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Editorial
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Editor's column

Dairy industry is a socially significant segment of the agro-industrial complex. Dairy products are one of the largest markets in Russia. Its contribution to the gross domestic product and economic growth can hardly be underestimated.

In 2020, global milk production reached 903 million tons. Around the world, 165 million tons of dairy by-products and 120 million tons of whey are produced every year. Last year, Russia produced 825 thousand tons of whey, which was by 21.6% more than the year before.

Whey is a by-product of cheese, curd, and casein production. However, its nutritional value is very high. Whey includes about 50% of milk solids, 95% of lactose, 20% of protein, and up to 10% of fat. All these valuable components can be used to produce baby foods, sport supplements, and special foods for particular nutritional purposes, not to mention forage for farm animals.

Whey, its components, and their derivatives are valuable raw materials. The rates of whey production are very high, which means that advanced technology of whey processing is a pressing issue. The contemporary biotechnology sees whey as an extremely advantageous component of various functional foods.

Professor Andrei G. Khramtsov, Member of the Russian Academy of Sciences, is the founder of *Living Systems*, which is the leading Russian scientific school of intensive bionanomembrane technologies in dairy processing. *Living Systems* studies a wide range of relevant issues, from domestic food safety to the special role of lactose and its derivatives in human life cycle. Professor Khramtsov and his team discovered the whey phenomenon, set up the physical and chemical foundations of milk sugar industry, developed the system of centralized lactose production, and invented lactulose with bifidogenic properties. They also designed nanobiomembrane methods of waste-free processing that recycle agricultural raw materials into such functional food products as probiotics, prebiotics, and synbiotics. Finally, they were the first to use whey proteins in order to produce nanotubes, which revolutionized the formation of food systems.

Living Systems achieved great results in developing advanced functional foods and rationalizing the use of dairy by-products, including whey. The team managed to implement the principles of smart economy by applying novel methods to industrial whey processing. Their revolutionary technologies increase food and fodder resources, reduce environmental pollution, raise income, and reduce risks. *Living Systems* used nanotechnology to produce functional foods via bio-, membrane, and biomembrane processes at the level of whey clusters, thus creating immense opportunities for multifaceted development of agro-industrial complex, biotechnology, and medicine. Today, the *Living Systems* school is as relevant as ever. Their innovative designs are a timely response to the many challenges posed by the high-tech era.

Professor Khramtsov is recognized worldwide for his outstanding contribution to global dairy industry. On behalf of *Foods and Raw Materials* and its editorial board, I would like to congratulate Professor Khramtsov on his milestone birthday and wish him many happy years of productive labor for the benefit of Russian dairy industry.

Editor-in-Chief,
Corresponding Member of the Russian Academy of Sciences,
Professor A. Yu. Prosekov

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Beet pulp dietary fiber exposed to an extremely low-frequency electromagnetic field: detoxification properties

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Abstract:

Introduction. The lack of dietary fiber in the Russian people diet contributes to the development of various diseases. In this regard, it seems worthwhile to enrich foods with dietary fiber obtained from various types of raw materials. In our experiments, we used beet pulp. This study aimed to develop a technology for obtaining combined dietary fiber using the electrophysical method and evaluate its detoxification properties.

Study objects and methods. Study objects were pectin substances and combined detoxicants from beet pulp obtained by extracting with succinic acid with and without an extremely low-frequency electromagnetic field (ELF EM) treatment. The profiles of combined detoxicants and pectin substances were identified by IR-Fourier spectrometry. Beet pectin, beet cellulose, and their combined detoxicants were tested for complexing (binding) capacity with respect to lead ions (Pb^{2+}). For this, we applied the trilonometric method with some modifications.

Results and discussion. The analysis of the absorption bands of carboxyl groups carbonyls revealed the presence of free carboxyl groups in the combined detoxicants. The combined detoxicant with a 1:0.5 ratio of cellulose and pectin substances showed a high complexing (binding) capacity (601 mg/Pb^{2+}) with respect to lead ions (Pb^{2+}).

Conclusion. We developed a technology for producing combined detoxicants with a high complexing capacity with respect to lead ions analysed microstructures of gels obtained during the interaction between the combined detoxicant and lead acetate solution.

Keywords: Pectin, cellulose, beet, detoxicant, extraction, IR spectra, binding capacity, complexation

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INTRODUCTION

In 2012, the General Assembly of the World Health Organization (WHO) adopted the Global action plan for the prevention and control of noncommunicable diseases 2013–2020, supported by the health ministers of all the member states. It promoted the idea that people's health is determined not only by working conditions, but also by social and anthropogenic factors.

Today, Russian people consume too many simple carbohydrates and animal fats and not enough fruits and vegetables. The lack of dietary fiber contributes to various diseases, including diabetes, colon cancer, hernia of the oesophageal opening of the diaphragm, obesity, and gallstone disease. A sufficient amount of dietary fiber helps prevent dental caries and reduces the level of cholesterol in the body. Dietary fiber is involved in metabolic processes, controls gastric emptying, binds heavy metals and carcinogens and excretes them from the body [1].

Therefore, using dietary fibers obtained from various types of raw materials appears to be a promising way of enriching foods. Several recent studies have aimed to create foods with plant-based dietary fiber, including fiber from non-traditional raw materials [2–4].

Dietary fiber can be produced from beet pulp – a by-product from the processing of sugar beet at sugar

factories and table beet at canning factories [3]. Beetroot is a good source of dietary fiber that is used as a dietary supplement in various food products [5, 6].

Beet pulp is the cheapest pectin-containing raw material [7]. There are over 130 pulp drying stations at sugar factories in the CIS (Commonwealth of Independent States) countries. Their total capacity is 10 000 t of dried pulp per day. Thus, with an average yearly operation for 100 days, they can produce at least 800 000 t of dried pulp per year [8].

The cell walls of sugar beet pulp contain such dietary fibers as cellulose, hemicellulose, and pectin substances [9]. These compounds bind together, forming a so-called "pectin and cellulose complex" in a plant cell. However, there are no conditions for their detoxification properties to fully manifest, since their functional groups – involved in complexation (binding) reactions and ion-exchange processes – are difficult to access [10, 11].

Cellulose is an insoluble dietary fiber with almost no functional groups allowing for the sorption of heavy metal ions during complexation. Due to its mechanical detoxification properties, cellulose sorbs mainly high molecular weight compounds.

Pectin substances are soluble dietary fibers. Their detoxification properties are determined by free functional carboxyl groups [12]. Due to these groups, pectin substances sorb low molecular weight compounds and participate in complexation with respect to heavy metal ions [13, 14].

It seems worthwhile to recover pectin substances separately from cellulose, when destroying the pectin and cellulose complex, to release the functional groups of their molecules. Since they manifest different binding activity with respect to heavy metal ions, as well as different detoxification properties, it is advisable to obtain a combined detoxicant from these substances.

A number of studies, both in Russia and abroad, have aimed to optimise the production of dietary fiber with detoxification properties from beet processing by-products.

One of the studies proposed a technology of treating sugar beet pulp with high-voltage electric discharges (HVED) prior to pectin extraction [15]. After the HVED pre-treatment, pectin was extracted in acidified water with varied pH and temperature. The results showed an increase in pectin yield from 42.6% for untreated sugar beet pulp to 53.4% for HVED-treated sugar beet pulp under optimal extraction conditions (t = 90°C, pH = 2, duration = 1 h). The Fourier-transform infrared spectroscopy and gas chromatography-mass spectrometry showed similar functional groups and chemical composition between the standard of sugar beet pectin and the extracted molecules from untreated and HVED pre-treated sugar beet pulp.

Another study investigated the effect of beet pulp particle size on the extraction and physicochemical properties of pectin [16]. Reducing particle size from 406.33 to 24.93 μm increased pectin yield from 15.81 to 20.50% and the content of galacturonic acid from 38.51 to 59.97%. Larger particle size enhanced pectin's rheological properties (kinematic and dynamic viscosity, and activation energy). All pectin solutions showed the same linear viscoelastic properties. Beet pulp particle size affected the dynamic moduli of extracted pectin.

A method of gradual ethanol precipitation of sugar beet pectin from an acidic extract was offered by [17, 18]. The procedure was developed to purify sugar beet pectin from a pectin-containing aqueous extract. Five fractions with different chemical and molecular characteristics were obtained by gradually increasing the concentration of ethanol in the precipitating medium from 50 to 80%. The analysis of chemical and macromolecular characteristics of the fractions indirectly showed that the ability of pectin to dissolve in the ethanol-water binary mixture strongly depended on the polymer structure. The fractions rich in neutral sugars were precipitated at relatively high concentrations of ethanol, probably due to increased interaction between pectin chains and solvent molecules. The authors found that gradual ethanol precipitation was more selective in relation to pectin's structural features and surface properties than single-stage ethanol precipitation.

