed that the spores were stained green and the cells, red. To establish the presence of flagella, we studied the mobility of cultures in the "squashed straw" preparations [33].

The antibiotic resistance was determined by the zones of growth inhibition for the isolates with antibiotic discs. For this, we inoculated isolate cells onto a temporary medium using the spread plate technique,

Table 1 Antimicropial activity of natural microorganism isolates by the unrusion method (solid nutrient me

Microorganism isolates	Lysis zone diameter, mm						
	<i>Escherichia</i> <i>coli</i> ATCC 25922 ⁻	Salmonella enterica ATCC 14028 ⁻	Staphylo- coccus aureus ATCC 25923 ⁺	Pseudo- monas aeruginosa B6643 ⁻	Bacillus mycoides EMTC 9 ⁺	Alcaligenes faecalis EMTC 1882⁻	Proteus vulgaris ATCC 63 ⁻
Control	0	0	0	0	0	0	0
Ciprofloxacin (C)	21.0 ± 1.1	24.0 ± 1.2	19.0 ± 1.0	22.0 ± 1.1	25.0 ± 1.3	23.0 ± 1.2	20.0 ± 1.0
Bacillus subtilis	18.0 ± 0.9	20.0 ± 1.0	17.0 ± 0.9	20.0 ± 1.0	22.0 ± 1.1	20.0 ± 1.0	17.0 ± 0.9
Penicillium glabrum	0	0	5.0 ± 0.3	0	0	0	7.0 ± 0.4
Penicillium lagena	6.0 ± 0.3	10.0 ± 0.5	0	0	0	0	0
Pseudomonas koreenis	12.0 ± 0.6	5.0 ± 0.3	18.0 ± 0.9	0	0	17.0 ± 0.9	15.0 ± 0.8
Penicillium ochrochloron	0	0	0	0	0	6.0 ± 0.3	0
Leuconostoc lactis	20.0 ± 1.0	22.0 ± 1.1	17.0 ± 0.9	21.0 ± 1.1	24.0 ± 1.2	21.0 ± 1.1	18.0 ± 0.9
Lactobacillus plantarum	19.0 ± 1.0	18.0 ± 0.9	15.0 ± 0.8	19.0 ± 1.0	22.0 ± 1.1	21.0 ± 1.1	17.0 ± 0.9
Leuconostoc mesenteroides	17.0 ± 0.9	20.0 ± 1.0	16.0 ± 0.8	19.0 ± 1.0	22.0 ± 1.1	20.0 ± 1.0	18.0 ± 0.9
Pediococcus acidilactici	20.0 ± 1.0	21.0 ± 1.1	17.0 ± 0.9	19.0 ± 1.0	23.0 ± 1.2	21.0 ± 1.1	18.0 ± 0.9
Leuconostoc mesenteroides	15.0 ± 0.8	18.0 ± 0.9	14.0 ± 0.7	17.0 ± 0.9	20.0 ± 1.0	17.0 ± 0.9	15.0 ± 0.8
Pediococcus pentosaceus	21.0 ± 1.1	20.0 ± 1.0	17.0 ± 0.9	19.0 ± 1.0	24.0 ± 1.2	22.0 ± 1.1	18.0 ± 0.9
Lactobacillus casei	18.0 ± 0.9	19.0 ± 1.0	15.0 ± 0.8	10.0 ± 0.5	20.0 ± 1.0	17.0 ± 0.9	15.0 ± 0.8
Lactobacillus fermentum	15.0 ± 0.8	18.0 ± 0.9	14.0 ± 0.7	19.0 ± 1.0	21.0 ± 1.1	17.0 ± 0.9	15.0 ± 0.8
Bacteroides hypermegas	12.0 ± 0.6	10.0 ± 0.5	0	14.0 ± 0.7	0	9.0 ± 0.5	11.0 ± 0.6
Bacteroides ruminicola	7.0 ± 0.4	11.0 ± 0.6	0	12.0 ± 0.6	0	7.0 ± 0.4	10.0 ± 0.5
Pediococcus damnosus	17.0 ± 0.9	22.0 ± 1.1	16.0 ± 0.8	20.0 ± 1.0	23.0 ± 1.2	19.0 ± 1.0	18.0 ± 0.9
Bacteroides paurosaccharolyticus	15.0 ± 0.8	13.0 ± 0.7	0	16.0 ± 0.8	0	12.0 ± 0.6	14.0 ± 0.7
Halobacillus profundi	0	0	11.0 ± 0.6	0	14.0 ± 0.7	0	0
Geobacillus stearothermophilus	20.0 ± 1.0	22.0 ± 1.1	18.0 ± 0.9	19.0 ± 1.0	22.0 ± 1.1	20.0 ± 1.0	18.0 ± 0.9
Bacillus caldotenax	18.0 ± 0.9	23.0 ± 1.2	17.0 ± 0.9	20.0 ± 1.0	21.0 ± 1.1	22.0 ± 1.1	19.0 ± 1.0

Table 2 Antibiotic resistance of Bacillus subtilis isolate

Antibiotic	Diameter of a growth inhibition zone, mm							
	Content of bacteria in 1 mL of strain culture							
	1×107	5×107	1×10 ⁸	5×10 ⁸	1×10 ⁹	5×10 ⁹		
Ampicillin	0	0	0	0	0	0		
Benzylpenicillin	1.5 ± 0.5	1.5 ± 0.5	1.5 ± 0.5	1.5 ± 0.5	1.5 ± 0.5	1.5 ± 0.5		
Carbenicillin	19.0 ± 1.0	19.0 ± 1.0	19.0 ± 1.0	19.0 ± 1.0	19.0 ± 1.0	19.0 ± 1.0		
Polymyxin	0	0	0	0	0	0		
Streptomycin	13.5 ± 2.5	13.5 ± 2.5	13.5 ± 2.5	13.5 ± 2.5	13.5 ± 2.5	13.5 ± 2.5		
Gentamicin	0	0	0	0	0	0		
Clotrimazole	6.0 ± 1.0	6.0 ± 1.0	6.0 ± 1.0	6.0 ± 1.0	6.0 ± 1.0	6.0 ± 1.0		
Levomycitin	19.0 ± 1.0	19.0 ± 1.0	19.0 ± 1.0	19.0 ± 1.0	19.0 ± 1.0	19.0 ± 1.0		
Tetracycline	0	0	0	0	0	0		
Monomycin	1.5 ± 0.5	1.5 ± 0.5	1.5 ± 0.5	1.5 ± 0.5	1.5 ± 0.5	1.5 ± 0.5		
Neomycin	22.5 ± 2.5	22.5 ± 2.5	22.5 ± 2.5	22.5 ± 2.5	22.5 ± 2.5	22.5 ± 2.5		
Ceporin	17.5 ± 1.5	17.5 ± 1.5	17.5 ± 1.5	17.5 ± 1.5	17.5 ± 1.5	17.5 ± 1.5		
Kanamycin	18.5 ± 2.5	18.5 ± 2.5	18.5 ± 2.5	18.5 ± 2.5	18.5 ± 2.5	18.5 ± 2.5		
Novogramon	22.5 ± 2.5	22.5 ± 2.5	22.5 ± 2.5	22.5 ± 2.5	22.5 ± 2.5	22.5 ± 2.5		

with antibiotic discs on the agar. The experimental results were recorded after 24 h of cultivation in the incubator at 28° C [31].

RESULTS AND DISCUSSION

Table 1 shows the results of using the diffusion method to assess the antimicrobial properties of lactic

acid bacteria and other microorganisms isolated from natural sources in the Kemerovo region.

Of the twenty microorganism strains under study, eleven exhibited high antimicrobial activity against all test strains of pathogenic and opportunistic microorganisms (*Bacillus subtilis, Leuconostoc lactis, Lactobacillus plantarum, Leuconostoc*

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Antibiotic	Diameter of a growth inhibition zone, mm								
	Content of bacteria in 1 mL of strain culture								
	1×10 ⁷	5×107	1×10 ⁸	5×10 ⁸	1×109	5×10 ⁹			
Ampicillin	29.0 ± 1.0	29.0 ± 1.0	29.0 ± 1.0	29.0 ± 1.0	29.0 ± 1.0	29.0 ± 1.0			
Benzylpenicillin	21.5 ± 1.5	21.5 ± 1.5	21.5 ± 1.5	21.5 ± 1.5	21.5 ± 1.5	21.5 ± 1.5			
Carbenicillin	19.0 ± 2.0	19.0 ± 2.0	19.0 ± 2.0	19.0 ± 2.0	19.0 ± 2.0	19.0 ± 2.0			
Polymyxin	16.5 ± 1.5	16.5 ± 1.5	16.5 ± 1.5	16.5 ± 1.5	16.5 ± 1.5	16.5 ± 1.5			
Streptomycin	0	0	0	0	0	0			
Gentamicin	22.5 ± 2.5	22.5 ± 2.5	22.5 ± 2.5	22.5 ± 2.5	22.5 ± 2.5	22.5 ± 2.5			
Clotrimazole	4.0 ± 1.0	4.0 ± 1.0	4.0 ± 1.0	4.0 ± 1.0	4.0 ± 1.0	4.0 ± 1.0			
Levomycitin	18.0 ± 2.0	18.0 ± 2.0	18.0 ± 2.0	18.0 ± 2.0	18.0 ± 2.0	18.0 ± 2.0			
Tetracycline	0	0	0	0	0	0			
Monomycin	6.0 ± 1.0	6.0 ± 1.0	6.0 ± 1.0	6.0 ± 1.0	6.0 ± 1.0	6.0 ± 1.0			
Neomycin	11.0 ± 1.0	11.0 ± 1.0	11.0 ± 1.0	11.0 ± 1.0	11.0 ± 1.0	11.0 ± 1.0			
Ceporin	20.0 ± 2.0	20.0 ± 2.0	20.0 ± 2.0	20.0 ± 2.0	20.0 ± 2.0	20.0 ± 2.0			
Kanamycin	0	0	0	0	0	0			
Novogramon	21.5 ± 1.5	21.5 ± 1.5	21.5 ± 1.5	21.5 ± 1.5	21.5 ± 1.5	21.5 ± 1.5			

Table 3 Antibiotic resistance of	of <i>Leuconostoc l</i>	<i>lactis</i> isolate
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Table 4 Antibiotic resistance of Lactobacillus plantarum isolate

Antibiotic	Diameter of a growth inhibition zone, mm							
	Content of bacteria in 1 mL of strain culture							
	1×107	5×10 ⁷	1×10^{8}	5×10 ⁸	1×10 ⁹	5×10 ⁹		
Ampicillin	25.0 ± 2.0	25.0 ± 2.0	25.0 ± 2.0	25.0 ± 2.0	25.0 ± 2.0	25.0 ± 2.0		
Benzylpenicillin	28.5 ± 1.5	28.5 ± 1.5	28.5 ± 1.5	28.5 ± 1.5	28.5 ± 1.5	28.5 ± 1.5		
Carbenicillin	20.0 ± 2.0	20.0 ± 2.0	20.0 ± 2.0	20.0 ± 2.0	20.0 ± 2.0	20.0 ± 2.0		
Polymyxin	21.5 ± 1.5	21.5 ± 1.5	21.5 ± 1.5	21.5 ± 1.5	21.5 ± 1.5	21.5 ± 1.5		
Streptomycin	0	0	0	0	0	0		
Gentamicin	16.5 ± 1.5	16.5 ± 1.5	16.5 ± 1.5	16.5 ± 1.5	16.5 ± 1.5	16.5 ± 1.5		
Clotrimazole	0	0	0	0	0	0		
Levomycitin	27.0 ± 1.0	27.0 ± 1.0	27.0 ± 1.0	27.0 ± 1.0	27.0 ± 1.0	27.0 ± 1.0		
Tetracycline	30.0 ± 2.0	30.0 ± 2.0	30.0 ± 2.0	30.0 ± 2.0	30.0 ± 2.0	30.0 ± 2.0		
Monomycin	15.0 ± 2.0	15.0 ± 2.0	15.0 ± 2.0	15.0 ± 2.0	15.0 ± 2.0	15.0 ± 2.0		
Neomycin	10.0 ± 2.0	10.0 ± 2.0	10.0 ± 2.0	10.0 ± 2.0	10.0 ± 2.0	10.0 ± 2.0		
Ceporin	0	0	0	0	0	0		
Kanamycin	0	0	0	0	0	0		
Novogramon	10.0 ± 1.0	10.0 ± 1.0	10.0 ± 1.0	10.0 ± 1.0	10.0 ± 1.0	10.0 ± 1.0		

mesenteroides, Pediococcus acidilactici, Pediococcus pentosaceus, Lactobacillus casei, Lactobacillus fermentum, Pediococcus damnosus, Geobacillus stearothermophilus, Bacillus caldotenax).

Bacteroides hypermegas, Bacteroides ruminicola, and Bacteroides paurosaccharolyticus showed insignificant antimicrobial activity against Gramnegative test strains, while Halobacillus profundi had an inhibitory effect on Gram-positive species only. Penicillium glabrum had a slight antimicrobial effect on Staphylococcus aureus, Proteus vulgaris, and Shigella flexneri; Penicillium lagena, on the test strains of Escherichia coli, Salmonella enterica, Shigella flexneri, Aspergillus flavus, and Penicillium citrinum; Penicillium ochrochloron, on the test strains of Alcaligenes faecalis and Listeria monocytogenes.

For further studies of antibiotic resistance, we selected four isolates with maximum antimicrobial activity against pathogenic and opportunistic test

strains, namely *Bacillus subtilis*, *Leuconostoc lactis*, *Lactobacillus plantarum*, and *Leuconostoc mesenteroides*.

These isolates were tested for antibiotic resistance, i.e. resistance of a strain to one or more antibacterial drugs, or decreased sensitivity (immunity) of a culture to the action of an antibacterial substance.

Antibiotic resistance can develop as a result of natural selection through random mutations and/ or antibiotic exposure. Microorganisms are able to transmit genetic information about antibiotic resistance through horizontal gene transfer. In addition, antibiotic resistance can be induced artificially by genetic transformation, for example, by introducing artificial genes into the genome of a microorganism [13].

Tables 2–5 show the results of studying the antibiotic resistance of microorganisms isolated from natural sources in the Kemerovo region.

Antibiotic		Diameter of a growth inhibition zone, mm							
	Content of bacteria in 1 mL of strain culture								
	1×107	5×107	1×10 ⁸	5×10 ⁸	1×109	5×10 ⁹			
Ampicillin	26.0 ± 1.0	26.0 ± 1.0	26.0 ± 1.0	26.0 ± 1.0	26.0 ± 1.0	26.0 ± 1.0			
Benzylpenicillin	19.5 ± 1.5	19.5 ± 1.5	19.5 ± 1.5	19.5 ± 1.5	19.5 ± 1.5	19.5 ± 1.5			
Carbenicillin	16.0 ± 2.0	16.0 ± 2.0	16.0 ± 2.0	16.0 ± 2.0	16.0 ± 2.0	16.0 ± 2.0			
Polymyxin	18.0 ± 1.0	18.0 ± 1.0	18.0 ± 1.0	18.0 ± 1.0	18.0 ± 1.0	18.0 ± 1.0			
Streptomycin	0	0	0	0	0	0			
Gentamicin	20.5 ± 1.5	20.5 ± 1.5	20.5 ± 1.5	20.5 ± 1.5	20.5 ± 1.5	20.5 ± 1.5			
Clotrimazole	5.5 ± 1.5	5.5 ± 1.5	5.5 ± 1.5	5.5 ± 1.5	5.5 ± 1.5	5.5 ± 1.5			
Levomycitin	19.5 ± 2.5	19.5 ± 2.5	19.5 ± 2.5	19.5 ± 2.5	19.5 ± 2.5	19.5 ± 2.5			
Tetracycline	0	0	0	0	0	0			
Monomycin	11.0 ± 1.0	11.0 ± 1.0	11.0 ± 1.0	11.0 ± 1.0	11.0 ± 1.0	11.0 ± 1.0			
Neomycin	16.5 ± 1.5	16.5 ± 1.5	16.5 ± 1.5	16.5 ± 1.5	16.5 ± 1.5	16.5 ± 1.5			
Ceporin	21.5 ± 1.5	21.5 ± 1.5	21.5 ± 1.5	21.5 ± 1.5	21.5 ± 1.5	21.5 ± 1.5			
Kanamycin	0	0	0	0	0	0			
Novogramon	19.0 ± 1.0	19.0 ± 1.0	19.0 ± 1.0	19.0 ± 1.0	19.0 ± 1.0	19.0 ± 1.0			

Table 5 Antibiotic resistance of Leuconostoc mesenteroides isolate

As we can see, *Bacillus subtilis* proved to be resistant to ampicillin, gentamicin, and tetracycline. It exhibited high sensitivity to neomycin, novogramon, kanamycin, carbenicillin, levomycitin, and ceporin, but low sensitivity to benzylpenicillin, monomycin and clotrimazole.

Leuconostoc lactis was highly sensitive to ampicillin, gentamicin, benzylpenicillin, and novogramon, insensitive to clotrimazole and monomycin, and resistant to streptomycin, tetracycline and kanamycin.

Lactobacillus plantarum showed resistance to streptomycin, clotrimazole, ceporin and kanamycin, high sensitivity to tetracycline, benzylpenicillin, and levomycitin, and low sensitivity to neomycin and novogramon.

Leuconostoc mesenteroides was resistant to streptomycin, tetracycline, and kanamycin, insensitive to clotrimazole and monomycin, and highly sensitive to ampicillin, ceporin, benzylpenicillin, gentamicin, levomycitin, and novogramon.

We found that the isolates with different concentrations of microorganisms displayed the same antibiotic resistance. The diameter of the growth inhibition zone was the same for all concentrations of microorganisms.

CONCLUSION

Thus, we studied the antibiotic activity and resistance of lactic acid bacteria and other antagonist microorganisms isolated from natural sources in the Kemerovo region. We established a correlation between the type of isolate and the type of antibiotic. According to the study, eleven microorganisms out of twenty exhibited high antimicrobial activity, while the rest of the strains had an insignificant effect on the test strains and opportunistic microorganisms.

We found that all the isolates showed some degree of resistance to the following antibiotics used to treat infectious diseases: ampicillin, benzylpenicillin, carbenicillin, polymyxin, streptomycin, gentamicin, clotrimazole, levomycitin, tetracycline, monomycin, neomycin, ceporin, kanamycin, and novogramon.

The progressive resistance of the studied bacteriocinproducing microorganisms to antibiotics allows for their use in the production of pharmaceutical antibiotic drugs.

CONTRIBUTION

The authors were equally involved in the writing of the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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Instant tea from *Condonopsis javanica* L. root extract via spray drying

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Abstract:

Introduction. Codonopsis javanica L. root is a gingsen-like medicinal material with valuable bioactive compounds and alkaloids in its composition. However, the diversification of commercial products from *Codonopsis javanica* root extract is limited and poorly represented on the market. This study presents a new production process of an instant tea product from *Codonopsis javanica* root extract, which involved spray drying with maltodextrin as a drying additive.

Study objects and methods. The research featured different process parameters including a drying additive concentration, a drying temperature, and a feed flow rate. Moisture content and drying yield were selected as the main outcomes.

Results and discussion. In general, the improved drying yield was associated with an increased drying additive concentration, a lower drying temperature, and a higher feed flow rate. The best drying yield (78.35%) was obtained at the drying additive concentration of 30% (w/w), the drying temperature of 140° C, and the feed flow rate of 300 mL/h. The total saponin content in the product was 0.29% (w/w), and the ABTS free radical scavenging ability reached 59.48 µgAA/g. The obtained powder was spherical and exhibited fairly uniform particle morphology with shriveled and concave outer surface.

Conclusion. The research results justified the use of *Codonopsis javanica* as an ingredient in beverage industry and suggested maltodextrin as an appropriate substrate for spray-drying natural extracts.