Guo *et al.* established the efficacy of purifying sugar beet pectin from non-pectin components by metal precipitation [19]. To assess the selectivity of copper ions in relation to binding pectin or non-pectin compounds, two fractions were fractionated from sugar beet pectin, namely copper-precipitated pectin and copper-unprecipitated pectin. The chemical analysis revealed certain structural differences between the fractions. In particular, copper-unprecipitated pectin contained a markedly higher amount of neutral sugars and proteins, but a lower content of galacturonic acid and acetyl ester groups, compared to copper-precipitated pectin. However, there was no appreciable difference in the degree of methylation. In addition, AFM analysis showed that copper-precipitated pectin displayed branched fibrous structures, while copper-unprecipitated pectin had distinguishable granular shapes. The authors concluded that copper ions selectively bound the anionic regions among pectin chains, thereby separating pectin saccharides from non-uronide compounds, i.e. low molecular weight carbohydrates and free proteins.

The process of ethanol precipitation of sugar beet pectin with and without counter ions was investigated by [20]. In aqueous solution, when cations bound to sugar beet pectin, the negative charges on the pectin chains reduced and the hydrodynamic radius of the whole polymer decreased. Cation-bound pectin were more prone to ethanol precipitation because they were less solvated by water molecules. In the absence of cations, partially-ionized beet pectin actively interacted

with water molecules at the initial precipitation pH (I-pH) range of 3.26–2.83, which made it difficult to precipitate pectin by adding ethanol. However, in the presence of cations, the precipitation of sugar beet pectin was straightforward due to cation binding and ethanol dehydration. Protonated pectin and their salts were obtained. The authors found that no conformational transition occurred in cation-bound pectin after ethanol precipitation. This indicated the importance of the electrostatic nature of the caution-beet pulp interaction during the precipitation process.

The effect of pH on the ethanolic precipitation of sugar beet pectin was studied by [21]. Sugar beet pectin was precipitated from a purified extract in a 75% ethanol solution at an initial extract pH (I-pH) ranging from 2.0 to 4.5. The authors studied the effect of I-pH on pectin yield and pectin-cation interactions. The lowest pectin yield was obtained by precipitating the acidic pectin extract with ethanol at I-pH 4.5. Pectin yield increased to a maximum value as I-pH decreased from 4.5 to 3.0. The results indicated that decreased electrostatic repulsion between pectin chain segments enhanced pectin chain-chain interactions, thereby improving the precipitation effect. A decrease in pectin yield was observed as I-pH decreased below 3.0. The cation content of various samples was measured by high-performance cation-exchange chromatography in order to determine the content of pectin-bound cations during precipitation. The cation content and degree of cation binding were measured for pectin precipitated at I-pH 3.0, 2.5, and 2.0. The results revealed that a decrease in pectin yield accompanied a decrease in cation-pectin interactions. This suggested that the precipitation of beet pectin substances from an aqueous extract involved complex interactions between cations, solvent molecules, and pectin chain segments. The larger precipitation effect observed with divalent ions, compared to monovalent ions, might be due to enhanced inter-chain interactions between pectin molecules, probably via the formation of intermolecular bonds.

Most developments in this field offer ways to increase dietary fiber yield from raw materials. Fewer studies aim to develop technologies for improving its complexing capacity, which is becoming increasingly relevant today.

Important risk factors for many diseases include disturbed nutrition, lack of macro- and micronutrients, exposure to antibiotics, and a wide range of chemical and biological contaminants in food. Metabolic disorders are increasingly being treated with food products, including specialised foods containing soluble and insoluble dietary fibers [22].

In order to fight disease and promote health, we need to develop scientifically based approaches to obtaining functional ingredients with high detoxification and sorption activity.

This study aimed to develop a technology for obtaining combined dietary fibers using electrophysical methods and evaluate their detoxification properties.

STUDY OBJECTS AND METHODS

The study objects were samples of pectin substances and combined detoxicants obtained from beet pulp.

The modified technology for obtaining pectin substances and combined detoxicants from beet pulp included the following steps:

– soaking granulated sugar beet pulp in water at 30 ± 2°C;
– hydrolysis-extraction of the pulp in an aqueous solution of succinic acid at pH 2 and 80°C for 65 min in an extremely low-frequency (29 Hz) electromagnetic field (ELF EMF) to obtain pectin extract and pulp waste;
– reaching a 5% content of pectin substances and their coagulation with 96% ethanol for 10 min;
– double washing of the coagulum in 80% ethanol;
– infrared drying of the coagulum under vacuum at 0.06 MPa and 35°C to a pectin moisture content of max. 7%;
– powdering the pectin to a particle size of max. 200 μm and double washing of the pulp waste in water;
– infrared drying of the pulp under vacuum at 0.06 MPa and 35°C to a moisture content of max. 7% to obtain cellulose;
– powdering the cellulose to a particle size of max. 200 μm; and
– further mechanical mixing of the cellulose and pectin substances at a ratio of 1:0.05 to 1:0.5.

The following samples were obtained:

– combined detoxicant obtained by extracting with succinic acid in an ELF EMF;
– combined detoxicant obtained by extracting with succinic acid;
– pectin obtained by extracting with succinic acid in an ELF EMF (without cellulose in the powder); and
– pectin obtained by extracting with succinic acid (without cellulose).

The dry powders and their profiles were identified by IR-Fourier spectrometry on an Agilent Cary 660 spectrometer. The infrared spectra were interpreted using [23].

The microstructure of the samples was determined using an XSP 10-640x microscope with a MOV-1-16x ocular micrometre.

We determined the binding capacity of dietary fibers by the trilonometric method with some modifications. In particular, 30.0 cm^3 of a working 0.1 M lead acetate solution was poured into a 250 cm^3 conical flask, to which 25.0 cm^3 of the model solution was added. After being held in an AVU-6s shaker, the mixture was divided into fractions with a TsLM 1–12 centrifugal apparatus. The precipitate was washed in distilled water until it had a negative qualitative reaction to lead ions

with potassium bichromate. The centrifugate and the wash waters were combined and mixed with distilled water to reach the mark in a 250 cm^3 flask. An aliquot of 10 cm^3 of the solution was titrated with 0.01 mol/dm^3 ethylene diamine tetraacetate (EDTA) solution at pH 9–10 using Eriochrome Black T until the colour changed from violet to blue.

The binding capacity was calculated as a metal binding percentage using the following equation:

$$BC = \frac{A_1 - A_2}{A_1} \cdot 100 \quad (1)$$

where A_1 is the total metal mass, g; A_2 is the remaining metal mass in the solution, g. A_1 and A_2 in Eq. (1) were calculated using the results of the titrometric tests as:

$$A_1 = \frac{C_{edta} \cdot V_1 \cdot M}{1000} \quad (2)$$

$$A_2 = \frac{C \cdot 250 \cdot V_{avg} \cdot M}{1000 \cdot V_2} \quad (3)$$

where C is the concentration of the initial metal solution, mol/dm^3; V_1 is the initial volume of metal solution, cm^3; V_2 is the aliquot centrifugate solution taken for titration, cm^3; V_{avg} is the average volume of EDTA taken for titration, cm^3; M is the molecular mass of metal, Da.

RESULTS AND DISCUSSION

Figures 1–4 show the results of identifying the profiles of the combined detoxicants and pectin substances obtained from beet pulp. The absorption bands of carbonyls in carboxyl groups were analyzed in the IR spectra of the samples.

As shown in Figs. 1–4, the absorption intensity varied in the spectral range of 800–4000 cm^{-1} depending on the type of dietary fiber and the method of its preparation. The bands in the range of 100–1200 cm^{-1} did not show such a dependence. The absorption bands for the valence vibrations of carboxyl group carbonyls were in the range of 1695–1710 cm^{-1}; those for ester groups, in the range of 1720–1740 cm^{-1}. Beet pectin substances had a large content of hydrolysed ester compounds, while combined detoxicants had a prevalent content of carboxyl group carbonyls, which presumably indicated their increased ability to form complexes.

We know that the complexing properties of pectin substances depend on the content of free carboxyl groups, i.e. the degree of esterification of carboxyl groups with methanol or ethanol [24, 25]. The degree of esterification determines the linear charge density of the macromolecule and, consequently, the strength

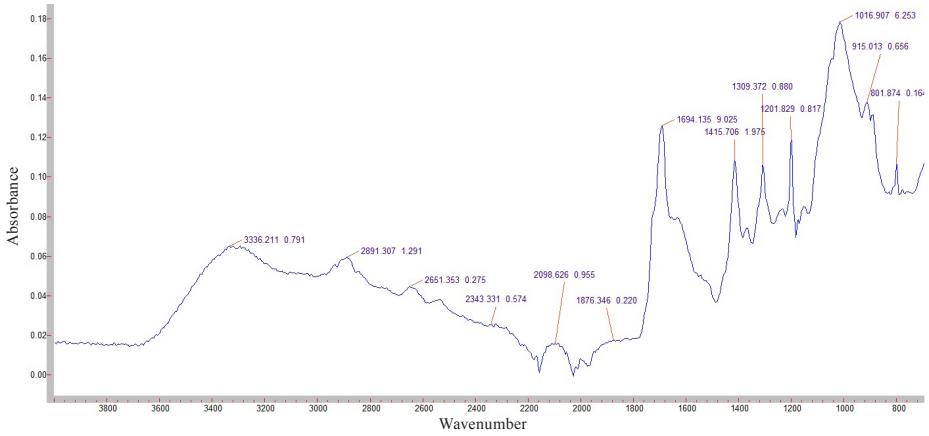


Figure 1 IR spectrum of a combined detoxicant obtained by extracting with succinic acid in an ELF EMF

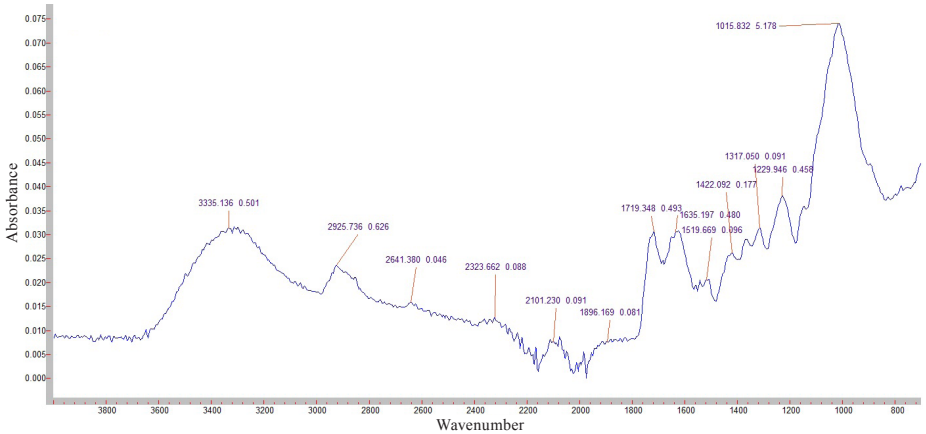


Figure 2 IR spectrum of a combined detoxicant obtained by extracting with succinic acid

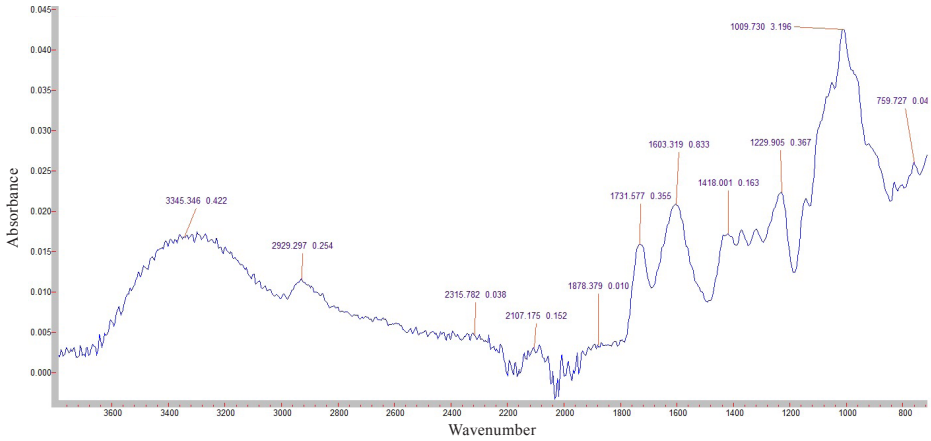


Figure 3 IR spectrum of pectin obtained by extracting with succinic acid in an ELF EMF

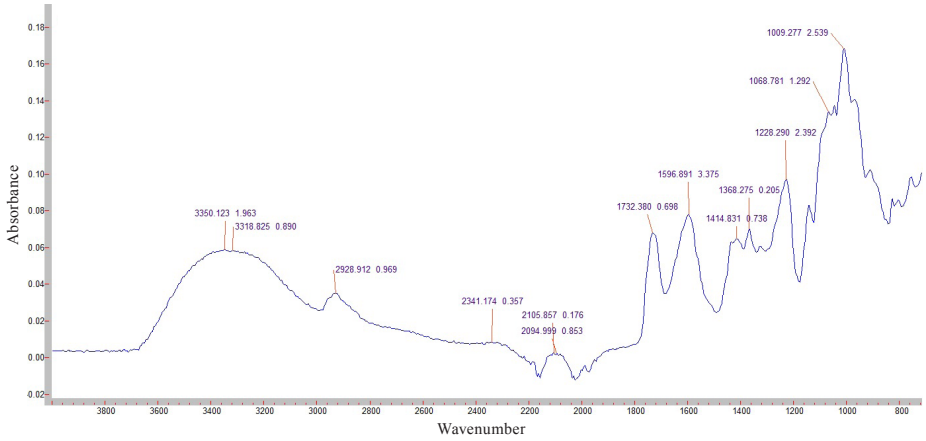


Figure 4 IR spectrum of pectin obtained by extracting with succinic acid

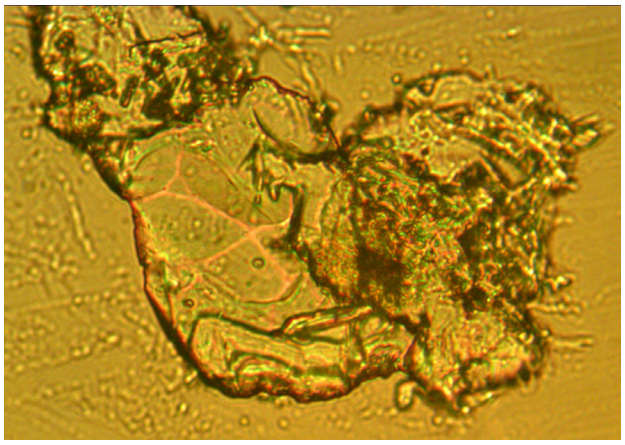


Figure 5 Microstructure of a combined detoxicant from beet pulp (400× magnification)

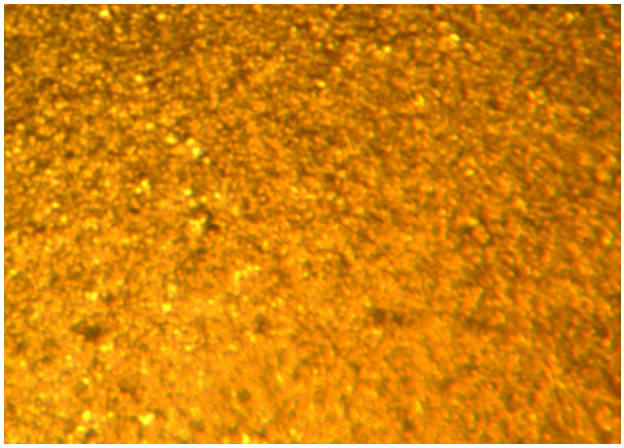


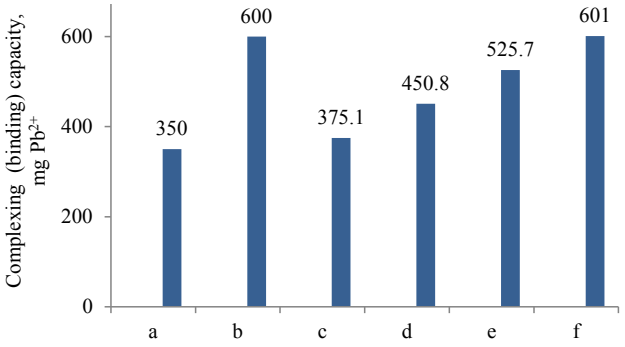
Figure 6 Microstructure of a pectin solution (100× magnification)

and method of cations bonding [26, 27][1]. Since the detoxification activity of pectin substances is inversely proportional to its degree of esterification, we assumed that the combined detoxicants obtained through ELF EMF treatment and extraction with organic acid would have a higher binding capacity than pectin substances without cellulose.