Keywords: Codonopsis javanica, root extract, instant tea, spray drying, maltodextrin, process optimization, antioxidant activity, saponin

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INTRODUCTION

Codonopsis javanica L., known in Vietnamese as "Dangsam", is a member of the *Campanulaceae* family. It grows in the shade of trees and produces bell-shaped flowers [1–3]. *C. javanica* is a popular traditional herbal medicine in China. In Vietnam, it can be found in 14 mountainous Northern provinces, particularly in Lang Son, Cao Bang, Ha Giang, Lao Cai, and Son La, at the height of 500–1600 m above the sea level. It also grows

in the highland areas of Southern provinces, including Quang Nam, Lam Dong, and Kon Tum, at an altitude of 1500 m [4, 5]. The habitats include pastureland, woodland edge in mountainous regions, hill slopes, and upland areas [6].

C. javanica contains valuable bioactive compounds and exhibits numerous pharmaceutical properties. Its root is known to contain glucose, essential oil, fatty substances, and alkaloids [7]. Past studies that employed nuclear magnetic resonance also

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registered codotubulosins A and B, adenosine and 5-(hydroxymethyl) furfural in quaternary ammonium alkaloids in the *C. javanica* roots [8]. Codonopsis roots contain such substances as polysaccharides, saponins, alkaloids, and phytosteroids, which significantly contribute to the pharmacological efficacy of the plant material [9, 10].

Extracts of *C. Javanica* or other species of codonopsis were used to treat diabetes and other illnesses [11–13]. They also possess strong antifatigue, antioxidant, antimicrobial, antitumor, and immune-boosting properties [14–17]. In *in vitro* experiments, *C. javanica* extract showed mutagenic, antimutagenic, anticancer, and antitumor properties against various human cell lines [18]. Polysaccharides from *C. javanica* were demonstrated to protect mice with cerebral ischemia-reperfusion injury [19]. Another experiment also proved the antilarval properties of *C. javanica* aqueous extract against *Aedes albopictus* pupae, a vector of Dengue fever [20].

In Vietnamese traditional medicine, *C. javanica* root is used to treat a number of disorders related to digestive and respiratory system [7]. Similar uses of *C. javanica* root were also reported in Chinese traditional medicine, the most popular preparation method being decoction or tea brewing [21]. As a result of the recent interest in health beneficial natural ingredients, plant extracts with functional properties are often included in instant tea formulations [22]. Instant tea formulation has the advantage of favorable aroma, stimulating effect, and convenience. To avoid degradation, the final moisture content of instant tea powder samples is approximately 3-5% [23].

The research objective was to investigate the parameters of instant tea production from *C. javanica* root extracts by spray drying. The parameters under analysis included moisture, drying yield, total saponin content, and antioxidant activity.

STUDY OBJECTS AND METHODS

Condonopsis javanica L. roots were purchased from the local farmers in the province of Kon Tum, Vietnam. They were harvested during the winter season at the age of two years. Then the roots were cut into smaller pieces, and their moisture content was reduced from 80.16% to 8.17% in a drying oven (Memmert UN110, Germany). The dried roots were mechanically powdered. Afterwards, 60% ethanol by volume was added to the powder in the amount of 40 mL per 1 g. The suspension was then subjected to hydrodistillation for 4 h at 60°C. Water was removed with a rotary evaporator until the weight of the solid in the extract was 40.3%. We obtained 65.75 g of dried extract from 100 g of input root powder. After multiple runs, the accumulated extract was stored in a cooler for spray drying.

To obtain instant tea, a drying additive (maltodextrin) was completely dissolved in 500 mL of

distilled water and left at room temperature overnight. The solution was then mixed with the prepared C. javanica root extract at an appropriate ratio and then with Tween 80. The amount of the added Tween 80 equaled 5% of the weight of the prepared C. javanica root extract. After that, the mix was stirred at 6000 rpm for 20 min in a rotor-stator blender to allow emulsion formation. About 800 mL of the mix was then put into a lab-scale spray dryer (Pilotech YC-015 Mini Spray Dryer). The first single-factor investigation involved the effect of drying additive concentration on the properties of the product. The main spray drying parameters were the following: drying temperature = 140° C, feed rate = 120 mL/h. The dry powder collected was placed in the airtight glass bottle at 25°C for further examination.

The moisture content of the product was determined using the AOAC International (AOAC, 2007) method. The sample was dried in an oven at 105°C till constant weight. The dried sample was then measured for weight loss (%) and the moisture content (%) [24].

To determine drying yield, we used the following formula [25]:

$$DY(\%) = \frac{m_2 \times (1-y)}{m_1 \times x} \times 100$$

where m_1 is the weight of the feed solution, g; m_2 is the weight of the powder obtained by spray drying; x is solids, %; and y is the moisture content of the obtained powder product.

ABTS scavenging activity was determined using the method previously described by Pham *et al.* and Mradu *et al.* [26, 27]. To prepare the stock solution, 10 mL of 7.4 mM ABTS solution was dropped to 10 mL of 2.6 mM $K_2S_2O_8$ and kept at room temperature without exposure to light for 15 h for subsequent use. One milliliter of stock solution was diluted with 60 mL of methanol to get an absorbance value of 1.1 ± 0.02 at 734 nm to produce the working solution. Then 0.5 mL of the extract was added to 1.5 mL of the working solution and kept in darkness for 30 min at room temperature. A UV-VIS spectrophotometer recorded the absorbance of the mix at 734 nm. Ascorbic acid was used as a standard, and the results were expressed as µg ascorbic acid equivalents per gram of dried sample (µgAA/g).

To determine saponin content, 1 g of dried sample was finely powdered and solubilized in 20 mL of 20% isopropanol. The mixture was then heated in a microwave at 86°C for 20 min. The obtained mix was then filtered using Whatman paper for further quantitative purpose.

The saponin content was assessed spectrophotometrically as reported by Jennifer *et al.*, with minor modifications [28]. Briefly, 3.5 mL of the Liebermann – Burchards (LB) reagent, consisting of a 1:5 mix of acetic acid and sulfuric acid, was added to 1 mL of sample solution. If saponins were present, the sample solution fluoresced with yellow. The saponin

content in the solution was then quantified by measuring its absorbance at 580 nm. The following calibration curve describes the relationship between absorbance and saponin concentration:

Absorbance $(mg/mL) = 4.5725 \times Concentration of saponins (mg/mL) + 0.0164.$

Total saponins were calculated on the fresh weight basis.

The morphology of the spray-dried powder was studied by a scanning electron microscope (JSM 6300 SEM). The samples were mounted directly on aluminum SEM stubs in carbon conductive tape and covered by gold sputtering with a thin layer of gold.

Each measurement was carried out in triplicate. Statgraphic statistics software was used to evaluate the statistical data (Statpoint Technologies, version 20,

Table 1 Moisture and texture of C. javanica instant tea at various maltodextrin concentrations



Inc., Warrenton, VA, USA). The variance analysis (ANOVA) and the least significant difference (LSD) were calculated to compare the mean value of the film properties with P = 0.05.

RESULTS AND DISCUSSION

We determined the moisture and texture of powdered tea from *Condopopsis javanica* L. root extract obtained at various maltodextrin concentrations (Table 1). High concentrations seemed to result in the product with lower moisture and minor agglomerate formation.

Fig. 1 shows the dependence of drying yield on maltodextrin concentration. These impacts on drying yield were statistically significant (P < 0.05), as displayed by the one-way ANOVA analysis. Further LSD multiple range tests for drying yield values pointed out differences among the yields obtained at five distinct concentrations (15, 20, 25, 30, 35%). The highest drying yield (75.68%) was attained at the 30% concentration of maltodextrin. Generally, DY was directly proportional to the concentration that rose from 15% to 30%. This can be explained by the effect of exterior-active carbohydrates of maltodextrin, which attach with volatile compounds in the extracts [29]. As a result, higher concentrations of drying additives could support the remaining volatiles and simultaneously increase spray drying yield. As noted by Nunes and Mercadante, the high concentration of the drying additive (35% w/w) resulted in a caramelization reaction that produced furanones, furans, pyrones, and carbocyclic, thus reducing drying yield [30]. Due to the economical characteristic of maltodextrin, we used 30% of maltodextrin in the subsequent tests.

Table 2 shows the texture and moisture of the microcapsules obtained at different drying temperatures. Since an elevated temperature led to products with lower moisture content, we examined an effect of drying temperature on drying yield (Fig. 2). The results were statistically significant (P < 0.05), as displayed by



Figure 1 Drying yield of instant tea from *C. javanica* root extract at different maltodextrin concentrations

Table 2 Moisture and texture of instant tea from *C. javanica* root extract at different drying temperatures

 Table 3 Moisture and texture of instant tea from C. javanica

 root extract at different feed flow rates

Drying temperature, °C	Texture	Moisture, %	Feed flow rate, mL/h	Texture	Moisture, %
140		6.77 ± 0.05	120		6.77 ± 0.05
160		6.6 ± 0.075	180		7.05 ± 0.076
180		6.01 ± 0.09	240		8.23 ± 0.112
200		5.013 ± 0.1	300	A CARLE	8.71 ± 0.13

the one-way ANOVA analysis. Further LSD multiple range tests for drying yield pointed out well-defined differences among the yields obtained at different temperatures (140, 160, 180, 200°C). The greatest drying yield (75.68%) was achieved at 140°C. As the temperature rose from 140 to 200°C, drying yield decreased.

As previously mentioned, high inlet/outlet temperature (140°C) led to a caramelization reaction,





Figure 2 Drying yield of instant tea from *C. javanica* root extract at different drying temperatures



Figure 3 Drying yield of instant tea from *C. javanica* root extract at different feed flow rates



Figure 4 SEM images of instant tea from *C. javanica* root extract

 Table 4 Total saponin content and free radical scavenging ability in instant tea from C. javanica root extract

Product	Saponin,	ABTS,
	%	µgAA/g
Condonopis javanica root extract	0.41	168.88
Instant tea	0.29	59.48
Performance recovery	69.00	35.22

Table 3 demonstrates the moisture and texture of the instant tea at different feed flow rates. An increased feed flow rate improved the moisture in the obtained product. We then examined these differences of feed flow rate with respect to drying yields, as shown in Fig. 3. These impacts on drying yield were statistically significant (P < 0.05), as indicated by the one-way ANOVA analysis. In addition, the yields obtained at different particular feed flow rates (120, 180, 240, 300 mL/h) were statistically different. The largest drying yield (79.47%) was achieved at 300 mL/h. Generally, as the feed flow rate rose from 120 to 300 mL/h, drying yield increased.

Jumah *et al.* showed that the feed flow rate was faster at droplet atomization stage, which led to larger droplets. These droplets contained a high content of water and, subsequently, resulted in high moisture content in the powdered product [33]. In addition, a higher feed flow rate increased drying yield. This could be explained by the fact that a higher feed flow rate and higher drying rates could reduce the dehydration time of the powder. On the other hand, a low moisture powder is usually mixed with exhaust air, presenting difficulties for cyclonic separation [35]. These results are similar to the studies conducted by Suzana F. Alves *et al.* and Tomazelli Júnior *et al.* [36, 37]. Considering the drying yield results, we chose the feed flow rate of 300 mL/h as optimal for further experiments.

Fig. 4 demonstrates the SEM photographs shrunk to microscopic scale of *C. javanica* instant tea obtained with 30% (w/w) concentration of maltodextrin at 140°C. The particles had a comparatively regular shape and no visible breaks or ruptures were observed,

proposing a satisfactory core retention and barrier of the microcapsules. At low drying temperature, the shape of the obtained particles was typically spherical with a shriveled and concave outer surface, indicating that the low drying temperature clearly provides a better core ingredient protection [38, 39]. Some particles demonstrated a smooth and rigid outer surface due to quick evaporation. Therefore, the optimal drying temperature for instant tea production from *C. Javanica* root extracts using maltodextrin as a drying additive was 140°C.

We evaluated the saponin content and free radical scavenging ability of the *C. javanica* extract and its powder obtained by spray drying (Table 4). The results showed that the saponin content in the extract was higher than that in the powdered tea by 0.29%. The original extract appeared to exert more scavenging activity on ABTS free positive radicals with the total antioxidant value at 168.88 μ gAA/g. Meanwhile, after spray drying, the total antioxidant value decreased, as expressed by the reduced free radical capture activity. This implies that saponin in the *C. Javanica* extract had the proton accept capacity and could serve as inhibitor of free radical and, probably, as a primary antioxidant [1].

CONCLUSION

In the present study, we produced instant tea from *Condopopsis javanica* L. root extract via spray drying. The maximum yield reached 78.35% at the concentration of maltodextrin used as a drying additive of 30% (w/w), the drying temperature of 140°C, and the feed rate of 300 mL/h. The resulting instant tea products had a high total saponin content (0.29%, w/w) and a good free radical scavenging ability (59.48 μ gAA/g). Therefore, the using of *C. javanica* root extract to produce instant tea is beneficial to commercialize the products for the beverage market. Further studies are required to evaluate the sensory properties of the powdered product and examine the economic feasibility of the spray drying process.

CONTRIBUTION

Nguyen Phu Thuong Nhan and Nguyen Duong Vu conceived and designed the analysis. Le Van Thanh, Nguyen Phu Thuong Nhan, and Than Thi Minh Phuong performed the experiment and collected the data. Long Giang Bach and Tran Quoc Toan supervised the research and wrote the paper.

CONFLICTS OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this article.

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Quality characteristics of snacks produced from nixtamalized corn flours of new drought-tolerant yellow corn hybrids

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Abstract:

Introduction. Producing new maize cultivars in areas with limited water resources is the main task of plant breeders. However, there is little information regarding their technological characteristics and industrial potential. Besides, snacks have gained worldwide acceptability and become part of modern food culture, especially among young people and children. Thus, our study aimed to produce corn snacks from new yellow corn hybrids planted under water stress in Delta region, Egypt.

Study objects and methods. We investigated healthy processing techniques and used nixtamalization and baking instead of frying. We also evaluated the chemical composition and starch crystallinity of flour, the rheological properties of dough, as well as color attributes and sensory characteristics of baked snacks.

Results and discussion. Significant differences (P < 0.05) were found between all corn genotypes in their fat, protein, ash, crude fiber, and carbohydrate contents. The experimental drought conditions caused higher protein and fat contents compared to normal conditions. X-ray diffraction indicated that nixtamalization decreased starch crystallinity. Also, X-ray and rapid visco analysis showed that Y2 genotype exhibited the highest crystallinity and the lowest pasting properties, while Y3 and Y5 had the lowest crystallinity and the highest pasting properties. Baked snacks made from nixtamalized corn flour of genotypes planted under drought conditions had comparable quality characteristics in terms of color and sensory properties to the control snacks made from SC178 genotype planted under normal conditions.

Conclusion. The new corn hybrids grown in limited water conditions and the developed snacks represent a healthy alternative to corn-based fried snacks.

Keywords: Drought-tolerant plants, nixtamalization, X-ray, snacks, sensory evaluation, corn

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INTRODUCTION

In recent years, snack foods have gained importance and popularity worldwide and become part of modern food culture. However, they have a low nutritional value due to high carbohydrate and fat contents and a low protein content [1]. Moreover, the dependence on convenience snacks has exposed consumers to a higher risk of obesity, cardio-vascular disease, and cancer [2]. Recently, with the revolution in food marketing and the consumer trend towards healthier low sodium, low oil, and low calorie foods, it is necessary to develop new products that offer quality, variety, cost-efficiency, convenience, and nutritive value [3].

The current trend in the food industry is to develop more nutritive snack foods, rather than eliminate snacks from the diet, largely due to their economic value [1]. Snacks has become very popular all over the world, especially among children [4]. Tortilla chips and corn chips are the most popular corn-based snack products. Corn chips are fried products made from corn flour, while tortilla chips are Mexican corn snacks traditionally manufactured from nixtamalized corn grains with or without frying [5]. The resulting snacks, even fried after baking, have a lower oil content, firmer texture, and a stronger alkaline flavor compared to corn chips. They are convenient, ready-to eat, and inexpensive corn products with digestive and dietary principles of vital importance [6].

Nixtamalization is a process that involves alkaline cooking and steeping of corn kernels, which are then washed and ground to produce masa (soft and moist dough). Corn masa is kneaded and molded, and then baked on a hot griddle for tortilla chips [7–8]. Nixtamalization provides nutritional, technological, and

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safety benefits to corn grains. The nutritional benefits include improved protein quality, increased calcium and B-vitamins availability, and reduced phytic acid and tannins contents [9]. Technologically, nixtamalized grains are more easily ground due to softer pericarp and endosperm, with gelatinized starch and improved aroma. In addition, nixtamalization reduces mycotoxin contents in corn grains [10].

Maize is a vital crop for both human food and livestock feed, and the demand for maize and its products grows day by day due to its versatile uses, including medicine, textile, and biofuel production [11–12]. By 2025, maize will be the most common crop produced all over the world [13]. Water and productive land limitation leads plant breeders to vertical expansion through improving the efficiency of water use and increasing unit area productivity [14–15].

In this regard, maize production programs are continuously trying to increase yield, quality, and stability under water deficit conditions [16]. While grain yield is a commonly investigated parameter, quality and technological parameters have less attention [17]. Therefore, we aimed to investigate the possibility of using nixtamalized corn flours – obtained from the best yellow corn hybrids based on grain yield under drought conditions – in baked corn snacks production.

STUDY OBJECTS AND METHODS

Raw materials. For this study, we used materials planted under normal and water stress (drought) conditions in the Experimental Farm of Agricultural Research Centre (ARC), Delta region, EL-Kalyubia Governorate, Egypt. We selected six of the best yellow maize crosses (Y –Y6) according to their superiority in grain yield under drought conditions in the field experiment (yield and irrigation data published in Esmail *et al.*) [14]. They were obtained from hybridization between the imported CIMMYET parental lines following the half-diallel crossing system. Single cross Giza 178 was used as a chick variety. Chemicals and other ingredients for ready-made snacks production were purchased from the local market.

Chemical composition. Moisture, ash, fiber, protein, and fat contents in corn hybrids were determined by methods recommended by the Association of Official Analytical Chemists [18]. Total carbohydrates were calculated by difference.

Preparation of nixtamalized corn flour. Nixtamalized corn flour was prepared according to the method of Quintanar-Guzman *et al.* with some modification [19]. In particular, corn kernels were boiled in a 1% calcium hydroxide solution (percent by grain weight) for 2 h, soaked in boiled water for 14 h, and washed with excess tap water followed by decantation using a sieve. The washed nixtamalized grains were dried for 8–10 h at 60°C and then cooled to 25°C. The dried grains were milled in an analytical mill (Brabender mill, Junior) to pass a 60 mesh screen (0.0028 in sieve opening), and a minimum of 0.102 ± 0.06 cm of free space between the shaft and the stationary body of the mill. The masa prepared from grains was packed in polyethylene bags and stored in a refrigerator (4°C) until use.

X-ray diffraction. Starch crystallinity was evaluated by X-ray diffraction patterns of the samples using monochromatic CuK radiation on a Philips X-ray diffract meter at 35 kv and 15 mA (Central Lab, National Research Centre, Egypt). Lyophilized samples were placed on the 1 cm² surface of a glass slide and equilibrated overnight at * a relative humidity of 91% and run at 2–32 θ (diffraction angle 2 θ). The spacing was computed according to Bragg's law [20].

Pasting properties of flours. Pasting properties of nixtamalized corn flours were determined using a rapid visco analyzer starch master R&D pack V 3.0 (Newport Scientific Narrabeen, Australia) according to the methods approved by the American Association of Cereal Chemists [21]. The measured parameters were pasting temperature, peak viscosity, trough viscosity, final viscosity, breakdown and setback viscosity.