Figure 5 shows the microstructure of the combined detoxicant obtained from beet pulp using our technology.

As we can see, the combined detoxicant had a voluminous porous structure with wide and narrow fragments and twisted zones. Unlike it, pure

[1] State Standard 29186-91. Pectin. Specifications. Moscow: Standards Publishing; 2004. 14 p.



(a) beet pulp cellulose; (b) beet pulp pectin substances; (c) cellulose and pectin substances 1:0.05; (d) cellulose and pectin substances 1:0.2; (e) cellulose and pectin substances 1:0.35; (f) cellulose and pectin substances 1:0.5

Figure 7 The complexing (binding) capacity of a combined detoxicant with respect to lead ions (Pb^{2+}) depending on its qualitative composition

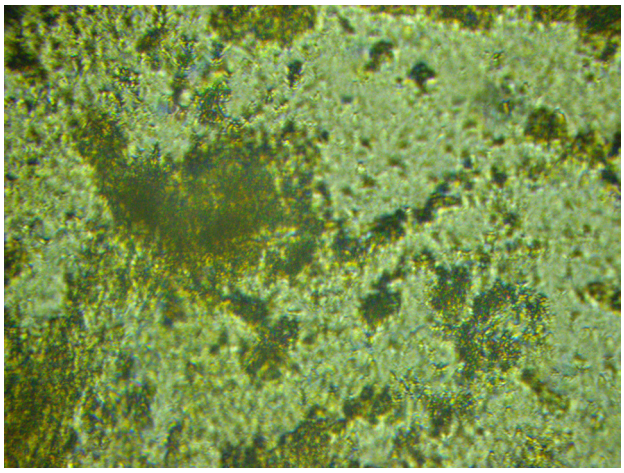


Figure 8 Microstructure of the dispersion system during interaction between the combined detoxicant and lead acetate solution

pectin (Fig. 6) and cellulose solutions had a monotonic structure and a weakly pronounced character.

Then we studied the binding capacity of the structure-forming agents with respect to lead ions. We found that cellulose produced from beet pulp using our technology had a binding capacity of 350 mg Pb^{2+}/g; and pectin substances, 600 mg Pb^{2+}/g.

The high complexing capacity of cellulose may be due to the maceration of plant tissue under the influence of an electromagnetic field, high temperature and pH, which makes it more accessible to metabolic processes. Another factor could be the presence of residual amounts of pectin substances.

Figure 7 shows the complexing capacity of a combined detoxicant with respect to lead ions (Pb^{2+}) depending on its qualitative and quantitative composition.

We found that the ratio of cellulose and pectin substances from 1:0.05 to 1:0.5 provided the combined detoxicant with a good complexing capacity. The sample with a ratio of 1:0.5 showed the highest binding capacity (601 mg Pb^{2+}/g), clearly indicating a synergetic effect when using a combined detoxifier (an over 20% increase in binding capacity).

The increase in binding capacity could be explained by the following factors. The presence of pectin in the combined solution let to a slight decrease in the pH of the medium. As a result, it destroyed cellulose micelles and released individual macromolecules, facilitating access to free hydroxyl groups involved in complexation.

The microstructure of the dispersion system during the interaction between the combined detoxicant and lead acetate solution is shown in Fig. 8.

We assumed that the dark areas of various sizes could indicate the presence of agglomerates resulting from the complexation of lead and active groups of pectin substances and cellulose contained in the combined detoxicant.

Table 1 Process parameters for producing combined detoxicants from beet pulp

Process stage and parameter	Value
Soaking granulated beet pulp:	
temperature, °C	30 ± 2
Hydrolysis-extraction of the pulp:	
pH of succinic acid, units	2
temperature, °C	80
duration, min	65
EMF frequency, Hz	29
Concentrating the pulp:	
content of pectin substances, %	5
Coagulating pectin substances with 96% ethanol:	
duration, min	10
Washing the coagulum in 80% ethanol:	
frequency	2
Infrared drying of the coagulum:	
pressure, MPa	0.06
temperature, °C	35
moisture, max., %	7
Powdering the pectin:	
particle size, μm	200
Washing the pulp waste:	
frequency	2
Infrared drying of the pulp:	
pressure, MPa	0.06
temperature, °C	35
moisture, max., %	7
Powdering the cellulose:	
particle size, μm	200
Mechanical mixing of pectin and cellulose:	
ratio	from 1:0.05 to 1:0.5

Based on the experimental results, we developed a technology for producing combined detoxicants from beet pulp. The process parameters are presented in Table 1.

CONCLUSION

As a result of the studies, we developed a technology for obtaining combined dietary fibers from beet pulp using the electrophysical method and evaluated their detoxification properties.

Identifying the profiles of the combined detoxicant samples and pectin substances obtained from beet pulp revealed that the combined detoxicants had an increased number of active groups reactive to effective complexation. A comparative evaluation of the microstructures of the combined detoxicants and pure samples of pectin and cellulose obtained from beet pulp suggested that the combined detoxicant provided a greater possibility for the chemisorption of metal ions.

The experiments established that 1:0.5 was the optimum ratio of cellulose and pectin substances in the combined detoxicant to ensure a high complexing capacity.

CONTRIBUTION

The authors were equally involved in the writing of the manuscript and are equally responsible for any potential plagiarism.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Hydrolysis products from sockeye (*Oncorhynchus nerka* L.) heads from the Kamchatka Peninsula produced by different methods: biological value

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Abstract:
Introduction. Sockeye salmon (*Oncorhynchus nerka* L.) is a valuable Pacific salmon. Sockeye heads are a significant share in processing sockeye salmon. Traditionally, fish by-products are used to make fishmeal. However, due to the high content of collagen proteins and fat in sockeye salmon heads, it is difficult to produce fishmeal from this raw material. Controlled enzymatic or combined hydrolysis allows protein, fat, and minerals to be extracted to supply the market with higher value products with desirable features. This research was aimed to analyze the chemical composition and biological value of hydrolysis products obtained from sockeye heads.
Study objects and methods. We investigated hydrolysis products of sockeye salmon heads, namely protein hydrolysates, fat and sludge. Thermal hydrolysis and enzymatic-thermal hydrolysis were used for the tests. Thermal hydrolysis was realized in reactor. For enzymatic-thermal hydrolysis, the raw material was pre-treated by proteolytic enzyme Alcalase. The hydrolysates obtained were investigated. Chemical composition was determined in accordance with State Standard 7636-85. HPLC was used for molecular weight and amino acid analysis. Gas chromatography was used for fatty acid analysis. Biological value of proteins was determined by the balance of the amino acid composition comparing it with the "ideal protein model".
Results and discussion. Thermal hydrolysis resulted in the production of protein hydrolysate powder with protein content of 92.0% dry matter and a protein recovery rate of 39.6%. Combined hydrolysis resulted in the production of protein hydrolysate powder with protein content of 92.6% and a protein recovery rate of 83%. All protein hydrolysates contained all essential amino acids. The biological value of protein hydrolysate obtained by thermal and combined hydrolysis was 80.1 and 82.8%, respectively.
Conclusion. Hydrolysed products obtained by thermal and enzymatic-thermal hydrolysis had a valuable chemical composition and could be recommended for food and feed use.

Keywords: Fish, by-products, sockeye, hydrolysis, amino-acid profile, peptides, protein

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INTRODUCTION

The highest actual salmon harvest – 676 thousand tons – was recorded last in 2018 [1]. In recent years, the average annual salmon harvest has been 280 thousand tons [2]. The total harvest of sockeye salmon of the Kamchatka Peninsula in 2018 was 41.1 thousand tons [3].

The average total annual sockeye salmon harvest worldwide is 140–180 thousand tons [4]. Sockeye salmon (*Oncorhynchus nerka* L.) is a valuable Pacific salmon. It is about 60 cm long with an average weight of 2 to 4 kg. In the Russian Far East, when processing sockeye salmon, by-products (heads, spines, etc.) account

for about 25% of the total weight, which is about 10 thousand tons a year [5]. By-products have a valuable chemical composition, but only about 20–30% of the total amount is used to produce fishmeal.