Preparation of snacks. Snacks were prepared according to Agrahar-Murugkar *et al.* by mixing 100 g NCF and 3 g salt in a planetary mixer for 2 min at a low speed using a flat blade, then adding 15 mL sunflower oil and mixing for another 6 min [2]. After this, we changed the mixer blade to a hook type, added 50 mL water, and mixed the dough for about 2 min at a low speed, followed by a medium speed for 2–4 min until soft, cohesive and pliable dough developed. The prepared dough was covered with wet muslin cloth and left to rest for 5 min at room temperature. Then, we sheeted it manually, cut in a circular shape (1.50 mm thick) and baked at 180°C for 8 min on one side and another 5 min on the other side. The chips were then dried for 1 h at 70°C and cooled to room temperature.

Color quality of processed snacks. The color parameters of snacks were evaluated using a Hunter color meter (Hunter Associates Lab Inc. (Model No: LabScan XE, USA). The instrument was calibrated with a white standard tile of Hunter Lab color standard (LX No. 16379): x = 77.26, y = 81.94 and z = 88.14 ($L^* = 92.43$, $a^* = -0.88$, $b^* = 0.21$). The results were expressed in accordance with the CIELAB system for L^* ($L^* = 0$ [black], $L^* = 100$ [white]), a^* ($-a^* =$ greenness, $+a^* =$ redness), and b^* ($-b^* =$ blueness, $+b^* =$ yellowness). In addition, the total color difference (ΔE) between the control snacks (made from SC178 planted under normal irrigation conditions) and those made from corn genotypes planted under drought conditions was calculated as follows:

$$\Delta \mathbf{E} = \left[(\Delta \mathbf{L})^2 + (\Delta \mathbf{a})^2 + (\Delta \mathbf{b})^2 \right]^{0.5}$$

Along with this, we calculated Hue angle, Chroma, and Browning Index (BI) using the following expression:

Chroma =
$$[(a^*)^2 + (b^*)^2]^{0.5}$$

Hue angle = $\tan^{-1}(b^*/a^*)$
Browning Index (BI) = $\frac{[100 (x - 0.31)]}{0.17}$
Where, $x = \frac{(a^* + 1.75L^*)}{(5.645L^* + a^* - 3.012b^*)}$

Sensory evaluation. Snacks were evaluated for their sensory characteristics by 15 trained panelists. The tested characteristics included color, flavor, taste, crispiness, appearance, and overall acceptability [22].

Statistical analysis. The obtained data were statistically analyzed using the SAS Systems for Windows software, version 6.12 TS020 (SAS, Statistical Analysis System, Institute Inc., Cary, NC, 1996). We performed analysis of variance (ANOVA) and the least significant difference (LSD) test (P < 0.05) to determine significant differences between the treatment means.

RESULTS AND DISCUSSION

Chemical composition of yellow corn hybrids. The chemical composition of tested corn samples planted under normal irrigation and drought conditions is presented in Table 1. We found significant genotype differences in moisture, protein, fat, fiber, ash, and carbohydrates. The moisture contents of corn genotypes varied in a narrow range from 11.33 to 12.70%. We noticed a slight decrement in moisture among all corn hybrids planted under drought conditions compared to normal conditions. This decrement was insignificant in some genotypes (SC178, Y1, Y2, and Y5) and significant in others (Y3, Y4, and Y6). The protein content, however, varied in a wide range: its highest value (13.28%) was found in Y4 genotype planted under drought conditions and the lowest (9.52%), in Y6 genotype planted under normal conditions. Also, the fat content varied from 4.28 to 5.50% for Y6 and Y2 genotypes planted under normal conditions, respectively.

Generally, we found that the corn genotypes planted under drought conditions had higher protein and fat contents compared to those planted under normal conditions. Each genotype showed higher protein and fat contents under water stress conditions compared to normal conditions. Carbohydrate contents, however, showed a reverse trend. Similar results were reported by Barutcular *et al.* for maize and Rharrabti *et al.* for wheat [12, 23]. Mousavi *et al.* reported that water stress, especially during the flowering stage, affected the photosynthesis process and thus greatly decreased the starch content while increasing protein and fat contents in the grains [24].

The fiber contents of corn genotypes varied from 2.95 to 3.30% for Y2 planted under water stress and SC178 planted under normal conditions, respectively. At the varietal level, there were no significant differences between the fiber contents of Y1, Y2, Y4, and Y5 genotypes under both irrigation conditions. Fiber contents of SC17 and Y6 genotypes showed a significant decrement under drought conditions compared to normal conditions. By contrast, Y3 genotype revealed a significant increment in fiber under drought conditions. Regarding ash, we found that SC178 showed the highest

 Table 1 Chemical composition of yellow corn genotypes (% on dry weight basis)

Genotype	Moisture	Protein	Fat	Fiber	Ash	Carbohydrates		
		Yellow corn h	ybrids planted und	er normal condition	IS	i		
SC178	11.82 ^{BCD}	10.15 ^F	4.39 ^F	3.30 ^A	1.65 ^A	80.51 ^{AB}		
Y1	11.95 ^{BC}	9.75 ^G	5.20 ^B	3.12 ^{BC}	1.44 ^{BC}	80.49 ^{AB}		
Y2	11.09 ^E	10.90 ^{CD}	5.21 ^B	3.19 ^{ABC}	1.30 ^D	79.40 ^{BCD}		
Y3	12.22 ^{AB}	10.65^{DE}	4.80 ^D	2.98^{EF}	1.31 ^{CD}	80.26 ^{AB}		
Y4	12.70 ^A	10.50^{EF}	4.50^{EF}	3.10^{CDE}	1.26^{DE}	80.64 ^{AB}		
Y5	11.70 ^{CD}	10.51^{E}	4.30 ^F	3.20 ^{ABC}	1.32 ^{CD}	80.67^{AB}		
Y6	12.50 ^A	9.52 ^G	4.28 ^F	3.29 ^A	1.19^{DEF}	81.72 ^A		
Yellow corn hybrids planted under drought conditions								
SC178	11.50 ^{CDE}	11.80 ^B	4.90 ^{CD}	3.10^{CDE}	1.12 ^F	79.08^{BCD}		
Y1	11.56 ^{CDE}	10.78^{CDE}	5.10 ^{BC}	3.10^{CDE}	1.49 ^B	79.53 ^{BCD}		
Y2	11.51 ^{CDE}	11.69 ^в	5.50 ^A	2.95 ^F	1.19^{DEF}	78.67 ^{CD}		
Y3	11.40^{DE}	13.01 ^A	4.50^{EF}	3.17 ^{ABC}	1.25^{DE}	78.17 ^{CD}		
Y4	11.33 ^{de}	13.28 ^A	4.70^{DE}	3.20 ^{ABC}	1.22^{DEF}	77.60 ^D		
Y5	11.65 ^{CD}	11.05 ^c	4.79 ^E	3.25 ^{AB}	1.30 ^D	79.61 ^{BC}		
Y6	11.59 ^{CDE}	10.89 ^{CD}	4.80 ^E	3.03^{DEF}	1.15^{EF}	80.13 ^{ABC}		
LSD	0.5103	0.3591	0.2536	0.1345	0.1397	1.9701		

SC178 = Single Cross Giza 178, Y1–Y6 = new yellow corn hybrids

Means with the same letters in the same column are not significantly different

value (1.65%) under normal irrigation and the lowest value (1.12%) under drought conditions. However, there were no significant differences between the ash contents of the six new genotypes under both irrigation conditions.

The high protein and fat yielding genotypes and the comparable fiber and ash contents under drought conditions may be due to the drought tolerance of the new hybrids. The chemical composition of yellow maize (on a dry weight basis) was previously reported by Watson as 71.7% starch, 9.5% protein, 4.3% fat, and 1.4% ash [25]. Compared to these data, all the corn genotypes in our study had high protein and fat contents. Similar values for these macronutrients were also found among 1245 corn samples from different locations all over the world [26]. Also, the reported values for moisture and fat contents of yellow corn are close to those reported by Yaseen et al. and Hussein et al., being 12.50 and 5.15%, respectively [27, 28]. However, they reported lower values for crude protein (7.88%), ash (0.5%), crude fiber (2.5%), and total carbohydrates (76.0%).

Starch crystallinity of yellow corn genotypes. The X-ray diffraction pattern diagrams for raw and nixtamalized corn samples are shown in Fig. 1a and B, respectively), and the respective crystallinities are illustrated in Fig. 1c. All raw corn genotypes planted under normal and drought conditions showed A-type diffraction peaks around 9.9, 5.8, 5.1 and 3.8 Å at 8.8°, 15.0° , 17.4° and 22.9° (at 20), respectively. There were no

clear differences between the diffractograms of yellow corn genotypes.

Similar results were previously reported in [29–32]. They stated that X-ray diffractions of native cereal starches showed pure "A" type peaks. In addition, Abd-Allah *et al.* mentioned that the calculated "d" spacing of yellow corn starch ranged between 5.4004 and 3.4767 Å [29]. Also, they assumed that symmetric X-ray diffraction patterns of the tested samples could be due to the fact that cereal starch is a homogeneous material mainly composed of amylose and amylopectin. On the other hand, the specified diffracting angle (at 20) for each peak in each starch type could be explained by the molecular weight and the amylose/amylopectin ratio variations.

As we can see in Fig. 1b, a diffraction peak at about 4.4 Å was developed in the nixtamalized samples. It is also clear that the specified peaks in the NCF diffractograms were characterized by decreased intensity and broad background compared to those in the raw samples (Fig. 1a). The peak at 4.4 is the first indication of a V-type amylose-lipid complex pattern [33].

Arambula *et al.* revealed that an amylose–lipid complex developed as a result of starch gelatinization during extrusion or nixtamalization of corn flour [31]. Besides, Mondragon *et al.* mentioned that amylose–lipid complexes might develop during alkali steeping [34]. Finally, Agrahar-Murugkar *et al.* noted that the location of this peak was slightly displaced from the strong 4.4 Å



Figure 1 X-ray diffraction diagrams for raw corn samples (a) and nixtamalized corn samples (b), and crystallinity values for raw corn samples (c). NSC178 = Single Cross Giza 178 planted under normal conditions, DSC178 = Single Cross Giza 178 planted under drought conditions, Y1-Y6 = new yellow corn hybrids planted under drought conditions

to around 4.5–4.7 Å in the X-ray pattern of fried tortilla chips [2].

Beside the transition from pure "A" pattern in raw corn flour to "A + V" pattern in NCF, the decreased peak intensity and its broad background indicated the transition from the semi-crystalline phase to the amorphous phase resulting in a partial disruption of the crystalline starch structure [31, 34]. As we can see in Fig. 1c, Y2 genotype had the highest crystallinity value (87.11%), followed by Y6 and Y4 genotypes (64 and 60%, respectively). Y3 genotype had a lower crystallinity value (24.58%), with the lowest recorded for Y5 (21%). In general, starch crystallinity in corn flours may be affected by mechanical (milling process) and amylolytic activity, as it decreases with damage caused to starch granules [32, 35].

Pasting properties of hybrid nixtamalized corn flour. The pasting properties of NCF dough were rheologically evaluated by a rapid visco analyzer (Table 2). The results showed wide variations in peak viscosity, trough value, breakdown, final, and setback viscosity of yellow corn hybrids planted under drought conditions. However, all corn hybrids showed the same peak time. For instance, the peak and final viscosity values ranged from 151 cp to 660 cp and from 250 cp to 1186 cp for Y2 and Y3 genotypes, respectively. The trough value, breakdown and final viscosity ranged from 128 cp to 541 cp, from 23 cp to 119 cp, and from 122 cp to 645 cp for the same genotypes, respectively. All parameters were greater for Y3 genotype, while Y2 genotype had lower parameters.

Pasting properties are measurements of starch behavior (gelatinization and retrogradation) during processing [36]. These properties could be affected by the molecular structure of amylopectin (branch chain length and distribution) [37] and the granule size [38]. Amylopectin contributes to swelling and pasting of starch during heating. Amylose contributes to starch retrogradation during the cooling stage through its aggregation by hydrogen bonds [39].

It was indicated that the presence of lipids restricts the swelling of starch granules and amylose leaching, resulting in reduced viscosity of the corn flour paste during gelatinization, whereas amylose and lipids inhibit the swelling [40, 41]. In our study, the lower viscosity values of Y2 hybrid could be due to its high fat content (Table 1) and a higher crystallinity degree (Fig. 1c). On the other hand, Sefa-Dedeh *et al.* reported a drastic reduction in the pasting properties of NCF compared to raw flour [9]. They attributed the reduction in viscosity, especially during the cooling stage, to the saturation of hydroxyl groups on the starch molecules with calcium ions (Ca₂). The resulting Ca(OH) ions prevent any further association of the starch molecules in the cooked paste viscosity.

Color attributes of corn snacks. The color of nixtamalized corn flour-based products is an important quality parameter which directly influences the consumer's acceptability of the product. Table 3 and Fig. 2 show the color quality of snacks manufactured from NCF of SC178 genotype planted under normal and water stress conditions, as well as the new hybrids (Y1–Y6) planted under water stress conditions. We found a wide range of significant differences for all color parameters of the snacks: 61.58-69.91, 3.35-9.17 and 25.02-32.12 for lightness (L^*), redness (a^*) and yellowness (b^*), respectively.

Noteworthily, the snacks produced from SC178 genotype planted under normal irrigation conditions showed the lowest L^* and the highest a^* and b^* values. The highest L^* was recorded for snacks produced from Y1, while the lowest a^* and b^* values were recorded for snacks produced from Y4 genotype. The total color differences (ΔE), chroma (C^*), hue angle (H^*) and browning index (B.I.) varied between 8.23–11.96, 25.24–33.40, 74.05–83.32 and 47.36–82.16, respectively.

The snacks produced from corn hybrids planted under drought conditions tended to have higher L* and H^* values and lower a^* , b^* , C^* and BI values, compared to those produced from SC178 planted under normal conditions. Similar previous studies stated that the color of NCF ranged from white to dark yellow, depending on the alkali concentration, processing conditions, and corn type [2, 42, 43]. In addition, Sefa-Dedeh *et al.* stated that the yellowish color in NCF-based products, even when produced from white corn, was closely related to the

Sample	Peak Time, min	Peak Viscosity, c.p.	Trough, c.p.	Breakdown, c.p.	Final Viscosity, c.p.	Setback, c.p.
NSC178	7.0	535	430	93	932	502
DSC178	7.0	569	471	98	952	481
Y1	7.0	430	354	76	784	430
Y2	6.9	151	128	23	250	122
Y3	7.0	660	541	119	1186	645
Y4	7.0	214	172	42	344	172
Y5	7.0	577	482	95	980	498
Y6	7.0	177	145	32	287	142

Table 2 Pasting properties of nixtamalized corn flour

NSC178 = Single Cross Giza 178 planted under normal conditions, DSC178 = Single Cross Giza 178 planted under drought conditions, Y1-Y6 = new yellow corn hybrids planted under drought conditions

Samples	Lightness (L*)	Redness (<i>a</i> *)	Yellowness (b*)	Total color differences (ΔE)	Chroma (<i>C</i> *)	Hue angle (<i>H</i> *)	Browning index (B.I.)
NSC178	61.58 ^D	9.17 ^A	32.12 ^A	0.00 ^E	33.40 ^A	74.05 ^D	82.16 ^A
DSC178	69.53 ^{AB}	3.33 ^G	28.45 ^D	10.53 ^B	28.64 ^D	83.32 ^A	54.75 ^D
Y1	69.91 ^A	3.82^{E}	29.53 ^c	10.23 ^B	29.78 ^c	82.63 ^A	57.42 ^c
Y2	69.03 ^{AB}	4.99 ^в	26.24 ^F	10.37 ^B	26.71 ^F	79.24 ^c	52.09 ^E
Y3	66.93 ^c	3.55 ^F	27.26^{E}	9.16 ^c	27.49^{E}	82.58 ^A	54.85 ^D
Y4	69.25 ^{AB}	3.35 ^G	25.02 ^G	11.96 ^A	25.24 ^G	82.38 ^A	47.36 ^F
Y5	68.58 ^B	4.88 ^c	30.06 ^B	8.46 ^D	30.45 ^B	80.78^{B}	61.34 ^B
Y6	66.37 ^c	4.49 ^D	27.34^{E}	8.23 ^D	27.71 ^E	80.68 ^B	56.74 ^c
LSD	1.1735	0.0859	0.4883	0.6242	0.4982	1.3986	1.0263

Table	3 Color	attributes	of snacks	from	drought-tol	lerant corn	genotypes
							0

NSC178 = Single Cross Giza 178 planted under normal conditions, DSC178 = Single Cross Giza 178 planted under drought conditions, Y1-Y6 = new yellow corn hybrids planted under drought conditions



NSC178

DSC178







Figure 2 Snacks processed from drought-tolerant corn genotypes. NSC178 = Single Cross Giza 178 planted under normal conditions, DSC178 = Single Cross Giza 178 planted under drought conditions, Y1-Y6 = new yellow corn hybrids planted under drought conditions

Genotypes	Color	Crispiness	Odor	Taste	Appearance	Overall
51	(10)	(10)	(10)	(10)	(10)	acceptability (10)
NSC178	9.1 ^A	9.1 ^A	9.4 ^A	9.0 ^A	8.9 ^A	8.8 ^A
DSC178	8.3 ^{BCD}	8.5 ^{BC}	8.6 ^B	8.3 ^{BC}	8.6 ^{AB}	8.4 ^{AB}
Y1	6.4 ^E	8.1 ^{CD}	8.4 ^B	7.9 ^{CD}	8.2 ^{BC}	8.1 ^{BC}
Y2	6.0 ^E	7.7 ^D	8.1 ^B	7.5 ^D	7.8 ^c	7.6 ^c
Y3	8.6 ^{ABC}	8.0 ^{CD}	8.3 ^B	7.8 ^{CD}	8.1 ^{BC}	7.9 ^{BC}
Y4	8.0 ^D	8.2^{CD}	8.5 ^B	8.0 ^{CD}	8.3 ^{BC}	8.1 ^{BC}
Y5	8.8 ^{AB}	8.9 ^{AB}	9.2 ^A	8.7 ^{AB}	9.0 ^A	8.9 ^A
Y6	8.2 ^{CD}	7.9 ^D	8.2 ^B	7.7 ^D	8.0 [°]	7.8 ^c
LSD	0.552	0.576	0.596	0.564	0.579	0.558

Table 4 Sensory characteristics of snacks from drought-tolerant corn genotypes

NSC178 = Single Cross Giza 178 planted under normal conditions, DSC178 = Single Cross Giza 178 planted under drought conditions, Y1-Y6 = new yellow corn hybrids planted under drought conditions

lime concentration [9]. This observation could be due to the varietal performance of yellow corn hybrids (yellow pigments content) under drought conditions.

Browning index (BI) is the most important color attribute in baked products because it affects their final quality [44]. With respect to the yellow pigments content, browning coloration could be due to both enzymatic and non-enzymatic reactions. During nixtamalization, once cell walls and cellular membranes lose their integrity, enzymatic oxidation of phenolic compounds rapidly takes place by polyphenols oxidase [45]. However, the non-enzymatic Maillard reaction takes place between reducing sugars and proteins during the baking process.

Sensory evaluation of corn snacks. The mean scores of sensory characteristics (Table 4) showed significant differences ($P \le 0.05$) between the genotypes for color, crispiness, odor, taste, appearance, and overall acceptability. The snacks produced from SC178 planted under normal conditions and Y5 planted under drought conditions were rated highest in all sensory attributes, while those produced from Y2 were rated lowest. As we said above, color is a very important quality parameter of baked products that reflects raw material formulation and processing.

The brown-yellow color measured by the Hunter instrument (Table 3) for the snack samples manufactured from SC178 and Y5 NCF confirmed the results of sensory analysis. The favorable taste and aroma of these samples could be due to the Millard reaction that takes place during baking. In a similar work by AgraharMurugkar *et al.*, nixtamalization improved the sensory properties of chips [2]. Further, in a study to identify the market demand for corn-based snacks, Menis-Henrique *et al.* found a need for snacks with a lower fat content and a better nutritional value [46]. Therefore, we can conclude that nixtamalized corn flour is organoleptically superior and this technology could be used on a commercial scale.

CONCLUSION

We found that Y3 and Y5 genotypes grown under water stress conditions provide corn grains with superior quality that can be used in snack production. Also, we can conclude that baked snacks made from nixtamalized corn flour are a healthy alternative to fried snacks. Finally, these findings could contribute to achieve both food and nutritional security, especially in water scarce areas.

CONTRIBUTION

The authors were equally involved in designing the research plan. Prof. Ramadan Esmail was involved in the production and cultivation of new yellow corn hybrids. Ahmed Hussein and Ayman Mohammad took part in the production of NCF, as well as in the manufacture and evaluation of snacks. Attia Yaseen and Ayman Mohammad were involved in writing the manuscript, and Ayman Mohammad checked it for plagiarism.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Kashk with caper (*Capparis spinosa* L.) extract: quality during storage

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Abstract:

Introduction. Dairy products are an important part of the diet. Kashk is a traditional Iranian dairy product rich in protein. However, kashk has a high water content and is a good medium for the growth of microorganisms. The aim of this study was to investigate the effect of the ethanolic extract of caper fruit (*Capparis spinosa* L.) on reducing the microbial burden of kashk.

Study objects and methods. The study objects were three kashk samples. The control sample was kashk without caper extract. Two experimental samples included kashk with 0.211 and kashk with 0.350 mg/mL of ethanolic caper extract. All the samples were tested for pH, sensory and antioxidant properties, colorimetric parameters, and microbial population. The experiments were performed on days 0, 7, 14, 21 and 28 of storage.

Results and discussion. The results showed all the samples had pH within the standard values during the entire shelf life (3.96 to 4.53). The samples with 0.350 mg/mL of the caper extract had the lowest EC_{50} (12.05 µg/mL), i.e. the highest antioxidant activity. The increased concentration of the extract and storage time resulted in a decrease in *L** and increase in *b**, while did not impact *a**. *Staphylococcus aureus* population increased more rapidly than *Clostridium botulinum* during the storage time, and the overall sensory acceptability of the kashk samples on days 0 and 7 received the highest score.

Conclusion. The kashk samples containing 0.350 mg/mL of caper extract had an improved antimicrobial, antioxidant and antifungal properties and can be produced and consumed as a new functional product.

Keywords: Dairy products, plant extract, microbial population, antioxidant activity, sensory properties, shelf life

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INTRODUCTION

Nowadays, a demand for healthier food containing essential nutrients is growing so that digestive health is considered a key factor in producing functional food products [1–3]. Dairy products are an important part of the diet [4–6]. Kashk, a by-product of milk processing, is traditionally obtained by boiling, condensing, or drying buttermilk after buttering or lean yogurt [7]. The chemical composition of kashk includes 84.25% of dry matter, 8.57% fat, 95.9% salt, 53.60% total protein, 11.08% ash, and 1.06% lactose. Kashk also contains amino acids and such minerals as calcium, magnesium, iron, sodium, and potassium [8, 9].

Generally, kashk is used as a flavoring agent [10]. Kashk results from soaking curd, adding water and salt, grinding and sanitizing [11]. Kashk has a high microbial contamination potential due to its high moisture and protein content, and if contaminated, it can be very dangerous and even lead to fatal cases. This product

is mostly exposed to *Staphylococcus aureus* and *Clostridium botulinum* contamination [9, 12, 13].

Controlling foodborne pathogenic bacteria and ensuring food safety is the most important issue for those involved in food processing [14]. Due to the detrimental effects of chemical preservatives, the need for research into the antimicrobial effects of natural preservatives and plant essential oils on the growth of microorganisms in food models under laboratory conditions has increased [13]. On the other hand, any preservatives in kashk is prohibited to be used, while the use of natural aromatic extracts or plants as a flavoring agent is allowed [16].

Caper (*Capparis spinosa* L.) is a medicinal plant from the *Capparidaceae* family and different species of caper have different uses. Antimicrobial effects of some species of caper on *Staphylococcus aureus*, *Streptococcus pyogenes*, *Helicobacter pylori*, *Escherichia coli*, and *Bacillus cereus* have been

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documented [15, 16]. The presence of stachydrine and spermidine alkaloids, such as capparispine and cadabicin, in the seeds, roots, flowers, and dried fruits of caper allows using this plant as a nutritional or pharmaceutical supplement worldwide [19–21].

The hydrophobic properties of caper extracts increase its permeability into the cell membrane of microorganisms, which disrupts all vital activities and ultimately causes cell death of the microorganisms. In addition, the extract can damage the enzymes involved in energy regulation and synthesize constituents that inactivate or destroy genetic materials [22, 23]. Its antimicrobial properties are also due to the presence of hydroxyl (OH) groups [24]. The aim of the present study was to investigate the effect of caper fruit extract on the quality and antimicrobial properties of kashk.

STUDY OBJECTS AND METHODS

Extraction of caper (*Capparis spinosa* L.). In this study, caper fruit was collected and identified in Khuzestan Province in southwestern Iran in 2020 (Fig. 1). The fruits were washed, dried and powdered by using an electric mill. Then, 7 kg of dried caper was extracted with 40 L of 70% ethanol for 24 h at room temperature using an electric mixer. The extract was then filtered with filter paper No.1 (repeated on the remaining sediment). Next, all solutions were concentrated in a vacuum rotary evaporator at 40°C, and extraction efficiency was calculated based on [25].

Composition and antioxidant properties of caper extract. The total amount of phenols was determined by the Folin & Ciocalteu's reagent, flavonoids by aluminum chloride method, and antioxidant activity by DPPH radical scavenging assay. Caper fruit extracts were analyzed for quantitative and qualitative determination of polyphenols and flavonoids using high performance liquid chromatography reversal and diode array detection. The apparatus was equipped with a detector, a C18 reverse phase column (Prodigy ODS-3, 4.6×150 mm, 5 µm; Phemomenex, Torrance, CA) and a linear converter unit. The column temperature was set at $30 \pm 1^{\circ}$ C. Rinsing with acetonitrile aqueous solution (97:3 ratio, both with 3% acetic acid) was performed as the initial step. The lyophilized extract was mixed with



Figure 1 Caper fruit under study

1 mL of mobile phase prior to analysis. Further preparation was performed by centrifugation for 5 min at 12 rpm. Then, 20 μ L of the solution was injected directly into the high-performance liquid chromatography system [26].

Minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) of caper extract. *Staphylococcus aureus* stock culture (ATCC 9144) and dried *Clostridium perfringens* (ATCC13124) were taken from the Microorganism Collection Center of Iran Scientific and Industrial Research Organization. The stoke cultures were transferred into 10 mL Brain Heart Infusion (BHI) broth and incubated in a shaking incubator at 37°C for 24 h. Then, 0.1 mL of the culture was transferred into 10 mL of BHI medium and incubated at 37°C for another 24 h until the end of the progressive phase.

Subsequently, diluted cultures were used to inoculate plate agar and industrial kashk for subsequent target inoculations. In a 96-well plate, 100 µL of the culture medium (Müller Hinton broth) was added to all the wells, and then 100 μ L of the sample extract was added to the first well. After mixing the culture medium and the sample in the first well, 100 µL of it was added to the second well and dilution continued until the concentration of the extract in the wells was reduced by half. Then, a uniform suspension of Staphylococcus aureus and Clostridium perfringens half McFarland 1.5×108 CFU/mL were added to all the wells and the 96-well plate was incubated at 37°C for 24 h. At the end, one well before the well in which turbidity was considered minimum bactericidal concentration and the first concentration in which turbidity was not observed was minimum inhibitory concentration [27].

Preparation and quality evaluation of kashk samples. Pasteurized kashk was randomly purchased from a local manufacturer in Karaj. The experimental samples were kashk with minimum bactericidal and minimum inhibitory concentrations of the caper extract. Kashk without the extract was used as control. Then, qualitative tests of the kashk samples were performed on days 0, 7, 14 and 28 of storage. The qualitative tests included pH (with the help of a pH meter based on AOAC 2000 standard), antioxidant properties (by using DPPH radical scavenging assay), and colorimetric test (by a HunterLab spectrophotometer based on CIELAB system) [26, 28]. Table 1 shows the kashk samples under study.

Microbial population in kashk during storage. In this method, each specimen was infected with *Staphylococcus aureus* and *Clostridium perfringens* with a microbial population of 10⁵ CFU/mL and kept at refrigerator temperature. To determine the population of *Staphylococcus aureus*, a certain amount of sample was diluted and sterilized on a plate. Baird-Parker agar was added to the plate with egg yolk emulsion with tellurite and incubated at 37°C for 72 h. The number of

 Table 1 Kashk samples under study

CK ₀	Control kashk on day 0 (the day of production)
CK ₇	Control kashk on day 7 of storage
CK ₁₄	Control kashk on day 14 of storage
CK ₂₁	Control kashk on day 21 of storage
Ck ₂₈	Control kashk on day 28 of storage
EKI ₀	Experimental kashk with MIC on day 0 (the day
	of production)
EKI ₇	Experimental kashk with MIC on day 7 of storage
EKI ₁₄	Experimental kashk with MIC on day 14 of storage
EKI ₂₁	Experimental kashk with MIC on day 21 of storage
EKI ₂₈	Experimental kashk with MIC on day 28 of storage
EKB ₀	Experimental kashk with MBC on day 0 (the day
	of production)
EKB ₇	Experimental kashk with MBC on day 7 of storage
EKB ₁₄	Experimental kashk with MBC on day 14 of storage
EKB ₂₁	Experimental kashk with MBC on day 21 of storage
EKB_{28}	Experimental kashk with MBC on day 28 of storage

MBC is minimum bactericidal concentration MIC is minimum inhibitory concentration

Staphylococcus aureus per was calculated taking into account the dilutions and volumes used to determine the colony count in the plates. To count *Clostridium perfringens* in the samples, we used Sulfite Polymyxin Sulfadiazine (SPS) agar. Bacterial plates were incubated in an anaerobic chamber at 37°C for 72 h and finally colonies were counted [13].

Sensory properties of kashk samples. After basic training, 15 individuals (aged 25–30 years) evaluated the sensory characteristics of the kashk samples with the help of a five-point Hedonic scale. The characteristics under study were texture, odor, taste, oral sensation, adhesion, and general acceptance. The maximum (5) and the minimum (1) scores implied a high and low quality of a sample, respectively [29].

Statistical analysis. At first, normality test was performed for all the data, and then the data were analyzed by factorial test in a completely randomized design with the mean and standard deviation. Duncan's multiple range test was used to determine the difference between the mean values at the 0.05 level. Minitab software was used for statistical analysis.

RESULTS AND DISCUSSION

Extraction efficiency of caper (*Capparis spinosa* **L.) extract.** The extraction methods and the antioxidant activity of the extracts correlated with each other, due to the different polarity of the compounds. A reduced

polarity of the solution undermines extraction efficiency. In particular, organic solvents are commonly used as antioxidants to extract phenolic compounds [30]. Twocomponent solvents are more effective in extracting phenolic compounds from plant samples than singlecomponent solvents [31].

According to Table 2, the average extraction efficiency of the caper extract was 23.23%, which was similar to results obtained by Hosseini *et al.* (24%) who used water and ethanol solvents [25].

In our study, the total amount of phenolic and flavonoid compounds in the target extract was 20.01 (mg gallic acid per g) and 12.16 (mg catechin per g), respectively. M. Mahboubi and A. Mahboubi extracted phenolic and flavonoid compounds from caper with water, ethanol, methanol, and ethyl acetate solvents. The phenolic content was 17.2, 31.7, 34.2 and 30 mg gallic acid, and flavonoid content was 0.06, 1.4, 17.1, and 96.5 mg/kg catechin, respectively [17]. Stefanucci et al. reported that phenolic and flavonoid contents in caper extracts varied depending on the geographical area. The total phenols were in the range of 9.63 to 24.17 (mg gallic acid per g) and flavonoid compounds, 5.02 to 23.50 (mg catechin per g) [26]. Mollica et al. used three methods to obtain caper extract, namely sedimentation, microwave, and solvent application. The phenolic compounds were within the range of 14.27 to 17.96 (gallic acid per g), and the flavonoid compounds were within the range of 10.17 to 11.67 (mg catechin per gram) [34].

Low EC_{50} values indicate an increase in antioxidant activity. Vahid *et al.* found that the amount of phenolic and flavonoid compounds is an important factor in determining the antioxidant capacity of plant extracts [32]. Ehsanifar *et al.* reported that ethanol as a polar solvent extracted flavonoids, glycoside, catechol, and tannin from crude plant tissue [36]. Aqueousalcoholic solvents soften the plant cell wall tissue and are associated with an increased solubility of bonded phenolic compounds. Since these compounds are highly soluble in polar solvents, they enter the solvent phase under the influence of concentration gradients.

Caper extracts contain phenolic and flavonoid compounds which can neutralize free radicals due to hydroxyl groups. A determining factor in antioxidant activity is not the number of hydroxyl groups present in the aromatic ring, but rather the position of the hydroxyl groups and the presence of other functional groups such as double bonds, the combination of hydroxyl groups and the presence of ketone groups play an important role. The negative correlation between total phenol

Table 2 Phenolic, flavonoid and EC₅₀ properties of caper extract

Treatment	Extraction	Total content of phenolic	Flavonoid content,	EC ₅₀ ,
	efficiency, %	compounds, mg gallic acid per g	mg catechin per g	mg/mL
Extract with 70% of ethanol	23.20 ± 0.14	$0.07 \pm 2 \ 0.01$	0.20 ± 12.16	0.03 ± 1.48
and 30% of water				

 Table 3 Phenolic compounds of caper (Capparis spinosa L.)

 extract

Name	Amount, µg/mg of extract
Gallic acid	0.06
Catechin	0.46
Chlorogenic acid	0.11
<i>p</i> -OH benzoic acid	0.27
Vanillic acid	0.12
Syringic acid	0.04
3-OH benzoic acid	0.08
p-coumaric acid	0.23
Rutin	6.32
t-Ferulic acid	0.03
Naringin	0.28
o-Coumaric acid	0.02
Quercetin	3.45

content and EC₅₀ values in DPPH free radical scavenging assay indicates the direct effect of phenols on antioxidant activity [24]. M. Mahboubi and A. Mahboubi extracted *Capparis spinosa* extract with water, ethanol, methanol and ethyl acetate solvents and reported the EC₅₀ values of 500, 560, 340 and 2000 (μ g/mL), while in our study this value was 1.48 (mg/mL), indicating the antioxidant properties of caper. Other studies also showed that caper has a significant antioxidant activity [17, 26, 34].

Composition and antioxidant properties of caper extract. In recent years, phenolic compounds have received special attention because of their biological activities. We analyzed phenolic compounds by means of high-performance liquid chromatography and diode array detection (Table 3).

Four previously phenolic compounds of caper extract, such as catechin, gallic, chlorogenic, and o-coumaric acid, reported in [32] were also observed in this study. Previous studies have reported rutin as one of the main components of the extract, which was confirmed in this study as well. Rutin has a wide range of biological activities such as antioxidant, antimicrobial, antidiabetic and cholesterol-lowering effects [26, 33]. Therefore, the remarkable biological effects of caper can be attributed to rutin, which can be a source of bioactive compounds.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of caper extract. The microbial properties of kashk are defined by the limit of *Staphylococcus aureus* and sulfite reductive *Clostridia*. The results of MIC and MBC of *Capparis spinosa* extract for the two target microorganisms are shown in Table 4.

In the present study, the MBC level for *Staphylococcus aureus* (0.283 mg/mL) was lower than that for *Clostridium perfringens* (0.350 mg/mL). *Staphylococcus aureus* lacks capsules and spores but is resistant to drought and withstands salt up to 10%.

Table 4 Minimum inhibitory concentration (MIC)and minimum bactericidal concentration (MBC)

Target microorganisms	MIC, mg/mL	MBC, mg/mL
Staphylococcus aureus	0.148	0.283
Clostridium perfringens	0.211	0.350

Clostridium perfringens is a Gram-positive, sporozoic, and usually capsulogenic bacterium, with a higher resistance to the extract than *Staphylococcus aureus*. Various mechanisms have been proposed so far for the antimicrobial activity of the extracts. The most accurate theory suggests that the number and position of hydroxyl groups are a key factor in the antimicrobial activity of phenolic compounds, flavonoids, quercetin and their derivatives.

Other mechanisms, such as flavonoids as a result of the ability to form complexes with the cell wall and inhibit the growth of microorganisms, or phenolic compounds by enzymatic activity through reaction with sulfhydryl groups or nonspecific interactions with proteins prevent enzymatic and thus exhibit their antimicrobial activity. Polyphenols are also able to form high molecular weight soluble complexes with proteins, thereby attaching to the bacteria and destroying the receptors present on the bacterial cell surface [37]. Quercetin and its derivatives also inhibit bacterial growth through the DNA gyrase inhibition [27].

Rahnavard and Razavi showed that caper extracts demonstrated the antibacterial activity against a variety of Gram-positive and negative bacteria, including Staphylococcus epidermis, Staphylococcus faecalis, Staphylococcus aureus, Micrococcus luteus, and Bacillus cereus [21]. The aqueous extract of caper fruit did not show any antibacterial activity, while the ethanolic extract had the antimicrobial effect on a variety of Streptococcus spp. and Gram-The comparing of results negative bacteria [17]. from different studies in this case seems complicated because the results are influenced by factors such as the composition and type of culture medium, microorganism growth phase, the volume of culture medium, pH, temperature, and incubation time. The chemical composition, type, and the mechanism of action of phenolic compounds also play a part in antimicrobial activity [38]. Since one of the aims of the present study was to increase the kashk shelf life, we used MIC and MBC of Clostridium perfringens, which were high, as the amount of extract used in the kashk samples.

pH determination. The pH test of the kashk samples is shown in Table 5. Statistical analysis of pH-dependent parameters showed that the concentration of extract, time, and extract × time had a significant effect on kashk pH (P < 0.05), i.e., pH reduced with time. On the other hand, the addition of the extract increased the pH value. Based on standard, pH higher than 4.5 is considered

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Time, days		pH		EC ₅₀ , µg/mL			
	СК	EKI	EKB	СК	EKI	EKB	
0	$0.03\pm4.53^{\rm ab}$	$0.02\pm4.54^{\rm a}$	$0.04\pm4.56^{\rm a}$	$0.5\pm32.74^{\circ}$	0.7 ± 15.11^{j}	$0.5\pm12.05^{\rm k}$	
7	$0.02\pm4.36^{\circ}$	$0.01\pm4.51^{\rm b}$	0.03 ± 4.53^{ab}	$0.2\pm33.28^{\rm c}$	$0.5\pm17.10^{\rm f}$	$0.2\pm14.07^{\rm h}$	
14	$0.01\pm3.99^{\rm f}$	$0.03\pm4.41^{\circ}$	$0.05\pm4.42^{\circ}$	$0.7\pm33.47^{\rm c}$	$0.9\pm17.40^{\rm f}$	$0.4\pm14.24^{\rm h}$	
21	$0.01\pm3.29^{\rm j}$	$0.04\pm4.05^{\rm e}$	$0.02\pm4.11^{\text{d}}$	$1.1\pm35.03^{\rm b}$	$1.3\pm18.05^{\rm e}$	$0.5\pm15.10^{\rm j}$	
28	$0.04\pm2.96^{\rm h}$	$0.01\pm3.94^{\rm f}$	$0.01\pm3.96^{\rm f}$	$0.8\pm40.12^{\rm a}$	$0.7\pm20.15^{\text{d}}$	$0.8\pm17.05^{\rm f}$	

 Table 5 pH and DPPH radical scavenging ability of kashk samples during 28-day storage

CK: control kashk, EKI: experimental kashk with MIC (0.211 mg/mL of caper extract), and EKB: experimental kashk with MBC (0.350 mg/mL of caper extract)

Letters a-h indicate significant differences

undesirable because it causes an undesirable taste in the product and reduces its customer acceptibility [39].

The pH of all samples was within the standard range (3.96 to 4.53) during 28-day storage. Samples without extract (control samples) had lower pH than the experimental samples, and samples with two different concentrations of extract increased pH. It was due to interaction between extract and lactic acid bacteria.