One of the hardest parts to process is the sockeye salmon head, which, on average, accounts for 14.8–17.9% of the total fish weight and 67–69% of the total waste amount. Sockeye salmon heads contain a high amount of collagen proteins (26–38% of the total mass of proteins), fat (15–18% of the total weight), and mineral substances (3.8–5.1% of the total weight) [6]. When cooking such raw materials, a colloidal emulsion mass with increased viscosity forms, which is hard to separate and dry. Therefore, final products do not meet the standard requirements for fishmeal, including in terms of physical characteristics, water and fat contents, while fat is rapidly oxidized, which leads to intoxication and product damage [7]. These semi-finished products cannot be used as food.

To use the valuable biological potential of sockeye salmon heads, hydrolysis methods were proposed that make it possible to separate fractions from organic raw materials – proteins and peptides, fats and fat-soluble compounds, mineral compounds with water insoluble protein components [8]. Mild hydrolysis makes it possible to preserve the valuable chemical composition of each fraction, and hydrolysis products can be recommended even for food purposes. Chemical hydrolysis (acidic or alkaline) damages certain essential amino acids, so waste water purification is necessary because of the high concentration of chemical components [9].

This research proposed and investigated two different hydrolysis, thermal and enzymatic-thermal. These two methods allow for deep hydrolysis of the complex protein-lipid-mineral system of sockeye salmon heads with maximum preservation of valuable amino acids and unsaturated fatty acids. Thermal hydrolysis is based on using high temperatures and pressure in an aqueous medium. Enzymatic-thermal hydrolysis includes pre-treatment of raw material with proteolytic enzymes followed by thermal hydrolysis [10]. Depending on the hydrolysis method and its parameters, it is possible to produce protein products with a different amino acid composition and molecular weight, fatty products with different fractional lipid composition, as well as protein-mineral or mineral-protein by-products from sludge with different biological values. The content of these biologically active substances predetermines the uses of the final products of hydrolysis.

STUDY OBJECTS AND METHODS

Frozen sockeye (*Oncorhynchus nerka* L.) heads were provided by LSC Ozernovskiy fish cannery factory № 55 (the Kamchatka region, Russia) in September 2016. In plastic boxes, they were delivered by plane within 24 h to the laboratory and then stored in a freezer at –18°C for one week until tests. Sockeye was caught in the southwestern part of the Kamchatka Peninsula, in one of the largest spawning grounds of sockeye salmon in the world [11].

Chemical composition of sockeye heads was determined in accordance with State Standard 7636-85[1]. The mass fraction of fat was determined in previously dried samples by extraction with diethyl ether according to the Soxhlet method. Nitrogen content was determined by the Kjeldahl method using a UDK 127 analyzer (VELP Scientifica, Italy) with pre-burning of the samples in sulfuric acid in the presence of hydrogen peroxide and mineral catalyst.

Thermal and enzymatic-thermal hydrolysis. Hydrolysis tests were carried out at the technology company ANiMOX (Adlershof, Berlin, Germany). Defrosted sockeye heads were minced and subjected to thermal and enzymatic-thermal hydrolysis. Hydrolyzed mixture was cooled down and separated by centrifugation into three fractions: protein, fat and sludge (protein-mineral or mineral-protein by-products). The aqueous fraction (water-soluble proteins) was freeze-dried to produce a protein hydrolysate. The sludge was subjected to convectional drying to obtain a protein-mineral or mineral-protein product.

Thermal hydrolysis (T-hydrolysis) of minced raw materials was realized in a reactor for 60 min at 130°C and pressure 0.20 MPa (pH 7.0). After hydrolysis, the organic mixture was separated using a centrifuge at 3500 rpm for 10 min.

During enzymatic-thermal hydrolysis (ET-hydrolysis), the raw materials were at first treated by proteolytic enzyme Alcalase 2.5 L (Novozymes) for 6 h at 50°C and 130 rpm (pH 8.0). Minced raw material was mixed with hot water at a 1:1 ratio. Subsequent fractionation was carried out by centrifugation with parameters described above for T-hydrolysis.

The chosen hydrolysis parameters showed good results with relatively fatty sardine cannery by-products in our previous investigations [10]. We applied T-hydrolysis to produce simultaneously basic protein-mineral fish meal and high value added fish protein hydrolysate with mostly medium molecular weight of proteins between 10 kDa and 100 kDa. ET-hydrolysis was used to produce high value added fish protein hydrolysate with mostly low molecular weight of proteins under 10 kDa.

Molecular weight distribution. The molecular weight profile of protein hydrolysates was estimated by the SEC method on a Merck/Hitachi HPLC system (detection UV 213 nm) with a Phenomenex Yarra SEC-2000 column (300×7.8 mm). Degassed 0.1 M potassium phosphate buffer with pH of 6.8 was used as a mobile phase. Ten microliters of the previously prepared material with 0.45-micron pore size filtered

[1] State Standard 7636-85. Fish, marine mammals, invertebrates and products of their processing. Methods of analysis. Moscow: Standartinform; 2010. 86 p.

Table 1 Chemical composition of sockeye heads

Material	Dry matter, %	Protein (% of dry matter)	Fat (% of dry matter)	Ash (% of dry matter)
Sockeye heads	34.1	12.5 (36.7)	16.9 (49.6)	4.47 (13.1)

sample at a concentration of 0.2–0.3% dry matter was used for each measurement. Calibration was carried out in triplicate using the BIO-RAD Gel Filtration Standard 151-1901. The mean retention time and molecular weight were plotted on a half-logarithmic scale. The linear correlation was used to interpolate the molecular weight ranges under analysis. Accordingly, the sample chromatograms were integrated piecewise between the retention time estimated.

Amino-acid profile. The amino acid profile of proteins was determined at the UBF laboratory (Altlandsberg, Germany) using HPLC after hydrolysis of proteins in 6 N boiling hydrochloric acid for 48 h. O-phthalaldehyde was used for derivatization. HPLC detection was performed using Agilent 1200 Series G1379A for UV-detection, G1312A for fluorescence detection, and G1329A for diode-array detection.

Fatty acid composition. The fatty acid composition of sockeye head hydrolysis products was analyzed at the UBF laboratory (Altlandsberg, Germany) by gas chromatography. Transesterification was carried out by DGF standard procedure using TMSH (trimethylsulfonium hydroxide) in tert-butyl-methylether. Quantification was done by reference standard mixtures (Supelco, Merck). An analytical system was GC 2010 from Shimadzu (Kyoto, Japan) equipped with flame ionization detection and computer system. The gas chromatography values were quantified by response corrected total area principal. Phase separation was performed using SP2380 (Supelco), 0.2 µm film thickness, 0.5 mm diameter, 25 m. Detector: 250°C (temperature program starting at 75°C with 5 K/min to 125°C, followed by 2 K/min to 225°C). Equilibration time was 5 min.

Table 2 Chemical composition of raw materials and fractions obtained after thermal hydrolysis of sockeye heads

Sample	Dry matter, kg/100 kg	Protein, kg/100 kg	Fat, kg/100 kg	Ash, kg/100 kg	Share of fraction, %
Sockeye heads and water	17.0	6.27 (37.0)	8.46 (49.9)	2.24 (13.2)	100
Protein fraction	3.89	3.58 (92.0)	0.03 (0.69)	0.29 (7.34)	69.5
Sludge (protein-mineral fraction)	29.9	16.3 (54.7)	4.74 (15.9)	8.80 (29.4)	23.2
Fat fraction	100	nd	nd	nd	7.34

Values in brackets are used in relation to the dry matter

nd = not detected

Table 3 Chemical composition of raw materials and fractions obtained after enzymatic-thermal hydrolysis of sockeye heads

Sample	Dry matter, kg/100 kg	Protein, kg/100 kg	Fat, kg/100 kg	Ash, kg/100 kg	Share of fraction, %
Sockeye heads and water	17.0	6.27 (37.0)	8.46 (49.9)	2.24 (13.2)	100
Protein fraction	6.71	6.21 (92.5)	0.07 (1.01)	0.43 (6.47)	83.8
Sludge (mineral-protein fraction)	44.7	12.3 (27.4)	10.9 (24.5)	21.5 (48.1)	8.70
Fat fraction	100	nd	nd	nd	7.45