Faraji *et al.* used ethanolic extract of *Allium stipitatum* L. in different concentrations (0.5 to 2%) in kashk for 28 days. The results showed that the pH of samples increased with increasing the concentration of extract, and time had a diminishing effect on pHr, which is consistent with the present findings [40].

The extent of DPPH free radical scavenging ability. Statistical analysis showed that increasing the concentration of the extract, time and extract × time had a significant effect on EC_{50} in kashk samples (P < 0.05). The increased time and decreased amount of the extract resulted in an increase in EC_{50} in all the samples. The ability of the extracts to inhibit free radicals also increased as the concentration increased (Table 5).

Analysis of variance and comparison of mean EC_{50} of the samples showed that the kashk with the highest amount of extract (EKB, 0.350 mg/mL) had the lowest EC_{50} on day 0 of storage (the day of production) and thus had the highest potential for DPPH radical trapping. Since that sample contained the highest amount of the extract, the control sample assessed on day 28 of storage had the highest EC_{50} (40.12 mg/mL) and the lowest ability to trap DPPH radicals compared to the other samples. This is due to phenolic compounds present in

kashk (EC₅₀ 74.32 mg/mL) as scientists have found that kashk is capable of absorbing oxidative compounds due to its glutathione precursor (a type of protein) [41]. On the other hand, the effect of storage time on the DPPH trapping ability was significant (P < 0.05), which decreased in all the samples.

Jayasena and Jo reported a high correlation between the ability to trap free radicals and the amount of phenolic compounds in grains, fruits, vegetables, and beverages [24]. A review article by Mollica *et al.* showed the antioxidant activity of dairy products, namely of raw milk (EC₅₀ 26.41 mg/mL), yogurt (32.4 mg/mL), and cheese (23.5 µg/mL), but no data on the antioxidant activity of kashk was found [34]. In this study, we determined that the antioxidant activity of the control sample on day 0 of storage was 32.74 mg/mL, and it significantly increased in the samples with the caper extract because the extract itself had an antioxidant activity (EC₅₀ 1.48 mg/mL).

Colorimetric Analysis of Kashk Samples. The increased concentration of the extract on days 0 and 7 of storage had no significant effect on brightness (L^*) in the kashk samples, but with increasing the storage duration up to 28 days the effect of the extract on the samples was becoming significant (P < 0.05).

The samples showed the highest lightness on day 0, and the control sample had the lowest lightness (60.15) on day 28 (Table 6). The extract and time had also no effect on redness (a^*), and the addition of the extract and an increased storage time led to an increase yellowness (b^*) in the product. Thus all the three samples had the lowest yellowness on day 0, and the control sample had the most yellowness (27.20) on day 28.

 Table 6 Colorimetric characteristics of kashk samples

Time,		L*			a*			b^*	
days	СК	EKI	EKB	СК	EKI	EKB	CK	EKI	EKB
0	$0.4\pm67.20^{\rm a}$	$0.5\pm67.17^{\rm a}$	$1.2\pm67.15^{\rm a}$	$0.06\pm3.05^{\rm a}$	$0.06\pm3.05^{\rm a}$	$0.05\pm3.05^{\rm a}$	$1.2\pm22.66^{\text{e}}$	$1.0\pm22.55^{\text{e}}$	$1.4 \pm 22.50^{\text{e}}$
7	$0.9\pm67.08^{\rm a}$	$1.4\pm67.07^{\text{a}}$	$1.0\pm67.07^{\text{a}}$	$0.10\pm3.00^{\rm a}$	$0.02\pm3.09^{\rm a}$	$0.01\pm3.06^{\rm a}$	$0.6\pm24.39^{\text{d}}$	$0.4\pm24.41^{\text{ce}}$	$0.5\pm23.35^{\text{de}}$
14	$0.6\pm65.17^{\circ}$	$0.8\pm 66.06^{\rm b}$	$0.4\pm 66.26^{\rm b}$	$0.04\pm3.07^{\rm a}$	$0.04\pm3.11^{\rm a}$	$0.03\pm3.09^{\rm a}$	$1.3\pm24.99^{\text{dc}}$	$0.5\pm25.20^{\circ}$	$0.9\pm24.05^{\text{d}}$
21	$0.5\pm65.10^{\rm c}$	$0.9\pm65.05^{\circ}$	$1.3\pm65.07^{\rm c}$	$0.03\pm3.03^{\rm a}$	$0.02\pm3.12^{\text{a}}$	$0.05\pm3.10^{\rm a}$	$0.7\pm25.15^{\circ}$	$0.9\pm25.75^{\rm bc}$	$1.1\pm25.65^{\rm bc}$
28	1.1 ± 60.15^{e}	$2.4\pm61.10^{\text{d}}$	$7.1\pm61.44^{\rm d}$	$0.08\pm3.10^{\rm a}$	$0.03\pm3.00^{\rm a}$	$0.04\pm3.04^{\rm a}$	$1.0\pm27.20^{\rm a}$	$0.4\pm26.04^{\text{bc}}$	$0.6\pm26.20^{\text{b}}$

CK: control kashk, EKI: experimental kashk with MIC (0.211 mg/mL of caper extract), and EKB: experimental kashk with MBC (0.350 mg/mL of caper extract)

Letters a-e indicate significant differences



Figure 2 Dependence of *Staphylococcus aureus* concentration in kashk samples on storage time. CK: control kashk, EKI: experimental kashk with MIC (0.211 mg/mL of caper extract), and EKB: experimental kashk with MBC (0.350 mg/mL of caper extract)

Faraji *et al.* stated that addition of 0.5-2% ethanolic extract of *Allium stipitatum* over a 21-day storage resulted in an increase in L^* (from 53.3 to 70.2), no change in a^* (around 3.49), and increased b^* (from 17.05 to 49.56) [40]. Hosseini and Ansari reported that the addition of modified Tapioca starch to kashk over a 60-day storage increased L^* , but did not impact a^* and b^* [29].

The effect of microbial population on shelf life of kashk samples. Bacterial population changes of *Staphylococcus aureus* and *Clostridium perfringens* in the control and experimental kashk samples (with the caper extract) during 28 days of storage at 4° C are shown in Fig. 2. The initial concentration of *Staphylococcus aureus* in the control sample was 4.91 CFU/mL, which remained unchanged until the end of storage. By the end of storage (on day 28), the initial amount of *Staphylococcus aureus* had reduced to 0.6 CFU/mL in the experimental sample with MIC (0.211 mg/mL of the caper extract), and to 0.3 CFU/mL in the kashk with MBC (0.350 mg/mL).

The initial bacterial count of *Clostridium perfringens* in the control was 4.50 CFU/mL, which had not increased significantly by day 28 of storage. In the kashk with MIC (0.211 mg/mL of the caper extract), the initial amount of *Clostridium perfringens* reduced to 4 CFU/mL, and in the kashkl with MBC (0.350 mg/mL), to 3.50 CFU/mL. The high susceptibility of *Staphylococcus aureus* to the caper extract can be related to phenolic compounds present in the extract [11].

Although the extract under study also reduced the initial amount of *Clostridium perfringens* in kashk, the reduction was less than a logarithmic cycle indicating its resistance to the extract. The decrease in microbial load in the whole system could be due to the intrinsic acidic pH of kashk, but after day 21 a different trend was observed for *Clostridium perfringens* in the control sample. The overall results showed that with the increasing of storage time of the extract-



Figure 3 Dependence of *Clostridium perfringens* concentration in kashk samples on storage time. CK: control kashk, EKI: experimental kashk with MIC (0.211 mg/mL of caper extract), and EKB: experimental kashk with MBC (0.350 mg/mL of caper extract)

containing kashk samples, *Staphylococcus aureus* bacterial population decreased more rapidly than that of *Clostridium botulinum*, but none reached zero. Golestan *et al.* investigated the antimicrobial properties of the ethanolic extract of *Allium stipitatum* against *Clostridium botulinum* and *Staphylococcus aureus* in kashk. They found that *Staphylococcus aureus* count decreased more rapidly with increasing storage time, it had reached zero by the end of day 21 of storage [13].

Sensory properties of kashk. Figure 3 illustrates the effect of the caper extract on the sensory properties of the kashk samples based on the evaluation of panelists. The results showed that the highest scores of texture, smell, taste, oral feeling, adhesion, and general acceptability had the kashk samples on days 0, 7 and 14 of storage. With time, namely after 48 days of storage, the samples had the lowest overall acceptability.

The results also showed that the texture of the samples containing the extract received the highest score on day 14, while the control sample on day 14 had a lower score. However, the odor of the control sample had a higher score comparedc to that of the experimental kashk. There was no significant difference between the samples regarding taste and oral sensation. It is worth to note that the adhesion factor score remained maximal for all the samples during the storage time. The general acceptability of the kashk samples received the highest scores on days 0 and 7, and the lowest scores, on day 48 in all the three samples.

Golestan *et al.* demonstrated that peppermint essential oil at concentrations of 1500 and 2500 ppm and *Mentha pulegium* essential oil at a concentration of 2500 ppm had significant effects on taste of kashk [13]. Kashk samples containing 1500 ppm of *Mentha pulegium* essential oil and control samples were introduced as suitable samples, but kashk samples with



Figure 3 Effect of storage time (0 to 28 days) on sensory characteristics of kashk samples. CK: control kashk, EKI: experimental kashk with MIC (0.211 mg/mL of caper extract), and EKB: experimental kashk with MBC (0.350 mg/mL of caper extract)

Mentha pulegium essential oil at the concentrations of 1500 and 2500 ppm were acceptable for panelists.

Agulló *et al.* studied the effect of modified Tapioca starch on sensory properties (i.e., texture, smell, taste, oral sensation, adhesion and general acceptance) of kashk [29]. Their results showed that the type of starch affected the sensory properties. The sample with 1.5% hydroxypropylated tapioca starch had the best result and the control sample received the lowest score.

CONCLUSION

The results of the study showed that the caper (*Capparis spinose* L.) extract had no adverse effects on pH of kashk during storage time, and the kashk sample with the extract at the concentration of 0.350 mg/mL had the lowest EC_{50} (12.05 mg/mL), or the highest antioxidant activity on day 0 of storage. The increased extract concentration and storage time resulted in a decrease in L^* and an increase in b^* , while they did not influence a^* .

The number of bacteria had gradually decreased in the kashk samples with both concentrations of the extract by the end of a 28-day storage. With increasing the storage time, *Staphylococcus aureus* bacterial population declined compared to *Clostridium botulinum*.

The sensory evaluation results showed that the texture of the extract-containing samples had a higher score, which was even higher by day 14, and the control sample had a lower score on the same day. But in terms of smell the control samples were superior to the extract-containing samples.

In general, we can conclude that the kashk samples containing 0.350 mg/mL of caper extract had improved antimicrobial, antioxidant, and antifungal properties and can be considered as a new functional product.

CONTRIBUTION

Authors are equally related to the writing of the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Separation of gliadins from wheat flour by capillary gel electrophoresis: optimal conditions

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Abstract:

Introduction. Gliadin proteins are one of the gluten fractions. They are soluble in alcoholic solution and divided into four groups ($\alpha + \beta$, γ , ω 1.2, and ω 5-gliadins). In this paper gliadins were extracted from wheat flour, and optimal conditions for their separation were determined.

Study objects and methods. The separation was performed by capillary gel electrophoresis on Agilent apparatus, CE 7100 (a capillary with an inner diameter of 50 μ m, a total length of 33 cm, and an effective length of 23.50 cm). In order to determine the optimal conditions, different solvent concentrations (50, 60, and 70% ethanol), capillary temperatures (20, 25, 30, 35, and 40°C), and electrode voltages (-14.5, -16.5, -17.5 and -18.5 kV) were applied. Migration time and relative concentration of each protein molecules within gliadin fractions in the electrophoregram were analysed using Agilent ChemStation Software.

Results and discussion. The optimal conditions for gliadin separation were: solvent 70% (v/v) ethanol, capillary temperature of 25°C, and electrode voltage of -16.5 kV. Under these conditions, the total proteins were indetified as Xav = 23.50, including $\alpha + \beta$ gliadin fraction (Xav = 7.50 and relative concentration RC = 28.29%), γ -gliadins (Xav = 5.00, RC = 26.66%), ω 1.2-gliadins (Xav = 4.33, RC = 14.93%), and ω 5-gliadins (Xav = 6.67, RC = 30.98%).

Conclusion. The results of the research can be of fundamental importance in the study of gluten proteins and the influence of technological procedures on their change and the possibility of reducing the allergic effect of gluten during processing.

Keywords: Proteins, wheat, extraction, ethanol, electrophoresis, gluten

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INTRODUCTION

Gliadin proteins represent one of the gluten fractions. Most gliadin proteins are present as monomers. They affect the viscosity and extensibility of wheat flour [1, 2]. Gliadins are divided into four groups, namely α -, β -, γ and ω -gliadins. This division is based on mobility at low pH, i.e. in acidic conditions of A-PAGE electrophoresis medium (acid polyacrylamide gel electrophoresis). Based on research that was later conducted on amino acid sequences, α and β gliadins were classified in the same group (α/β) [3–5].

two-dimensional Modern methods. such as electrophoresis and high-pressure liquid chromatography with reversed phase, allow the separation of gliadin fractions into more than a hundred components. Based on the analysis of amino acid sequences (complete and partial), amino acid composition and molecular weight, gliadins are divided into: $\omega 5$, $\omega 1.2$, $\alpha + \beta$ and γ [3, 6-8]. ω -gliadins are characterized by a high content of glutamine, proline and phenylalanine. These amino acids together make up about 80% of the total ω gliadin composition. ω 5-gliadins have a higher molecular weight (\approx 50 000 Da) than ω 1.2 (\approx 40 000 Da). Most ω gliadins

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lack cysteine, so there is no possibility of disulfide binding. These proteins consist of repetitive sequences that are rich in glutamine and proline [3, 9, 10].

Molecular weights of $\alpha + \beta$ and γ -gliadins overlap (\approx 28 000–35 000 Da). The content of glutamine and proline is much lower compared to ω -gliadin. They differ in tyrosine content. Each of the two types has an N- and a C-terminal region [3, 11]. The N-terminal region (40-50% of total proteins) consists of repeating amino acid sequences that are rich in glutamine, proline, phenylalanine, and tyrosine. The repeating sequences of $\alpha + \beta$ gliadin are dodecapeptides. They are repeated five times. A typical unit of γ -gliadin is repeated up to 16 times. They are interspersed with additional remains [12, 13]. Within the C-terminal region $\alpha + \beta$ and γ -gliadins are homologous. The sequences are not repeting. They contain less glutamine and proline than the N-terminal region and have a more common composition. $\alpha + \beta$ and γ gliadins contain six or eight cysteine residues. These residues are located in the C-terminal region. They form intramolecular disulfide bonds [14, 15]. Although the content of total gliadin proteins depends on the type of wheat and growth conditions (soil, climate, fertilization), $\alpha + \beta$ and γ -gliadins are the highest components. Ω -gliadins are present in lower amounts [16-18].

To separate gliadin proteins, the following techniques are used: high performance liquid chromatography with reversed phase RP-HPLC, exclusion chromatography SE-HPLC, high performance capillary electrophoresis HPCE, sodium dodecyl sulphate polyacrylamide gel electrophoresis SDS-PAGE, and isoelectric focusing IEF [19]. One of the newer techniques for gliadin separation is the highperformance SDS-GCE, which is based on the difference in electrophoretic mobility of ions in solution within the capillary. The molecule size affects the mobility of ions [20].

The number of people who are allergic to gluten proteins from wheat is increasing, which makes food producers give their consumers a guarantee that products declared as "gluten free" really do not contain gluten. The aim of this study was to investigate optimal conditions (solvent concentrations, capillary temperature and voltage) for their separation by high performance capillary gel electrophoresis.

STUDY OBJECTS AND METHODS

Gliadin extraction. We analyzed gliadins in wheat flour samples (ash content: max 0.55%, moisture max: 15%, acidity: max 3, protein content 9.8 g/100 g) purchased on the market of the Republic of Srpska, Bosnia and Herzegovina by capillary gel electrophoresis.

Extraction of gliadin proteins was performed according to a modified Osborne method, as described by Lookhart and Bean [21]. After the albumins and globulins were removed (extraction was performed 3 times with 8 mL of deionized water each, it was obtained in laboratory conditions, on the apparatus Siemens water Technologies W3T199551, Siemens Ultra Clear, at a conductivity of 0.055 mS/cm and at a temperature of 20°C and 3 times with 8 mL of 2% solution of NaCl, NaCl, Lach-Ner, Czech Republic, high purity, \geq 99.00%) gliadin was extracted with 8 mL of ethanol of different concentrations (50, 60 and 70% v/v, refined REAHEM, 96% v/v ethyl alcohol, Srbobran, quality corresponds to the quality property for ethyl alcohol, contains a minimum of 96% v/v ethanol). Samples were homogenized on a vortex (Advanced Vortex Mixer ZX3, 3000 rpm) for 30 min. The samples were then centrifuged in a centrifuge (Rotina 380 R, Hettich Zentrifugen) for 5 min at 1000 rpm. The resulting supernatant was poured into a normal 25 mL vessel, and after the third extraction the normal vessel was made up to final volume with ethanol of various concentrations (50, 60 and 70% v/v). The precipitate was then washed with deionized water.

Samples preparation for analysis at GCE. Prior to analysis samples were diluted with sample buffer (SDS-MW sample buffer, PA 800 plus, Beckman Coulter, USA), so that the total volume was 95 μ L and the concentration was 1 mg/mL. Then 2 μ L of internal standard (10 kDa, PA 800 plus, Beckman Coulter, United States) and 5 μ L of 2-mercaptoethanol (high purity, 99.00%, Sigma-Aldrich Chemie GmbH, Germany) were added. The samples were then heated on a thermo-shaker (Thermo-Shaker, TS-100, Biosan) at 100°C for 3 min. After cooling to room temperature for 5 min, the samples were ready for analysis by capillary gel electrophoresis (Agilent, CE 7100).

Preparation SDS-MW standard for analysis by capillary gel electrophoresis. Prior to the preparation standard, based on the recommendation of the kit manufacturer, the standard was taken to room temperature for 15 min after removal from the refrigerator. It was then carefully stirred on a vortex (Advanced Vortex Mixer ZX3, 3000 rpm) for a few seconds. After that, 10 µL of standard (SDS-MW standard, PA 800 plus, Beckman Coulter, United States) was pipetted into the vial. Then 85 μ L of buffer (SDS-MW sample buffer, PA 800 plus, Beckman Coulter, USA) and 2 µL of internal standard (10 kDa, PA 800 plus, Beckman Coulter, USA) were added. Then 5 µL of 2-mercaptoethanol (Sigma-Aldrich Chemie GmbH, Germany, high purity, 99.00%) was added. Then, it was heated on a thermo-shaker (Thermo-Shaker, TS-100, Biosan), at a temperature of 100°C for 3 min. After heating, the standard vial was cooled to room temperature over 5 min. Prepared in this way, the standard is ready for analysis.

Gliadin proteins separation by capillary gel electrophoresis. Separation of gliadin proteins by capillary gel electrophoresis was performed on an Agilent apparatus, CE 7100, with a capillary inner

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Figure 1 Electrophoregram of MW standards of proteins of known molecular weight separated by capillary gel electrophoresis

diameter of 50 µm, a total length of 33 cm, and an effective length of 23.50 cm. The SDS-MW analysis kit, PA 800 plus (2015 Beckman Coulter, USA) was used for separation. SDS gel buffer (0.2% SDS, pH = 8) was used to fill the capillary. The kit contains the following chemicals: SDS-MW gel buffer (0.2% SDS, pH = 8), SDS-MW sample buffer (100 mM Tris-HCl, pH = 9, 1% SDS), internal standard (10 kDa), external standard (10 to 225 kDa), acid wash solution (0.1N HCl), base wash solution (0.1N NaOH), as well as two capillaries 57 cm long, 50 µm ID. According to the manufacturer's instructions, the kit is stored at room temperature after opening, except for the internal and external standards, which are stored at a temperature of 2-6°C. Preparation of the capillary electrophoresis (CE) instrument was done according recommendations Agilent Technologies [22-24].

Statistical data processing. Statistical data processing was performed in IBM SPSS, Statistics 26. Descriptive statistical analysis calculated the average value, standard deviation and 95% confidence interval of the average value. Variance analysis of different groups was used to evaluate the effect of solvent concentrations, capillary temperature and electrode voltage on the number of detected proteins and the relative concentration of each gliadin proteins.

RESULTS AND DISCUSSION

In order to determine molecular weights unknown proteins, a calibration curve was obtained using 7 proteins in SDS-MW size standard.

Electrophoregram, the migration time, and the calibration curve of MW standard proteins with known molecular weight (10, 20, 35, 50, 100, 150 and 225 kDa) are presented in Fig. 1, Table 1, and Fig. 2, respectively. The proteins were separated by capillary gel electrophoresis (CE, Agilent, CE 7100, internal capillary diameter 50 μ m, total capillary length 33 cm, effective

capillary length 23.50 cm, capillary temperature 25° C, voltage -16.5 kV (reverse mode), duration of analysis 30 min, and absorbance measured at 220 nm).

The ratio of molecular weights (log MW) and migration time (*t*) of proteins is represented by the equation y = 0.08168x - 0.00098, where *y* represents logMW and *x* represents the migration time of proteins (*t*). R² shows the correlation coefficient (0.9847).

A calibration curve was used to estimate the molecular weight of unknown proteins. The coefficient of correlation shows a high dependence of the logarithm of the molecular weight of the protein and the migration time of the protein.

The number of proteins in each gliadin fraction and their relative concentration were obtained based on the total number of identified proteins and the total relative concentration.

Table 2 shows descriptive indicators of total proteins and the number of gliadin proteins after extraction with different concentrations of ethanol.

Descriptive analysis showed that the highest number of proteins (23.50) was obtained after extraction with 70% ethanol, by the method of Lookhart and Bean. The lowest number of proteins was obtained by extraction with 50% ethanol (18.67). One-factor analysis of the variance of different groups showed that there was a statistically significant difference in the number of

 Table 1 Migration time of proteins with known molecular weight separated by capillary gel electrophoresis

Molecular weight (MW), kDa	log MW	t, min
10	1.00	13.36 ± 0.21
20	1.30	15.77 ± 0.18
35	1.54	18.13 ± 0.26
50	1.70	20.15 ± 0.29
100	2.00	24.25 ± 0.10
150	2.18	26.78 ± 0.36
225	2.35	29.41 ± 0.15



Figure 2 Calibration curve obtained as the ratio of the logarithm of molecular weight and migration times of known proteins separated by capillary gel electrophoresis

proteins, F(2.15) = 23.70, Sig. = 0.000. The highest number of within $\alpha + \beta$ gliadin fractions was obtained after extraction with 60% ethanol (7.67). The lowest number of those proteins was obtained after extraction with 50% ethanol (6.00). A statistically significant difference was found in the number of proteins, F(2.15) = 8.58, Sig. = 0.003.

Extraction with 50 and 60% ethanol produced the highest and the lowest number of proteins within the γ -gliadins (5.33 and 4.67, respectively). There was no statistically significant difference in the number

of proteins, F(2.15) = 1.15, Sig. = 0.342. The highest amount of ω 1.2-gliadins was obtained after extraction with 60% ethanol (5.17), while the lowest after extraction with 50 and 70% ethanol (4.33). One-factor variance analysis showed no statistically significant difference, F(2.15) = 2.19, Sig. = 0.146. The highest number of ω 5-gliadins was obtained after extraction with 70% ethanol (6.67). The lowest amount was observed after extraction with 50% ethanol (3.83). A statistically significant difference in the number of proteins was found, F(2.15) = 6.77, Sig. = 0.008.

According to Table 2, an increasing ethanol concentration increased total proteins, increased and then slightly decreased $\alpha + \beta$ gliadin fraction, decreased and then increased γ -gliadins, increased and then decreased ω 1.2-gliadins, and increased ω 5 gliadin fractions.

Table 3 shows descriptive indicators of the total relative concentration and the relative concentration of gliadin proteins after extraction with different concentrations of ethanol.

Descriptive analysis showed the highest relative protein concentration of $\alpha + \beta$ gliadin fractions after extraction with 50% ethanol (31.25%) and the lowest concentration after extraction with 60% ethanol (17.69%). One-factor variance analysis revealed a statistically significant difference in the relative protein concentration, F(2.15) = 174.13, Sig. = 0.000.

Extraction with 50 and with 60% ethanol produced the highest and the lowest relative concentration of γ -gliadins (27.72 and 18.55%). A statistically significant

Table 2 Descriptive indicators of total proteins and gliadin proteins separated by fractions (Agilent, CE 7100, capillary inside diameter 50 μm, total capillary length 33 cm, effective capillary length 23.50 cm, capillary temperature 25°C, voltage –16.5 kV (reverse mode), duration 30 min, absorbance measured at 220 nm)

	Ethanol,	Ν	Xav	SD	Std.	95% confidence	e interval of average	Min	Max
	% (v/v)				error	Lower bound	Upper bound	_	
Total number	50	6	18.67	1.21	0.49	17.40	19.94	17	20
of proteins	60	6	23.00	1.67	0.68	21.24	24.76	21	25
	70	6	23.50	1.05	0.43	22.40	24.60	22	25
$\alpha + \beta$ gliadins	50	6	6.00	0.63	0.26	5.34	6.66	5	7
	60	6	7.67	0.82	0.33	6.81	8.52	7	9
	70	6	7.50	0.84	0.34	6.62	8.38	6	8
γ gliadins	50	6	5.33	0.82	0.33	4.48	6.19	4	6
	60	6	4.67	0.82	0.33	3.81	5.52	4	6
	70	6	5.00	0.63	0.26	4.34	5.66	4	6
ω1.2 gliadins	50	6	4.33	0.82	0.33	3.48	5.19	3	5
	60	6	5.17	0.75	0.31	4.38	5.96	4	6
	70	6	4.33	0.82	0.33	3.48	5.19	3	5
ω5 gliadins	50	6	3.83	0.98	0.40	2.80	4.87	3	5
	60	6	5.50	1.22	0.50	4.21	6.79	3	6
	70	6	6.67	0.52	0.21	5.12	7.22	5	7
ANOVA (TP)	F(2.15) =	23.70	, Sig. = 0.00	0, eta squar	e = 84.78/11	1.61 = 0.76			
ANOVA $(\alpha + \beta)$	F(2.15) =	8.58,	Sig. = 0.003	, eta square	= 10.11/18.9	94 = 0.53			
ANOVA (γ)	F(2.15) =	1.15,	Sig. = 0.342	> 0.05					
ANOVA ($\omega 1.2$)	F(2.15) =	2.19,	Sig. = 0.146	> 0.05					
ANOVA (005)	F(2 15) =	6 77	Sig = 0.008	eta square	= 12 33/26	00 = 0.47			

Table 3 Descriptive indicators of the total relative concentration and relative concentration of gliadin fractions (Agilent, CE 7100, capillary inside diameter 50 μ m, total capillary length 33 cm, effective capillary length 23.50 cm, capillary temperature 25°C, voltage –16.5 kV (reverse mode), duration 30 min, absorbance measured at 220 nm)

	Ethanol,	N	RC, %	SD	Std. error	95% confidence	e interval of average	Min	Max
	% (v/v)					Lower bound	Upper bound	-	
Total relative	50	6	100.00	0.00	0.00	100.00	100.00	100	100
concentration	60	6	100.00	0.00	0.00	100.00	100.00	100	100
	70	6	100.00	0.00	0.00	100.00	100.00	100	100
$\alpha + \beta$ gliadins	50	6	31.25	1.30	0.53	29.89	32.61	29.07	32.87
	60	6	17.69	1.27	0.52	16.36	19.03	16.11	19.61
	70	6	28.29	1.40	0.57	26.82	29.76	26.06	30.09
γ gliadins	50	6	27.72	1.15	0.47	26.51	28.92	26.50	29.57
	60	6	18.55	0.97	0.40	17.53	19.57	17.21	19.99
	70	6	26.66	1.35	0.55	25.25	28.08	24.75	28.13
ω1.2 gliadins	50	6	5.21	0.34	0.14	4.85	5.56	4.84	5.66
	60	6	4.82	0.21	0.09	4.59	5.04	4.58	5.08
	70	6	14.93	1.04	0.43	13.84	16.03	13.03	15.95
ω5 gliadins	50	6	36.16	0.70	0.29	35.42	36.90	34.97	37.01
	60	6	47.45	1.37	0.56	46.01	48.89	45.81	49.39
	70	6	30.98	3.13	1.28	27.70	34.27	29.55	37.36
ANOVA $(\alpha + \beta)$	F(2.15) =	= 174.13	, Sig. = 0.000), eta squar	e = 609.67/	635.93 = 0.96			
ANOVA (γ)	F(2.15) =	= 111.01	Sig. = 0.000), eta squar	e = 301.93/2	322.33 = 0.94			
ANOVA (ω 1.2)	F(2.15) =	472.47	, Sig. = 0.000), eta squar	e = 394.02/	400.27 = 0.98			
ANOVA ($\omega 5$)	F(2.15) =	= 104.83	, Sig. = 0.000), eta squar	e = 851.07/	911.96 = 0.93			

difference in relative concentration was found, F(2.15) = 111.01, Sig. = 0.000. The relative concentration of $\omega 1.2$ -gliadins was the highest after extraction with 70% ethanol (14.93%) and the lowest after extraction with 60% ethanol (4.82%). The one-factor analysis of the variance showed a statistically significant difference in the relative concentration, F(2.15) = 472.47, Sig. = 0.000. As for $\omega 5$ gliadin fractions, they were found in the highest concentration after extraction with 60% ethanol (47.45%) and the lowest after extraction with 70% ethanol (30.98%). There was a statistically significant difference in the relative concentration, F(2.15) = 104.83, Sig. = 0.000.

Based on the obtained results (Table 3), an increasing ethanol concentration decreased and then increased the relative concentration of $\alpha + \beta$, γ - and ω 1.2-gliadins and increased and then decreased that of ω 5-gliadins.

Table 4 shows descriptive indicators of the total number of proteins and number of gliadin proteins separated by fractions after extraction with 70% (v/v) ethanol and separated at a capillary temperature of 20, 25, 30, 35 and 40°C.

Descriptive analysis revealed that the highest number of proteins was obtained after extraction with 70% ethanol and at a capillary temperature of 25°C (23.50), while the lowest amount of proteins was observed at 35°C (18.83). The one-factor analysis of variance showed a statistically significant difference in the number of proteins, F(4.25) = 11.02, Sig. = 0.000.

The highest and the lowest numbers of $\alpha + \beta$ gliadin fractions were obtained at 20°C (10.00) and 25°C (7.50), respectively. There was a statistically significant

difference, F(4.25) = 6.24, Sig. = 0.001. The number of γ -gliadins was the highest at 25°C (5.00) and the lowest at 35°C (3.50). ANOVA test showed a statistically significant difference in the number of proteins, F(4.25) = 9.01, Sig. = 0.000. The highest amount of ω 1.2-gliadins was obtained at a capillary temperature of 25°C (4.33) and the lowest at 20 and 35°C (2.67). A statistically significant difference in the number of proteins was found, F(4.25) = 9.08, Sig. = 0.000. ω 5-gliadins were identified in the highest number at a capillary temperature of 25°C (6.67) and in the lowest number at 35°C (3.50). The one-factor analysis of variance revealed a statistically significant difference, F(4.25) = 5.63, Sig. = 0.002.

According to the results obtained, it can be seen that with increasing capillary temperature, total proteins increased, then decreased and increased slightly again. $\alpha + \beta$ gliadin fractions decreased, then increased and decreased slightly again. As for γ -, ω 1.2- and ω 5-gliadins, their fractions increased, then decreased and increased slightly again.

Table 5 shows descriptive indicators of the total relative concentration of proteins and relative concentration of gliadin proteins separated by fractions after extraction with 70% (v/v) ethanol and separated at different capillary temperatures.

According to the data, the highest relative concentration of $\alpha + \beta$ gliadin fractions was obtained after extraction with 70% ethanol and a capillary temperature of 40°C (47.55%). The lowest concentration was observed at 35°C (27.22%). One-factor variance analysis revealed a statistically significant difference

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Table 4 Descriptive indicators of total proteins and the number of gliadin fractions (70% ethanol, Agilent, CE 7100, capillary inside diameter 50 μ m, total capillary length 33 cm, effective capillary length 23.50 cm, voltage –16.5 kV (reverse mode), duration 30 min, absorbance measured at 220 nm)

	Column tem-	Ν	Xav	SD	Std.	95% confidence interval of average M		Min	Max		
	perature, °C				error	Lower Bound	Upper bound	_			
Total number	20	6	20.83	1.47	0.60	19.29	22.38	19	23		
of proteins	25	6	23.50	1.05	0.43	22.40	24.60	22	25		
	30	6	21.67	1.75	0.71	19.83	23.50	19	23		
	35	6	18.83	1.47	0.60	17.29	20.38	16	20		
	40	6	19.33	1.03	0.42	18.25	20.42	18	21		
$\alpha + \beta$ gliadins	20	6	10.00	1.09	0.45	8.85	11.15	8	11		
	25	6	7.50	0.84	0.34	6.62	8.38	6	8		
	30	6	8.50	0.84	0.34	7.62	9.38	7	9		
	35	6	8.83	0.75	0.31	8.04	9.62	8	10		
	40	6	8.67	0.82	0.33	7.81	9.52	8	10		
γ gliadins	20	6	4.00	0.00	0.00	4.00	4.00	4	4		
	25	6	5.00	0.63	0.26	4.34	5.66	4	6		
	30	6	4.50	0.55	0.22	3.93	5.07	4	5		
	35	6	3.50	0.55	0.22	2.93	4.07	3	4		
	40	6	3.67	0.52	0.21	3.12	4.21	3	4		
ω1,2 gliadins	20	6	2.67	0.52	0.21	2.12	3.21	2	3		
	25	6	4.33	0.82	0.33	3.48	5.19	3	5		
	30	6	3.17	0.41	0.17	2.74	3.60	3	4		
	35	6	2.67	0.52	0.21	2.12	3.21	2	3		
	40	6	3.17	0.41	0.17	2.74	3.60	3	4		
ω5 gliadins	20	6	4.50	0.84	0.34	3.62	5.38	4	6		
	25	6	6.67	0.52	0.21	5.12	7.22	5	7		
	30	6	4.83	1.17	0.48	3.61	6.06	3	6		
	35	6	3.50	0.55	0.22	2.93	4.07	3	4		
	40	6	4.33	0.82	0.33	3.48	5.19	3	5		
ANOVA (TP)	F(4.25) = 11.	02, Sig	s = 0.000, eta	a square =	84.33/132.1	7 = 0.64					
ANOVA $(\alpha + \beta)$	F(4.25) = 6.2	4, Sig.	= 0.001, eta	square $= 1$	9.13/38.30 =	= 0.50					
ANOVA (γ)	F(4.25) = 9.0	1, Sig.	= 0.000, eta	square $= 9$.13/15.47 =	0.59					
ANOVA ($\omega 1.2$)	F(4.25) = 9.0	8, Sig.	= 0.000, eta	square $= 1$	1.13/18.80 =	= 0.59					
ANOVA ($\omega 5$)	F(4.25) = 5.6	F(4.25) = 5.63, Sig. = 0.002, eta square = $14.87/31.37 = 0.47$									

in the relative concentration, F(4.25) = 193.61, Sig. = 0.000. The relative concentration of γ -gliadins was the highest at 20°C (43.88%) and the lowest at 30°C (24.48%). A statistically significant difference in the relative concentration of different groups was F(4.25) = 210.31, Sig. = 0.000. A capillary temperature of 35°C led to the highest relative concentration within the ω 1.2-group (27.21%), while 30°C provided the lowest (14.03%). There was a statistically significant difference in the relative concentration, F(4.25) = 165.39, Sig. = 0.000. The highest relative concentration of ω 5gliadins was obtained after extraction with 70% ethanol and at a capillary temperature of 25°C (30.98%) and the lowest at 20°C (5.42%). The effect of capillary temperature on relative protein concentration within $\omega 5$ gliadin fraction was examined by one-factor analysis of variance. A statistically significant difference in the relative concentration within the fraction was found, F(4.25) = 195.85, Sig. = 0.000.

Based on the obtained results (Table 5), it can be seen that with increasing capillary temperature, the relative concentration of $\alpha + \beta$ gliadins decreased, then increased, decreased, and increased again. Within γ -gliadins, the relative concentration decreased, then increased, and decreased again. The relative concentration of ω 1.2-gliadins increased, then decreased, increased again and finally decreased. Within the ω 5 gliadin fractions, the relative concentration increased and then decreased.

Table 6 shows descriptive indicators of total proteins and the number of gliadin proteins separated by fractions after extraction with 70% (v/v) ethanol and separated applying different electrode voltages (reverse mode).

The highest number of proteins was obtained after extraction with 70% ethanol, according to the method by Lookhart and Bean and electrophoretic separation at a voltage of -16.5 kV (23.50). The lowest number of proteins was obtained at -14.5 kV (14.83). It was found that there is a statistically significant difference in the number of proteins, F(3.20) = 46.16, Sig. = 0.000. The highest and the lowest amounts of proteins within

Table 5 Descriptive indicators of the total relative concentration of proteins and relative concentration of gliadin fractions (solvent 70% ethanol, Agilent, CE 7100, capillary inside diameter 50 μ m, total capillary length 33 cm, effective capillary length 23.50 cm, voltage –16.5 kV (reverse mode), duration 30 min, absorbance measured at 220 nm)

	Column tem-	Ν	RC, %	SD	Std.	95% confidence i	nterval of average	Min	Max
	perature, °C				error	Lower bound	Upper bound		
Total relative	20	6	100.00	0.00	0.00	100.00	100.00	100	100
concentration	25	6	100.00	0.00	0.00	100.00	100.00	100	100
	30	6	100.00	0.00	0.00	100.00	100.00	100	100
	35	6	100.00	0.00	0.00	100.00	100.00	100	100
	40	6	100.00	0.00	0.00	100.00	100.00	100	100
$\alpha + \beta$ gliadins	20	6	36.38	1.11	0.45	35.21	37.55	34.90	37.88
	25	6	28.29	1.40	0.57	26.82	29.76	26.06	30.09
	30	6	33.86	1.29	0.52	32.51	35.21	32.27	35.38
	35	6	27.22	2.11	0.86	25.01	29.44	26.00	31.49
	40	6	47.55	0.99	0.41	46.50	48.60	46.01	48.99
γ gliadins	20	6	43.88	1.14	0.47	42.68	45.08	42.39	45.37
	25	6	26.66	1.35	0.55	25.25	28.08	24.75	28.13
	30	6	24.48	1.59	0.65	22.81	26.15	22.24	26.77
	35	6	29.83	1.08	0.44	28.70	30.96	28.36	31.44
	40	6	27.18	1.34	0.55	25.77	28.59	25.07	28.89
ω1.2 gliadins	20	6	14.75	0.89	0.36	13.81	15.69	13.28	15.87
	25	6	14.93	1.04	0.43	13.84	16.03	13.03	15.95
	30	6	14.03	1.34	0.55	12.62	15.44	12.07	15.71
	35	6	27.21	1.35	0.55	25.80	28.63	25.10	28.95
	40	6	14.46	0.58	0.24	13.85	15.07	13.71	15.13
ω5 gliadins	20	6	5.42	0.34	0.14	5.06	5.77	5.02	5.90
	25	6	30.98	3.13	1.28	27.70	34.27	29.55	37.36
	30	6	27.28	2.04	0.83	25.14	29.42	24.49	29.61
	35	6	12.86	1.90	0.77	10.87	14.85	10.83	15.95
	40	6	10.67	1.07	0.44	9.55	11.80	9.20	12.22
ANOVA $(\alpha + \beta)$	F(4.25) = 193	.61, Sig	$g_{.} = 0.000, \epsilon$	eta square :	= 1593.94	/1645.39 = 0.97			
ANOVA (γ)	F(4.25) = 210).31, Sig	$g_{.} = 0.000, \epsilon$	eta square :	= 1448.88	/1491.94 = 0.97			
ANOVA (ω1.2)	F(4.25) = 165	5.39, Sig	$g_{.} = 0.000, \epsilon$	eta square	= 773.27/8	302.49 = 0.96			
ANOVA (ω5)	F(4.25) = 195	.85, Sig	$g_{.} = 0.000, e_{.}$	eta square	= 2949.03	/3043.13 = 0.97			

the $\alpha + \beta$ gliadin fractions were obtained at a voltage of -17.5 Kv and -14.5 kV (8.17 and 5.17, respectively). A statistically significant difference in the number of proteins was revealed, F(3.20) = 12.50, Sig. = 0.000. Within γ -gliadins, the highest amount of proteins was obtained at a voltage of -16.5 kV (5.00) and the lowest at -14.5 kV (2.50). ANOVA test showed a statistically significant difference in the number of proteins, F(3.20) = 26.82, Sig. = 0.000. The highest number of proteins within the $\omega 1.2$ gliadin fractions was obtained at a voltage of -16.5 kV (4.33). The lowest amount of $\omega 1.2$ gliadins was obtained at -17.5 kV (2.50). A statistically significant difference in the number of proteins was found, F(3.20) = 10.85, Sig. = 0.000. Descriptive analysis showed that the highest number of proteins within the $\omega 5$ gliadin fractions was at -16.5 kV (6.67) and the lowest at -17.5 kV (3.00). There was a statistically significant difference in the number of proteins, F(3.20) = 12.83, Sig. = 0.000.