Values in brackets are used in relation to the dry matter, kg/100 kg

nd = not detected

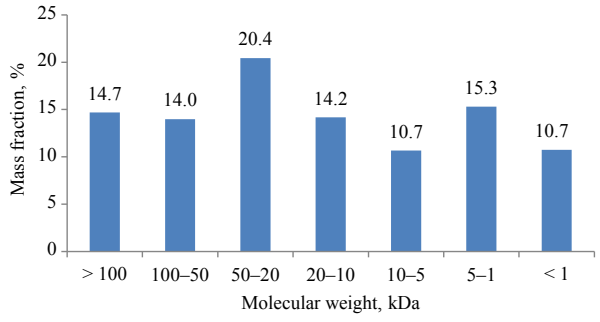

Figure 1 Protein distribution based on molecular weight of peptides and their share in protein hydrolysates from sockeye heads after thermal hydrolysis

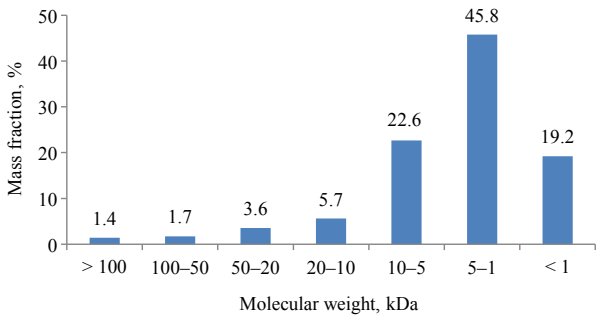

Figure 2 Protein distribution based on molecular weight of peptides and their share in protein hydrolysates from sockeye heads after thermo-enzymatic hydrolysis

Biological value. The biological value of proteins was determined by the balance of the amino acid composition by comparing it with the "ideal protein model" [12]. Amino-acid score was calculated according to the methodology of FAO/WHO using Eg. (1). The excessive amount of essential amino acids was calculated according to the formula of the coefficient of amino acid score difference (CASD) (Eg. (2)). Based on CASD biological value of proteins was calculated (Eq. (3)).

$$AS_i = \frac{AA_i}{AA_{i\,st}} \cdot 100 \quad (1)$$

where AS_i is the amino-acid score of the i-th essential amino acid, %; AA_i is the content of the i-th essential amino acid in 100 g of the analyzed protein, g; and is the content of the same essential amino acid in 100 g of the standard ("ideal") protein, g.

$$CASD = \frac{\sum \Delta DAS}{n} \quad (2)$$

where CASD is the coefficient of amino-acid score difference, %; is the difference of the amino-acid score of the i-th essential amino acid, %; AS_{min} is the minimal score for an essential amino acid in the analyzed protein, %; and *n* is the number of essential amino acids in the analyzed protein.

$$BV = 100 - CASD \quad (3)$$

where BV is biological value of the analyzed protein, %.

RESULTS AND DISCUSSION

Table 1 represents the chemical composition of sockeye heads (*Oncorhynchus nerka* L.).

Sockeye heads are a valuable raw material with great amounts of useful components.

Chemical composition of protein hydrolysates from sockeye heads after thermal hydrolysis. After thermal hydrolysis and separation of the organic suspension by centrifugation, three fractions were

Table 4 Amino-acid profile of lyophilized protein hydrolysates from sockeye heads produced by different hydrolysis methods

Indicators	T-hydrolysis		ET-hydrolysis		"Ideal" protein by FAO/WHO, g/100 g
	Content, g/100 g	Amino-acid score, %	Content, g/100 g	Amino-acid score, %	
Non-essential amino acids					
Alanine	6.68		5.51		
Arginine	7.01		4.79		
Asparagine	0		0.01		
Aspartic acid	5.08		5.27		
Citrullin	0.02		0.03		
Cystine	0.11		0.22		
Glutamine	0.02		0.02		
Glutamic acid	10.97		8.16		
Glycine	17.38		9.74		
Histidine	1.48	98.7	1.54	102.7	
Hydroxyproline	4.74		2.31		
Ornithine	0.10		0.11		
Proline	6.21		4.88		
Serine	3.35		3.98		
Taurine	2.40		1.20		
Tyrosine	0.85		1.49		
Essential amino acids					
Isoleucine	2.06	49.1	2.72	64.8	4.2
Leucine	2.84	59.2	4.49	93.5	4.8
Lysine	4.38	104.3	4.82	114.8	4.2
Methionine	2.24	77.2	2.06	71.0	2.9
Phenylalanine	2.44	87.1	2.21	78.9	2.8
Threonine	2.63	93.9	2.49	88.9	2.8
Tryptophan	n/a	n/a	n/a	n/a	n/a
Valine	no data	no data	2.79	66.4	4.2
Biological value of protein, %		80.13		82.79	
Protein content, g/100 g	90.2		90.7		
Dry matter, %	98.0		98.0		
Protein recovery rate, %	39.6		83.0		

Tryptophan is destroyed by the analysis method used therefore it was not taken into account in the calculation of amino-acid scores
n/a = not available

Table 5 Fractional composition of fats in protein hydrolysates from sockeye heads after thermal and enzymatic-thermal hydrolysis

Fractions of fatty acids	Fatty acid content, %	
	T-hydrolysis	ET-hydrolysis
Saturated fatty acids (SFAs)	29.3	32.7
Monounsaturated fatty acids (MUFAs)	39.2	43.7
Polyunsaturated fatty acids (PUFAs)	30.6	22.8
Trans fats	1.6	< 0.1
PUFAs:MUFAs:SFAs ratio	1:1.28:0.96	1:1.91:1.43
Mass fraction of fat,% of total mass of protein hydrolysate	0.68	0.99

Table 6 Fractional composition of fatty acids in fat from sockeye heads after thermal and enzymatic-thermal hydrolysis

Fractions of fatty acids	Fatty acid content, %	
	T-hydrolysis	ET-hydrolysis
Saturated fatty acids (SFAs)	22.3	23.2
Monounsaturated fatty acids (MUFAs)	54.8	42.9
Polyunsaturated fatty acids (PUFAs)	22.2	33.1
Trans fats	0.2	0.2
PUFAs:MUFAs:SFAs ratio	1:2.46:1	1:1.29:0.7
Mass fraction of fat,% of total mass of protein hydrolysate	86.8	88.1

produced, namely protein, fat, and sludge. Their chemical composition is presented in Table 2.

After thermal hydrolysis the protein fraction contained 92% of protein of dry matter. The fat was dry matter accounted for 100%. Rates of protein and fat extraction were 39.6 and 86.8%, respectively.

Molecular weight distribution of protein hydrolysates from sockeye heads after thermal hydrolysis. Figure 1 represents the distribution of proteins based on their molecular weight in protein hydrolysates after T-hydrolysis. The share of proteins with molecular weight over 20 kDa was 49.1%, while the share of proteins with molecular weight under 10 kDa (easily digestible peptides) was 36.7%. This shows that the major share of proteins after T-hydrolysis had a medium molecular weight.

Chemical composition of protein hydrolysates from sockeye heads after thermo-enzymatic hydrolysis. The chemical composition of fractions obtained by centrifugation of organic suspension after ET-hydrolysis is shown in Table 3.

After ET-hydrolysis, the protein fraction contained 92.5% of protein based on dry matter. The dry matter of fat accounted for 100%. Protein extraction rate was 83.0%, and the rate of fat extraction was 88.1%. ET-hydrolysis demonstrated a significantly increase in the extraction degree of biologically active protein and fat components from sockeye heads and a decrease in by-products compared to T-hydrolysis.

Molecular weight distribution in protein hydrolysates from sockeye heads after thermo-enzymatic hydrolysis. Figure 2 shows the distribution of proteins based on their molecular weight in protein hydrolysates after ET-hydrolysis. The share of proteins with molecular weight under 10 kDa (easily digestible peptides) was 87.6%. This implies that ET-hydrolysis allows producing protein hydrolysates with low molecular weight. This protein hydrolysate can be used as a highly digestible protein source for nutritional purposes.

Amino-acid profile of protein hydrolysates from sockeye heads. Table 4 shows amino acid profiles of freeze-dried protein hydrolysates from sockeye heads produced by different hydrolysis methods, as well as their biological value calculated based on amino acid balance using amino-acid score of essential amino acids and CASD (coefficient of amino acid score difference).