We can seen that with increasing voltage, total proteins increased, then decreased and increased slightly

again. Within the $\alpha + \beta$ and γ gliadin fractions, the number of proteins increased and then decreased. Within the fraction of ω 1.2- and ω 5-gliadins, the amount of proteins increased, then decreased and increased slightly again.

Table 7 shows descriptive indicators of the total relative concentration of proteins and relative concentration of gliadin proteins separated by fractions after extraction with 70% (v/v) ethanol and separated by applying different electrode voltages (reverse mode).

Descriptive analysis showed that the highest relative concentration of $\alpha + \beta$ gliadins was obtained at a voltage of -17.5 kV (65.13%). The lowest concentration within this fraction was at -16.5 kV (28.29%). A statistically significant difference in relative concentration was F(3.20) = 851.47, Sig. = 0.000. The highest and the lowest relative concentrations of γ -gliadins were obtained at -14.5 kV and at -18.5 kV (27.37 and 21.87%, respectively). A statistically significant difference in the relative protein concentration was found, F(3.20) = 20.47, Sig. = 0.000. A voltage of -14.5 kV

Table 6 Descriptive indicators of total proteins and the number of gliadin fractions (solvent 70% ethanol, Agilent, CE 7100, capillary inside diameter 50 μm, total capillary length 33 cm, effective capillary length 23.50 cm, capillary temperature 25°C, duration 30 min, absorbance measured at 220 nm)

	Voltage,	Ν	Xav	SD	Std.	95% confidenc	e interval of average	Min	Max
	kV				error	Lower bound	Upper bound	-	
Total number	-14.5	6	14.83	1.17	0.48	13.61	16.06	13	16
of proteins	-16.5	6	23.50	1.05	0.43	22.40	24.60	22	25
	-17.5	6	17.33	1.21	0.49	16.06	18.60	15	18
	-18.5	6	17.67	1.75	0.71	15.83	19.50	15	19
$\alpha + \beta$ gliadins	-14.5	6	5.17	0.75	0.31	4.38	5.96	4	6
	-16.5	6	7.50	0.84	0.34	6.62	8.38	6	8
	-17.5	6	8.17	1.17	0.48	6.94	9.39	6	9
	-18.5	6	7.17	0.75	0.31	6.38	7.96	6	8
γ gliadins	-14.5	6	2.50	0.55	0.22	1.93	3.07	2	3
	-16.5	6	5.00	0.63	0.26	4.34	5.66	4	6
	-17.5	6	3.00	0.63	0.26	2.34	3.66	2	4
	-18.5	6	3.00	0.00	0.00	3.00	3.00	3	3
ω1.2 gliadins	-14.5	6	3.00	0.00	0.00	3.00	3.00	3	3
	-16.5	6	4.33	0.82	0.33	3.48	5.19	3	5
	-17.5	6	2.50	0.55	0.22	1.93	3.07	2	3
	-18.5	6	3.00	0.63	0.26	2.34	3.66	2	4
ω5 gliadins	-14.5	6	4.17	0.75	0.31	3.38	4.96	3	5
	-16.5	6	6.67	0.52	0.21	5.12	7.22	5	7
	-17.5	6	3.00	0.63	0.26	2.34	3.66	2	4
	-18.5	6	4.67	1.03	0.42	3.58	5.75	3	6
ANOVA (TP)	F(3.20) = 4	46.16, 8	Sig. = 0.000,	eta square	= 242.33/2	77.33 = 0.87			
ANOVA $(\alpha + \beta)$	F(3.20) =	12.50, 8	Sig. = 0.000,	eta square	= 30.00/46	.00 = 0.65			
ANOVA (γ)	F(3.20) = 2	26.82, 8	Sig. = 0.000,	eta square	= 22.12/27	.62 = 0.80			
ANOVA (ω 1.2)	F(3.20) =	10.85, 8	Sig. = 0.000,	eta square	= 11.12/17	.96 = 0.62			
ANOVA (ω5)	F(3.20) =	12.83, 8	Sig. = 0.000,	eta square	= 22.12/33	.62 = 0.66			

caused the highest (26.73%) and –18.5 the lowest (3.91%) relative concentration of ω 1.2-gliadins. The one-factor analysis of variance showed a statistically significant difference, F(3.20) = 1316.91, Sig. = 0.000. Relative concentration of ω 5-gliadins obtained at a voltage of –18.5 kV was the highest (40.30%) and at –17.5 kV the lowest (4.91%). There was a statistically significant difference in the relative concentration, F(3.20) = 549.81, Sig. = 0.000.

According to the results obtained, the increasing voltage decreased then increased and decreased again the relative concentration of $\alpha + \beta$ gliadin proteins. Within the fraction of γ - and ω 1.2- gliadins the concentration decreased, and ω 5-gliadins increased then decreased and increased again.

Lookhart and Bean performed separation and characterization of wheat proteins by high-pressure capillary electrophoresis (HPCE) [21]. Gliadins were extracted with 70% (v/v) ethanol. Separation of proteins was performed at a voltage of 22 kV and at a temperature of 45°C. The detection wavelength was 200 nm. Based on the obtained results, the retention time of gliadin proteins was: α gliadins 3–4 min (molecular weight according to SDS-PAGE 35–38 kDa), β 4–6 min (37–43 kDa), γ 5–6 min (43–47 kDa), and ω 6.8–10 min (48–63 kDa).

Bietz and Schmalzried analyzed gliadins from wheat by capillary electrophoresis [25]. Gliadins were extracted with ethanol and methanol of different concentrations (30, 40, 50, 60 and 70% v/v), with and without the reducing agent dithioerythritol. The temperature of the capillary ranged from 30 to 50°C, and the voltage from 8 to 12 kV. The detection wavelength was 200 nm. Capillary temperature of 40°C and voltage 10 kV showed optimal conditions. Ethanol proved to be a better solvent than methanol.

Changing ethanol concentration (50, 60, and 70% v/v), capillary temperature (20, 25, 30, 35, and 40°C), and voltage (-14.5; -16.5, -17.5, and -18.5 kV), we found that the optimal conditions for separation of gliadin proteins were 70% ethanol concentration, a capillary temperature of 25°C, and a voltage of -16.5 kV (reverse mode) (Fig. 3).

Our results are in agreement with Lookhart and Bean and Bietz and Schmalzried [21, 25]. Although the mentioned authors separated gliadin proteins by using different techniques of capillary electrophoresis, 70% ethanol proved to be the optimal solvent, which lines up with our results [21]. The gliadin proteins in this work were separated in less than 10 min, which is in agreement with Lookhart and Bean [21].

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Table 7 Descriptive indicators of the relative concentration of proteins and relative concentration of gliadin fractions (solvent 70% ethanol, Agilent, CE 7100, capillary inside diameter 50 µm, total capillary length 33 cm, effective capillary length 23.50 cm, capillary temperature 25°C, duration 30 min, absorbance measured at 220 nm)

	Voltage,	N	RC, %	SD	Std.	95% confidence interval of ave		Min	Max			
	kV				error	Lower bound	Upper bound	-				
Total relative	-14.5	6	100.00	0.00	0,00	100.00	100.00	100	100			
concentration	-16.5	6	100.00	0.00	0,00	100.00	100.00	100	100			
	-17.5	6	100.00	0.00	0,00	100.00	100.00	100	100			
	-18.5	6	100.00	0.00	0,00	100.00	100.00	100	100			
$\alpha + \beta$ gliadins	-14.5	6	35.42	1.27	0,52	34.09	36.75	33.06	36.81			
	-16.5	6	28.29	1.40	0,57	26.82	29.76	26.06	30.09			
	-17.5	6	65.13	1.90	0,78	63.13	67.13	61.63	67.05			
	-18.5	6	34.08	0.73	0,29	33.31	34.85	33.09	34.95			
γ gliadins	-14.5	6	27.37	1.03	0,42	26.29	28.45	26.10	29.22			
	-16.5	6	26.66	1.35	0,55	25.25	28.08	24.75	28.13			
	-17.5	6	25.51	1.24	0,51	24.20	26.81	23.81	26.96			
	-18.5	6	21.87	1.61	0,66	20.18	23.56	20.02	24.38			
ω1.2	-14.5	6	26.73	0.74	0,30	25.95	27.51	25.98	28.10			
gliadins	-16.5	6	14.93	1.04	0,43	13.84	16.03	13.03	15.95			
	-17.5	6	5.34	0.37	0,15	4.95	5.73	4.99	5.89			
	-18.5	6	3.91	0.50	0,20	3.39	4.43	3.21	4.52			
ω5	-14.5	6	10.66	0.75	0,31	9.87	11.45	9.92	11.98			
gliadins	-16.5	6	30.98	3.13	1,28	27.70	34.27	29.55	37.36			
	-17.5	6	4.91	1.01	0,41	3.85	5.97	3.99	6.73			
	-18.5	6	40.30	0.88	0,36	39.37	41.22	38.88	41.25			
ANOVA $(\alpha + \beta)$	F(3.20) =	851.47	, Sig. = 0.00	0, eta squar	e = 4935.49	/4974.13 = 0.99						
ANOVA (γ)	F(3.20) =	20.47,	Sig. = 0.000	, eta square	= 107.68/14	42.75 = 0.75						
ANOVA (ω 1.2)	F(3.20) =	1316.9	1, Sig. = 0.0	00, eta squa	are = 2000.5	4/2010.67 = 0.99						
ANOVA (ω5)	F(3.20) =	549.81	F(3.20) = 549.81, Sig. = 0.000, eta square = 5014.18/5074.98 = 0.99									

Gliadin extraction with 70% (v/v) ethanol



Figure 3 Electrophoregram of gliadin proteins extracted from wheat flour using 70% (v/v) ethanol and separated by capillary gel electrophoresis at a capillary temperature of 25°C and at a voltage of -16.5 kV

CONCLUSION

Based on the results obtained, the optimal conditions for gladin separation were 70% ethanol concentration, a capillary temperature of 25°C, and a voltage of -16.5 kV (reverse mode). Under these conditions, total proteins were 23.5, including $\alpha + \beta$ gliadin proteins (Xav = 7.50, relative concentration 28.29%), γ -fractions (Xav = 5.00, RC = 26.66%), ω1.2-gliadins (Xav = 4.33, RC = 14.93%), and ω5-gliadins (Xav = 6.67, RC = 30.98%).

The results obtained in this paper can greatly contribute to the prevention of the incorrect declaration of the "gluten free" products, reduction of health risks for people who are sensitive to gluten proteins, as well as the cost of treating the ones with celiac disease. In addition, the results of the research are of fundamental importance in the study of gluten proteins and the influence of technological procedures on their change and the possibility of reducing the allergic effect of individuals gluten proteins, during processing.

CONTRIBUTION

Authors are equally related to the writing of the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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Substituting wheat flour with okara flour in biscuit production

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Abstract:

Introduction. High fiber bakery products can be a healthy snack option for consumers. Our study focused on the effect of replacing wheat flour with okara flour on the physicochemical, nutritional, textural, and sensory attributes of biscuits.

Study objects and methods. We used 2, 4, 6, and 8% w/w okara flour to prepare biscuits. Refined wheat flour (control), mixed flour (okara and wheat flour), dough, and biscuits were assessed for physicochemical, textural, and nutritional properties, as well as sensory characteristics. The volume of particles was higher in 8% okara flour (145 µm) compared to refined wheat flour (91 µm).

Results and discussion. 2, 4, 6, and 8% w/w okara flour biscuits showed significantly ($P \le 0.05$) lower spread ratio and weight loss than biscuits from wheat flour. Hardness, stickiness, and cohesiveness of 2, 4, 6, and 8% okara flour dough were significantly ($P \le 0.05$) lower compared to the control, resulting in decreased cutting strength and increased hardness of okara flour biscuits. Moisture, protein, ash, fat, and crude fiber contents of 2, 4, 6, and 8% okara biscuits were significantly ($P \le 0.05$) higher compared to the control suggested that 4% okara biscuits had higher consumer acceptability and were superior to the control and other okara biscuits.

Conclusion. Mixed flour biscuits made from okara and wheat flours were superior in physicochemical, nutritional, textural, and sensory attributes, which allows considering them as an alternative healthy snack.

Keywords: Flour, dough, particle size, texture, biscuits, nutrient content, sensory evaluation

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INTRODUCTION

Biscuit is a popular, versatile bakery item consumed by all levels of society worldwide due to its taste, affordability, convenience, and an extended shelf life [1]. Biscuits, or cookies, are usually low in fiber, vitamins or minerals, and are highly calorific [2]. Thus, they cannot be added to the group of healthy foods. However, consumers are seriously concerned about their health issues. For this reason, bakery products with a high fiber content may be a choice for health-conscious people. Biscuits have good consumer acceptance, just as snacks do, and a long shelf life. Therefore, there is scope for nutritional development and fortification [3].

Okara is a pulpy, fiber-rich by-product of tofu and soy milk processing. Soy okara is a rich source of fiber [4]. It is composed of cellulose, hemicellulose, and lignin, as well as protein, lipids, vitamins, phytochemicals, and phytosterols [5, 6]. According to Grizotto *et al.*, one ton of processed soybeans could produce about two tons of okara following soymilk production [7]. As a result, every year enormous quantities of okara create disposal problems. To solve them, okara may be used as a dietary additive. It can further be processed to convenient and useful forms such as powders or extrudates [8]. It can be used directly in soups or salads. In addition, we can now find many online recipes where freshly produced okara is used as a raw ingredient.

However, it is hard to find an industrial product of soy okara because of its high moisture content (around 85 g) and poor textural quality that may cause rapid deterioration [5]. Some possible applications of okara

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might include baked goods, beef patties, and coconut cookies due to its high amount of fiber and protein [8, 9]. Dietary fiber is a significant component in bakery products, confectionery, meat, beverage, and dairy items [8]. However, supplementing baked products with dietary fiber may change the flavor, texture, and taste of final products [3].

A number of studies have shown the application of okara flour in tortillas, cookies, roti and parata, and even in breakfast cereals [8, 10–12]. The use of okara as a gluten free all-purpose flour may add further value to this agro waste and bring significant nutritional benefits.

Our study aimed to identify the effect of okara flour on the properties and nutritional composition of biscuits.

STUDY OBJECTS AND METHODS

Soybeans and refined wheat flour were collected from K-R Market (Mymensingh, Bangladesh). Sodiumbi-carbonate (food grade) was supplied by Mitali Scientific Co. Ltd., Bangladesh.

Whole soybeans were soaked in a 0.5% NaHCO₃ solution (1:2) at 60°C for four hours in a water bath (Schufzart, Membart GmBH+ Co., Büchenbach, Germany). The water was discarded and the soaked beans were dehulled before grinding to remove unwanted substances using a dehuller. The hydrated soybeans were blanched at $90 \pm 2^{\circ}$ C for 10 min with the addition of 0.5% NaHCO₃ (w/v), and the solution was drained well. The beans were washed with potable water for three times [13]. The blanched beans were ground with the addition of hot water (100°C) [bean to water ratio = 1:4] using a super mass collider (Masuko Sangyo Co. Ltd., Kawaguchi, Japan).

Soy okara was collected after soy milk extraction by filtering through double layers of cheese cloth. Soy okara was dried in a cabinet drier (Dayton Electric MFG. Co. Ltd., USA) at 60°C for 24 h and ground using a grinder. The ground okara was sieved (420-micron mesh size) and kept in a desiccator to reduce the moisture content by up to 5% [11]. The powder was finally placed in a sealed polyethylene-laminated aluminum foil bag and kept at -20° C before analysis and further processing.

Moisture, protein, ash, and fat contents of soy okara flour, refined wheat flour, and biscuits were determined according to the AOAC method [14]. Genistein was determined by the HPLC method as modified by [15].

Particle size was measured according to [16]. Average particle sizes $(d_{3,2} - surface-weighted mean diameter, Sauter mean diameter and d_{4,3} - volume-weighted mean diameter, De Brouckere mean diameter) of refined wheat flour, as well as 2, 4, 6 and 8% soy okara flour were determined using a particle size analyzer (Malvern Zetasizer Nano ZS, UK) with the attachment of dry feed.$

Refined wheat flour (RWF), okara flour, and other ingredients were weighed according to Table 1 and mixed together. Fat was mixed with the ingredients and water was added to the mixer to form dough. The dough was kneaded, rolled to uniform thickness (5 mm) and cut in round shape biscuits of 4 cm in diameter. They were baked at 220°C for 10 min and cooled at room temperature. The biscuits were packed in HDPE and kept in desiccators for further analysis.

The spread ratio, an essential quality parameter of biscuits, was determined as follows:

Spread ratio =
$$D / T$$

where D is the average diameter and T is the average thickness of biscuits after baking, cm.

Weight loss (WL) of biscuits during baking was calculated by the following formula [17]:

$$WL = (W_{dough} - W_{biscuit}) / W_{dough} \times 100$$

where W_{dough} is the weight before baking and $W_{biscuit}$ is the weight after baking five samples, g.

The doughs made from different amounts of soy okara flour (2, 4, 6, and 8%) and only refined wheat flour (control) were tested for firmness by a penetration test. The dough was placed in a concentric cylinder (30 mm in diameter) under a cylindrical probe (5 mm) (Stable Micro Systems, UK). The test conditions included 2 mm/s pretest speed, 3 mm/s test speed, 10 mm/s posttest speed, 50 kg load, and 60% strain. When the probe penetrated 60% of the dough, it was found to gain its original position. The absolute peak force of the force-time curve was taken as dough firmness [18]. Each dough was tested three times.