Table 7 Fatty acid composition of fat in sockeye heads after thermal- and enzymatic-thermal hydrolysis

Fatty acids	Fatty acid content, %	
	T-hydrolysis	ET-hydrolysis
Myristic	4.54	4.60
Pentadecylic	0.47	0.48
Palmitic	14.76	15.19
Palmitoleic	6.12	6.13
Margaric	0.73	0.77
Margaroleic	0.35	0.35
Stearic	2.09	2.28
Oleic	18.65	18.88
Vaccenic	3.85	3.85
Linoelaidic	0.23	0.23
Linoleic	1.88	1.94
Gamma-linolenic	0.93	0.92
Arachidic	0.18	0.18
Alpha-linolenic	3.80	4.30
Gondoic	12.52	12.26
Eicosadienoic	0.38	0.38
Eicosatrienoic	1.25	12.41
Behenic	0.24	0.32
Erucic	12.26	0.37
Docosadienoic	7.79	7.30
Arachidonic	0.14	0.00
Eicosapentaenoic	0.67	0.65
Lignoceric	0.00	0.17
Nervonic	1.08	1.11
Docosahexaenoic	5.11	4.96
TOTAL	100.00	100.00
Total SFAs	22.27	23.21
Total MUFAs	54.83	42.94
Total PUFAs	22.17	33.08
Total trans fatty acids	0.23	0.23
Total omega-3 fatty acids	10.82	22.32

According to Table 4, amino acid profiles of protein hydrolysates produced by T-hydrolysis and ET-hydrolysis were very similar. Hydrolysates contained all the essential amino acids and could be called high value proteins. The main amino acids were glutamic acid (8.16–10.97% of protein), glycine (9.74–17.38%), and proline (4.88–6.21%). They are characteristic for collagen proteins [13]. The assessment of the balance of proteins by amino-acid score and biological value in relation to the "ideal" protein showed that the biological value of protein hydrolysates after T-hydrolysis was 80.13%, and that of protein hydrolysates after ET-hydrolysis was 82.79%.

In comparison with the market product Amizate made in Norway out of salmon raw materials the protein hydrolysate have similar amino acid profile, especially after ET-hydrloysis [14]. This shows a high utilization potential of the Russian salmon fish by-products.

Fatty acid composition of fats in protein hydrolysates from sockeye heads. Table 5 describes the composition of fats (present in small amounts 0.68–0.99% of mass) in protein hydrolysates after T- and ET-hydrolysis. T-hydrolysis separated fat a bit better than ET-hydrolysis (0.68% vs. 0.99%). The proportion of polyunsaturated fatty acids (PUFAs) after T-hydrolysisis was larger than that after ET-hydrolysis (30.6% vs 22.8% of fat). The advantage of ET-hydrolysis is the minimum content of trans fat in the fat fraction (less than 0.1%).

Fatty acid composition of fat from sockeye heads after hydrolysis. Fractional analysis of fat from sockeye heads after T- and ET-hydrolysis (Table 6) showed that the yield after ET-hydrolysis was higher than after T-hydrolysis, and a PUFAs:MUFAs:SFAs ratio was more preferable after ET-hydrolysis. According to recommendations of FAO/WHO experts, this ratio is approximately equal to 0.6–1:1:1 [15].

Table 8 Amino-acid profiles of sediments after hydrolysis of sockeye heads after thermal and enzymatic-thermal hydrolysis

Indicators	T-hydrolysis		ET-hydrolysis		"Ideal" protein by FAO/WHO, g/100 g
	Content, g/100 g	Amino-acid score, %	Content, g/100 g	Amino-acid score, %	
Non-essential amino acids					
Alanine	3.56		2.34		
Arginine	3.97		1.95		
Asparagine	4.11		2.22		
Aspartic acid	0		0		
Citrullin	0.01		0		
Cystine	0.38		0		
Glutamine	0.01		0		
Glutamic acid	6.99		3.19		
Glycine	6.41		3		
Histidine	1.49	99.3	0.67	44.5	1.5
Hydroxyproline	1.16		0.77		
Ornithine	0.05		0.06		
Proline	2.26		1.65		
Serine	2.79		0.56		
Taurine	2.15		0.33		
Tyrosine	0		0.52		
Essential amino acids					
Isoleucine	2.69	64.0	1.48	35.2	4.2
Leucine	2.38	49.6	1.90	39.6	4.8
Lysine	4.30	102.4	1.94	46.2	4.2
Methionine	1.83	63.1	0.57	19.7	2.9
Phenylalanine	2.79	99.6	1.32	47.1	2.8
Threonine	0.55	19.6	1.08	38.6	2.8
Tryptophan	n.a.		n.a.		n/a
Valine	1.65	39.3	1.76	41.9	4.2
Biological value of protein, %		66.51		41.24	
Protein content, g/100 g	53.6		26.9		
Dry matter, %	98		98		

Tryptophan is destroyed by used analysis method therefore it was not taken into account in the calculation of amino-acid scores
n.a. = not available

Table 9 Fractional composition of fatty acids in by-products from sockeye heads after thermal and enzymatic-thermal hydrolysis

Fractions of fatty acids	Fatty acid content, % fat	
	T-hydrolysis	ET-hydrolysis
Saturated fatty acids (SFAs)	25.5	25.2
Monounsaturated fatty acids (MUFAs)	58.8	49.1
Polyunsaturated fatty acids (PUFAs)	15.0	24.9
Trans fats	0.1	< 0.1
PUFAs:MUFAs:SFAs ratio	1:3.92:1.7	1:1.97:1.01
Mass fraction of fat, % of total mass of protein hydrolysate	15.9	24.5

Sockeye fat samples after T- and ET-hydrolysis had a valuable fatty acid composition and could be recommended for food and feed use (Table 7). Both fat samples were rich in monounsaturated omega-9 oleic and gondoic fatty acids and most common saturated palmitic fatty acid. Fat after T-hydrolysis contained significantly more monounsaturated omega-9 erucic fatty acid. Fat after ET-hydrolysis contained a significantly higher amount of the rare polyunsaturated omega-3 eicosatrienoic essential fatty acid. Total amount of omega-3 fatty acids in fat after ET-hydrolysis was twice as large as that after T-hydrolysis (22.32% vs. 10.82%). These fatty acids have beneficial bioactivities including prevention of atherosclerosis, protection against maniac–depressive illness and various other medicinal properties [16].

Preliminary defatting of salmon by-products using relatively low temperature methods helps extracting fat raw materials [17]. This saves some enzyme costs and improve the quality of fat products as they are not processed using enzymes and high temperatures.

Amino-acid profile of the sludge from sockeye heads. As a result of hydrolysis, water-insoluble proteins and minerals formed a protein-mineral or mineral-protein sludge product. The yield of the protein fraction essentially depended on the type of hydrolysis [18]. T-hydrolysis produced much more protein than ET-hydrolysis (53.6% vs. 26.9%). Amino acid profiles of the sludge fractions were different (Table 8).

These by-products contained all the essential amino acids but their contents were lower than in protein hydrolysates including glutamic acid and glycine. The biological value of the protein-mineral product after T-hydrolysis was much higher than that one of mineral-protein product after ET-hydrolysis (66.51% vs. 41.24%). This can be explained by the fact that during ET-hydrolysis there is a deeper splitting of the protein chains and their transition into a soluble state is more intense therefore non-hydrolyzed proteins in the sludge contain less essential amino acids. The biological value of protein-mineral and mineral-protein products was much lower than that of protein hydrolysates.

Table 10 Fatty acid composition of fats in by-products from sockeye heads after thermal and enzymatic-thermal hydrolysis

Fatty acid	By-product T-hydrolysis, %	By-product ET-hydrolysis, %
Myristic	4.86	5.05
Pentadecylic	0.52	0.52
Palmitic	17.11	16.76
Palmitoleic	6.58	7.08
Margaric	0.79	0.78
Margaroleic	0.67	0.69
Stearic	2.58	2.37
Oleic	20.59	22.20
Vaccenic	3.98	4.18
Linoelaidic	0.15	0.00
Linoleic	1.69	1.42
Gamma-linolenic	0.65	0.50
Arachidic	0.18	0.17
Alpha-linolenic	4.53	5.39
Gondoic	13.00	13.75
Eicosadienoic	0.34	0.30
Eicosatrienoic	1.41	13.71
Behenic	0.23	0.31
Erucic	12.64	0.00
Docosadienoic	0.13	0.14
Arachidonic	3.54	1.84
Eicosapentaenoic	0.29	0.62
Lignoceric	0.00	0.00
Nervonic	1.32	1.22
Docosapentaenoic	0.00	0.00
Docosahexaenoic	2.23	1.02
TOTAL	100.00	100.00
Total SFAs	25.47	25.17
Total MUFAs	58.78	49.12
Total PUFAs	14.95	24.93
Total trans fatty acids	0.15	0.00
Total omega-3 fatty acids	8.46	20.73

Fatty acid composition of sludge from sockeye heads. Table 9 represents fractional composition of fatty acids in by-products after T- and ET-hydrolysis. These products contained a higher amount of fat after ET-hydrolysis (29.4%) compared to that after T-hydrolysis (15.9%).