Dough strength, adhesion, and stickiness tests were carried out using an SMS/Chen-Hosney Stickiness Cell and Prespex cylinder probe (25 mm) (Stable Micro Systems, UK). The test conditions included 2 mm/s pretest speed, 2 mm/s test speed, 10 mm/s post-test speed, 40 g trigger force, 3 mm return distance, and 10 s contact time [19–21]. The positive peak constraint from the curve was considered as stickiness force. The area falling under this force-distance curve indicates the work of adhesion. The distance of sample extension

Table 1 Basic formulation for preparation of biscuits (on 100 g flour basis)

Ingredients, g	Samples						
	Control	Experimental			ıl		
	(wheat flour)	(with okara flour)					
Wheat flour	100	98	96	94	92		
Okara flour	-	2	4	6	8		
Sugar	50	50	50	50	50		
Oil	40	40	40	40	40		
Baking powder	1.5	1.5	1.5	1.5	1.5		
Milk powder	5	5	5	5	5		
Salt	0.5	0.5	0.5	0.5	0.5		
Egg	45	45	45	45	45		
Ammonium bicarbonate	0.5	0.5	0.5	0.5	0.5		

during prove return was considered as dough strength or cohesiveness [18].

The three-point bending test was carried out employing a 3-point bending rig (Stable Micro Systems, UK) connected to a texture analyzer. The test conditions included 10 mm/s pretest speed, 1 mm/s test speed, 10 mm/s post-test speed, 10 mm distance, and 50 kg load cell; descending development was continued till the biscuits broke. The most extreme constraint was recorded as the "hardness" of the biscuits [22].

The cutting strength of biscuits was measured using an HDP/BS blade-type texture analyzer (Stable Micro Systems, UK). The biscuits were set on the platform, and the blade was connected to the crosshead of the instruments. The test conditions included 2 mm/s pretest speed, 2 mm/s test speed, 10 mm/s post-test speed, and 5 mm distance. The outright peak force of the curve was recognized as the cutting strength of the biscuits [18, 21]. Textural properties of the dough and biscuits were determined by a TA-XT plus texture analyzer (Stable Micro Systems, UK) with Texture ExpertTM software.

The color of biscuits was analyzed by a colorimeter (Chroma Meter CR400, Konica Minolta, Japan) under illuminant: *C, D65 and space: LAB. It was determined in L^* , a^* and b^* system, where L^* is lightness (100: white, 0: black), a^* is redness (+)/greenness (-), and b^* indicates yellowness (+)/blueness (-). All analyses were performed in triplicate.

The sensory evaluation of the control and experimental samples included color, texture, flavor, and overall acceptability by ten semi-trained panelists on a 9-point hedonic scale (9 = like extremely, 8 = like very much, 7= like moderately, 6 = like slightly, 5 = neither like nor dislike, 4 = dislike slightly, 3 = dislike moderately, 2 = dislike very much and 1 = dislike extremely). The results were evaluated by analyses of variance (ANOVA) and Duncan's new multiple range test (DMRT) of the Statistical Analysis System (SAS).

The physicochemical, nutritional, and textural properties were determined in replicate and statistically analyzed by a two-way ANOVA using the Microsoft Excell-2010.

Table 2 Chemical composition of okara and refined wheat flour (RWF)

Components	Okara flour	Refined wheat flour
Moisture, %	$13.75^{a} \pm 1.25$	$12.65^{a} \pm 1.50$
Protein, %	$35.14^{a} \pm 2.00$	$13.00^{b} \pm 1.90$
Fat, %	$10.78^{\text{a}} \pm 1.40$	$1.80^{b} \pm 1.00$
Ash, %	$3.95^{a} \pm 0.50$	$1.47^{b} \pm 0.25$
Crude fiber, %	$30.01^{a} \pm 2.25$	$3.23^{\mathrm{b}} {\pm 0.50}$
Genistein, mg/100 g	5.05 ± 0.50	nd
of okara flour		

Mean \pm SD represents the average of three replicates for each analysis Different letters in the same row show significant differences (P < 0.05)

nd = not determined

RESULTS AND DISCUSSION

Table 2 shows the nutritional composition of okara and refined wheat flour (RWF). Okara flour had significantly higher contents of protein, fat, ash, and crude fiber compared to wheat flour. However, when making biscuits, it is important to add wheat flour as it contains gluten, which makes the dough adhesive and cohesive. Yet, gluten is also responsible for celiac diseases [23]. In our study, we substituted 2, 4, 6, and 8% of wheat flour with the same quantities of gluten-free okara flour.

Figure 1 illustrates the distribution of particle sizes in the mixed flour (2, 4, 6, and 8% okara flour mixed with wheat flour) and control flour (RWF). We identified two distinct peaks for all mixed flour samples, whereas the first peak in the control was not as distinct, showing that the particle size distribution of all flours was bimodal. The results were in agreement with [16] and [24]. We also found the maximum particle size to be \sim 100 µm, indicating a higher volume. The particle sizes for both the experimental and control samples ranged from 0.1 to 100 µm, but the volume of particles was the highest at $\sim 145 \,\mu\text{m}$ and 91 μm , respectively. The results indicated that refined wheat flour had lower protein and fiber contents [25]. Hard dough prepared with finer particle-size flour has higher density, resulting in less developed biscuits during baking [26]. Thus, coarser, mixed or composite, flour is most desirable for preparing hard dough biscuits.

The mean volume diameter and the surface mean diameter of refined wheat flour (70.05 and 27.5 μ m, respectively) were significantly (P < 0.05) smaller than those of all mixed flour samples (83.7 and 32.8 μ m, respectively). There was no significant difference among 2, 4, 6, and 8% okara flour samples (P > 0.05). The flour particle size often affects the biscuits' water absorption capacity, density, and spread ratio. When fine particle-



Figure 1 Particle size of refined wheat flour and wheat flour mixed with okara flour

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Table 3 Effect of okara flour on physical properties of biscuits

Biscuits	Weight, g	Diameter (D), cm	Thickness (T), cm	Spread ratio (D/T)	Weight loss, %
Control (refined wheat flour)	$4.95^{\rm b}\pm0.40$	$4.62^{\mathtt{a}}\pm0.32$	$0.62^{\text{e}} \pm 0.06$	$7.45^{\mathrm{a}}\pm0.80$	$11.46^{\mathrm{a}}\pm0.35$
2% okara flour	$5.94^{\rm a}\pm0.50$	$4.58^{\rm b}\pm0.50$	$0.63^{\text{d}}\pm0.05$	$7.27^{\mathrm{b}}\pm0.70$	$10.01^{\mathrm{b}}\pm0.55$
4% okara flour	$5.95^{\rm a}\pm0.35$	$4.56^{\rm c}\pm0.42$	$0.64^{\rm c}\pm0.02$	$7.13^{\circ}\pm0.50$	$10.03^{\mathrm{b}}\pm0.50$
6% okara flour	$5.94^{\rm a}\pm0.70$	$4.55^{\rm c}\pm0.45$	$0.65^{\rm b}\pm0.01$	$7.00^{\rm d}\pm0.90$	$10.01^{\rm b}\pm0.65$
8% okara flour	$5.93^{\rm a}\pm0.55$	$4.53^{\text{d}}\pm0.60$	$0.66^{\text{a}}\pm0.01$	$6.86^{\rm e}\pm0.80$	$10.09^{\rm b}\pm0.75$

Mean \pm SD represents the average of five replicates for each analysis

Different letters in the same column indicate significant difference (P < 0.05)



Figure 2 Nutritional components of biscuits from wheat flour and okara flour-enriched biscuits

size flour is used for hard dough biscuits, it usually results in higher density and less effective baking properties [26]. Therefore, coarser composite flour is more desirable.

The weight of okara flour biscuits was higher than that of biscuits prepared with only refined wheat flour (control). The biscuits prepared with coarser 2, 4, 6, and 8% okara flour mixes had a significantly (P < 0.05) higher thickness and a smaller diameter than the control (RWF) biscuits (Table 3) due to higher development during baking [26]. As a result, they had a reduced spread ratio compared to the control. The spread ratio correlates with texture, grain fineness, bite, and overall mouth feel of the biscuits [27]. Additionally, using flour high in protein and fiber in place of wheat flour provides a reduced spread ratio [28].

Among the experimental biscuits, 8% okara samples had a smaller diameter and a lower spread ratio compared to the others (Table 3). This might be due to comparatively higher protein and fiber contents in 8% okara biscuits. Further, mixed flour biscuits showed a significant (P < 0.05) reduction in weight loss compared to biscuits from wheat flour (Table 3). However, there was no significant difference (P > 0.05) in weight loss among 2, 4, 6, and 8% okara flour biscuits. The lower weight loss in the experimental biscuits might be due to better water absorption by the flour components due to protein hydration, with less water evaporated during baking [17].

Moisture, ash, protein, and fat contents in the biscuits prepared with okara flour were higher than those in the biscuits from control flour (RWF) (Fig. 2). Among mixed flours, 8% okara flour provided significantly ($P \le 0.05$) higher nutrient contents compared to the others. The biscuits made with 8% okara flour had a higher moisture content than the control or the other mixed-flour samples due to a greater volume of water required at the time of dough making for its high fiber and protein contents.



Figure 3 Color parameters of biscuits from wheat flour and okara flour-enriched biscuits

Table 4 Textural properties of dough and biscuits from refined wheat flour and okara flour

Samples		Do	ough		Bis	cuits
	Firmness, N	Stickiness, N Work of adhe- I		Dough	Cutting	Hardness, N
			sion (N-S)×10 ⁻³	strength, mm	strength, N	
Control (refined wheat flour)	$4.45^{\mathrm{a}}\pm0.05$	$0.28^{\rm a}\pm0.05$	$5.20^{\rm a}\pm0.5$	$0.65^{\rm a}\pm0.10$	$64.94^{\mathtt{a}}\pm2.00$	$15.20^{\text{c}}\pm19.05$
2% okara flour	$3.90^{\rm b}\pm0.09$	$0.21^{\rm b}\pm0.07$	$4.75^{\rm b}\pm0.3$	$0.54^{\rm b}\pm0.09$	$55.75^{\text{b}}\pm1.75$	$17.55^{\text{b}}\pm1.55$
4% okara flour	$3.79^{\text{c}}\pm0.07$	$0.18^{\rm b}\pm0.05$	$4.50^{\rm c}\pm0.4$	$0.50^{\rm b}\pm0.01$	$52.55^{\text{b}}\pm1.55$	$18.10^{\text{b}}\pm1.25$
6% okara flour	$3.66^{\text{cd}}\pm0.05$	$0.16^{\rm b}\pm0.03$	$4.25^{\rm c}\pm0.1$	$0.40^{\rm c}\pm0.03$	$43.90^{\circ}\pm2.50$	$18.76^{\mathrm{a}}\pm2.00$
8% okara flour	$3.45^{\rm d}\pm0.04$	$0.14^{\rm b}\pm0.05$	$3.50^{\text{d}}\pm0.7$	$0.35^{\rm c}\pm0.05$	$40.35^{\text{cd}}\pm2.75$	$19.05^{\text{a}}\pm2.05$

Mean \pm SD represents the average of five replicates for each analysis

Different letters in the same column indicate significant difference (P < 0.05)

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Biscuits	Sensory parameters			
	Color	Flavor	Texture	Overall acceptability
Control (refined wheat flour)	$6.93^{b} \pm 0.50$	$6.73^{b} \pm 0.65$	$6.47^{b} \pm 0.85$	$6.60^{b} \pm 0.80$
2% okara flour	$6.33^{b} \pm 0.43$	$6.20^{\circ} \pm 0.50$	$5.94^{\mathrm{bc}} \pm 0.69$	$6.47^{b} \pm 0.45$
4% okara flour	$7.20^{a} \pm 0.72$	$7.20^{a} \pm 0.75$	$6.93^{a} \pm 0.95$	$7.33^{a} \pm 0.75$
6% okara flour	$6.00^{b} \pm 0.660$	$6.53^{bc} \pm 0.95$	$5.73^{\circ} \pm 0.99$	$6.33^{b} \pm 0.95$
8% okara flour	$6.00^{b} \pm 0.85$	$6.27^{\circ} \pm 0.55$	$5.67^{\circ} \pm 0.45$	$6.13^{b} \pm 1.20$
LSD	0.577	0.447	0.566	0.574

Table 5 Sensory characteristics of biscuits from refined wheat flour and okara-enriched biscuits

Mean \pm SD represents the average of five replicates for each analysis

Different letters in the same column indicate significant difference ($P \le 0.05$)

Flours rich in protein require much water to make machinable dough as protein is not sufficiently hydrated to form a network [29].

Dough firmness, strength (cohesiveness), and stickiness were significantly lower in 2, 4, 6, and 8% okara flour samples compared to the control (wheat flour only) (Table 4). Decreased dough firmness is usually related to a high fat content in formulations. This disrupts the development of a gluten network by lubricating the complete matrix and making it hydrated [17, 30]. Okara flour is gluten-free, which also accounted for lower firmness in mixed flour dough compared to dough made from wheat flour only. Stickiness is a significant parameter of dough quality as it affects the handling convenience and may damage the apparatus [31]. Okara flour dough showed lower stickiness compared to the control, owing to a higher water absorption ability. Lower stickiness and adhesion of okara dough correlate with a greater water absorption capacity, a comparatively low gluten content, and a higher fat content [16].

The three-point bending test showed significantly lower ($P \le 0.05$) hardness for 2, 4, 6, and 8% okara flour biscuits compared to wheat biscuits (Table 4). This was due to a high protein content and a better water absorption capacity in the mixed flour. 8% okara flour biscuits seemed harder than those with 2, 4, and 6% okara flour due to a lower gluten content compared to the control and the other mixed flours.

There was a significant (P < 0.05) difference for all five types of biscuits in terms of color (Fig. 3). The L^* value of all composite flour biscuits was lower than that in the refined wheat flour biscuits due to the presence of natural anti-browning substance such as genistein in okara flour [32]. In particular, its genistein content was 5.05 ± 0.5 mg/100 g of okara flour (Table 2). 8% okara flour had the highest L^* value, indicating less brown pigment formation. The result suggested that okara flour could reduce brown pigments. We observed high positive a^* values (redness) for 2, 4, 6 and 8% okara flour biscuits. Positive b^* values (yellowness) were significantly (P < 0.05) higher in mixed flour biscuits compared to the control due to the presence of phytochemicals and crude fiber in okara flour [11].

The results of sensory evaluation of biscuits enriched with okara flour and control biscuits are shown in Table 5. The sample with 4% okara flour showed the finest sensory characteristics in terms of color, texture, flavor, and overall acceptability. However, the other samples were also found acceptable. A DMRT analysis revealed that 4% okara biscuits were significantly better in color, texture, flavor, and overall acceptability than other biscuits containing 2, 6, and 8% okara flour. However, increasing the amount of okara flour decreased the level of overall acceptability.

CONCLUSION

Biscuits prepared from mixed okara (2, 4, 6, and 8%) and refined wheat flour were found to outperform refined wheat flour biscuits in physicochemical, nutritional, textural, and sensory attributes. Okara flour biscuits had an inferior spread ratio, but higher fiber and protein contents. We also found them to have poor cutting strength and greater hardness. Okara flour biscuits had better color due to the presence of genistein. 4% okara biscuits had higher consumer acceptability on a 9-point hedonic scale. Decreased dough hardness due to okara flour diminished the cutting strength and increased hardness in the corresponding biscuits. Thus, we can conclude that biscuits prepared from okara flour can be considered as a healthy snack option.

CONTRIBUTIONS

Md. A. R. Mazumder and Thottiam V. Ranganathan conceptualized and supervised the work. Anjuman A. Begum and Md. F. Jubayer were involved in manuscript writing and data analysis. Md. A. Momin and Asmaul H. Nupur performed laboratory experiments and data collection.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Lisitsyn AB, Chernukha IM, Nikitina MA. Designing multicomponent food products. Moscow, 2020

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Human life largely depends on the way nutrition affects our health. This fact explains the relevance of modeling multicomponent food products with preplanned nutritional and energy value. *Designing Multicomponent Food Products* was written by A.B. Lisitsyn, I.M. Chernukha, and M.A. Nikitina (Moscow, 2020). The book features such urgent problem of modern biotechnology as the basic aspects of multicomponent food design. The publication is intended for specialists in the field of food industry, biotechnology, food chemistry, nutritional science, and biomedicine, as well as for experts involved in design and technology of healthy, preventive, and personalized diets.

Biotechnology is currently getting extremely popular both in Russia and abroad. The authors believe that breakthrough science-intensive technologies will be able to provide the population with healthy and proper nutrition. Studies of the interaction of various nutrients and their effect on human health make it possible to determine the specifics of proper nutrition and identify the patterns of vital biophysical, biochemical, and energy mechanisms.

The book owes its scientific novelty to the comprehensive analysis of theoretical and practical aspects of multicomponent food design. The authors used advanced mathematical models that regulate the stages of functional food development. The method allowed the team to describe the changes in the chemical composition of the formulations, depending on the ingredient ratio and standards. This approach makes it possible to replace technological experiments with mathematical modeling.

The modern food industry includes thousands of raw materials, each with its own characteristics and functional, technological, physicochemical, and organoleptic profile. These parameters require systematization in order to optimize the design process.

The research has a distinct interdisciplinary character and unites biomedicine with theoretical and practical (digital) biotechnology. The textbook presents a theoretical summary of various studies in the field of food combinatorics. The material presentation is well-structured; it moves from general theory of biotechnology and food combinatorics to specific examples of formulation design for certain food products according to preplanned criteria. The chapters are arranged in such a way that they provide a clear systematic view of the functional food design.

The first chapter is entitled *Issues of International Food Regulation and Related Laws*. It features the existing nutrition strategies in the European Union, Russia, Great Britain, and the USA. It introduces the reader to the main issues of the book, its basic theoretical principles and concepts. The chapter shows how these concepts are being implemented nowadays and what they lead to.

The second chapter is A Review of Design Methods for Multicomponent Food Products. It examines the main findings and research results in the development of domestic food combinatorics. It traces the history of this science from its origins to the works of contemporary scientists. The authors analyzed publications that feature the principles and methods of designing the formulations of balanced food products. The initial stage included the development of theoretical foundations and specific methods of balanced food production. This stage was associated with the formalization of qualitative and quantitative ideas about the rational use of essential amino acids in the technology of adequate exotrophy. Nowadays, food product development includes not only nutritional and biological value but also medical, technological, economic, and social factors.

The third chapter, *Principles for the Development* of Adequate Human Nutrition, describes the laws of nutritional science, or nutritiology. It introduces the basics of human nutrition, depending on social factors (age, gender, workload, etc.) and various noncommunicable diseases (alimentary-dependent diseases and socially significant non-communicable diseases). The authors see a need in a new scientific approach that they call digital nutrition. They believe that digital

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nutrition will deal with the digital transformation of data on physiological needs, biologically active substances, energy, and the chemical composition of basic food products. Specialists in digital nutrition will design computer programs that will develop personalized diet recommendations.

The fourth chapter, *Food Design*, outlines the main stages of multicomponent food design as a fundamentally new direction in food industry. It reveals the principles of food combinatorics and the stages of designing new formulations for multicomponent foods.

The fifth chapter is entitled *Cluster Analysis for Typology of Food and Dishes*. It introduces cluster analysis with its principles and methods.

The sixth chapter, *Examples of Computer-Aided Design of Multicomponent Food Products*, features designing a new formulation based on the optimal cost criterion, foods for anemia prevention, meat dietary products fortified with calcium, and curd products based on the energy value criterion.

The language of the book is clear, consistent, and laconic, which ensures effective comprehension. The authors use the essential terminology that shapes the scientific apparatus of future specialists. The good use of general vocabulary helps clarify the material and examples.

The information is well-structured; the main ideas and definitions are visually highlighted. The educational material includes a wide range of data visualization tools, namely diagrams, charts, tables, formulas, figures, infographics, etc.

The authors achieve their goals by analyzing theoretical material and giving specific examples of computer design of various multicomponent food products. The means and methods of material presentation correlate with the goals. The list of references and recommended sources includes relevant domestic and foreign scientific literature.

In general, *Designing Multicomponent Food Products* by A.B. Lisitsyn, I.M. Chernukha, and M.A. Nikitina provides its readers with a systematic understanding of the scientific and applied aspects of multicomponent food design. The book contributes to the fundamental training of professionally oriented specialists with deep knowledge in the field of biotechnology and nutritional science.

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