By-products from sockeye heads obtained after T- and ET-hydrolysis were rich in monounsaturated omega-9 oleic and gondoic fatty acids and most common saturated palmitic fatty acid (Table 10). By-product after T-hydrolysis contained a significant amount of monounsaturated omega-9 erucic fatty acid. By-product after ET-hydrolysis contained no erucic fatty acid nut contained significantly higher amount of polyunsaturated omega-3 eicosatrienoic essential fatty acid. Total amount of omega-3 fatty acids in the by-

product after ET-hydrolysis is more than double of that after T-hydrolysis (20.73% vs 8.46%).

The data showed a difference in the content of polyunsaturated fatty acids and omega-3 fatty acids in fat and by-product fractions. In the by-products, this content was lower, especially of docosahexaenoic acid. This means that high-temperature treatment, sedimentation, and contact with mineral substances of the sedimentary part led to the fat quality deterioration but demonstrated pretty good fatty acid composition.

CONCLUSION

Processing sockeye (*Oncorhynchus nerka* L.) salmon heads through thermal and enzymatic-thermal hydrolysis resulted in the production of three products with valuable chemical composition, namely protein hydrolysates, fish oil, and sludge. Highly concentrated protein hydrolysates were low molecular weight peptides (90.2–90.7% dry matter) with all essential amino acids in a well-balanced state. Fish oil contained a large amount of valuable poly- and monounsaturated fatty amino acids (extraction ratio of 86.8–88.1%) and omega-3 fatty acids (10.82 and 22.32%, depending on the type of hydrolysis). Sludge was a protein-mineral product containing water-insoluble proteins (53.6–26.9% dry matter), fat (15.6–24.0%) with valuable poly- and monounsaturated fatty amino acids (15.0–24.5%), a high amount of omega-3 fatty acids (8.46 and 20.73%, depending on the type of hydrolysis), as well as minerals, mostly calcium and phosphorus (29.4–48.1%).

Thermal hydrolysis, which is simpler and faster, allowed 39.6% of protein to be extracted from raw materials in the form of protein hydrolysate with a protein content of 92%. The protein fraction with a molecular weight of more than 20 kDa accounted for 49.1%, and less than 10 kDa, 36.7%. The fat extraction rate of lipids into fish oil was 86.8%. In the protein-mineral by-product, protein content was 53.6%, mineral content was 29.4% and fat content was 15.9%, with protein recovery rate of 60.6% and mineral recovery rate of 91.1%. Thermal hydrolysis resulted in a more balanced amino acid and fatty acid composition compared to enzymatic-thermal hydrolysis.

Enzymatic-thermal hydrolysis is more labor-intensive but made it possible to extract 83% of protein from raw materials in the form of protein hydrolysate with a protein content of 92.6%, which in terms of the amino acid and fatty acid balance exceeded similar products after thermal-hydrolysis. The fraction of proteins with a molecular weight of more than 20 kDa accounted 6.7% and with that of less than 10 kDa, 87.6%. The fat extraction rate was higher than during thermal hydrolysis, with 88.1% of lipids. The resulting protein-mineral by-product had a reduced biological value in terms of amino-acid and fatty acid composition compared to the by-product obtained by thermal hydrolysis.

The high nutritional value of all three products after thermal and combined hydrolysis allows them to be used as food and feed additives – sources of valuable amino acids, fatty acids and minerals. These additives should be used for nutritional purposes separately or as components of food products (bakery and confectionery products). For feed purposes, hydrolysis products are recommended to be introduced into the composition of fish, farm animals and poultry feed.

Additionally, there is a possibility of using unrefined small underutilized fishes with lower fat content through hydrolysis processing in novel food technologies to obtain protein products with high biological value [19]. Further research should be done in this field as well. Furthermore, combination of fish hydrolysates with plant origin protein and anti-oxidant component may improve functional and economic characteristics of new products with high biological value [20, 21].

CONTRIBUTION

The authors were equally involved in the writing of the manuscript and are equally responsible for any potential plagiarism.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Textural properties of low fat mayonnaise with whey protein concentrate and Tragacanth gum as egg and fat substitutes

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Abstract:
Introduction. Mayonnaise is a kind of oil-in-water emulsion that usually contains 70–80% of oil. However, modern food science keeps providing new knowledge about high-fat products, which makes it possible to solve the problems related to health concerns.
Study objects and methods. The research featured high-fat mayonnaise (20% of oil) with reduced oil stabilizer (1.75%) and without egg stabilizer. In experimental samples, egg stabilizer was replaced with 0.3, 0.4, and 0.5% of whey protein concentrate and 0.3, 0.5, and 1.0% of Tragacanth gum. Mayonnaise with 3.5% oil stabilizer and 0.3% egg stabilizer was used as control sample. The samples were tested for such textural attributes as firmness, consistency, adhesive force, and adhesiveness.
Results and discussion. The highest and the lowest textural values were demonstrated by the sample with 0.4% of whey protein concentrate and 0.5% of Tragacanth gum and the sample with 0.5% of whey protein concentrate and 1.0% of Tragacanth, respectively. The former showed textural characteristics similar to those of the control sample. The presence of hydrocolloids proved to affect the texture properties of mayonnaise, whereas Tragacanth gum reduced its elasticity. It formed a strong and complex gel-like structure in the continuous phase. As a result, oil droplets in the emulsion had a smaller diameter, which improved the texture properties of low-fat mayonnaise.
Conclusion. Whet protein concentrate and Tragacanth gum in amounts of 0.5% and 1.0%, respectively, can be used to replace egg stabilizer and reduce oil stabilizer in low-fat mayonnaise.

Keywords: Mayonnaise, textural attributes, whey protein, Tragacanth gum, protein concentrate

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INTRODUCTION

According to the Iranian food standards developed by the Iranian Institute of Standards and Industrial Research, mayonnaise is a kind of seasoning produced by emulsifying edible vegetable oils in a liquid phase containing vinegar. This oil-in-water emulsion is then stabilized by the emulsifier compounds in egg yolk. Fats are necessary for human body as an important source of energy for growth. They provide both essential fatty acids and fat-soluble vitamins. Consumers' concern with the fact that high-fat foods may cause cardiovascular diseases, hypertension, and obesity has encouraged the food industry to develop healthier low-fat food products.

The consumption of oils and fats has increased significantly in recent decades. One of the reasons for this increase is that oil is widely used in many food products to obtain the appropriate textural properties. Not only is high oil content bad for health but it is also unprofitable for food producers. The general policy of global food industry is to produce fat-free or low-fat foods with a flavor similar to that of a natural product. Therefore, the use of proper fat substitutes in low-fat foods is inevitable. Fat substitutes are extremely diverse and include substitutes based on proteins, fats, and carbohydrates. Carbohydrate-based fat substitutes are a group of compounds derived from cereals, legumes, and herbs with digestible or non-digestible carbohydrates, including Tragacanth gum [2, 6]. Dried Tragacanth gum is obtained from a species of *Astragalus* genus that contains both water-soluble (tragacanthin) and water-insoluble (bassorin) fractions.

The US Food and Drug Administration classifies Tragacanth gum as a healthy food additive, which can be used as a stabilizer, emulsifier, thickener, and fat substitute in the food industry [7, 15]. There has been a lot of research on the production of low-fat mayonnaise

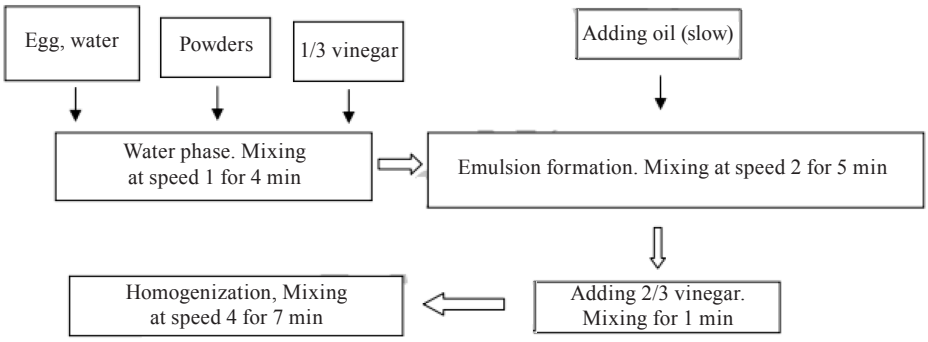
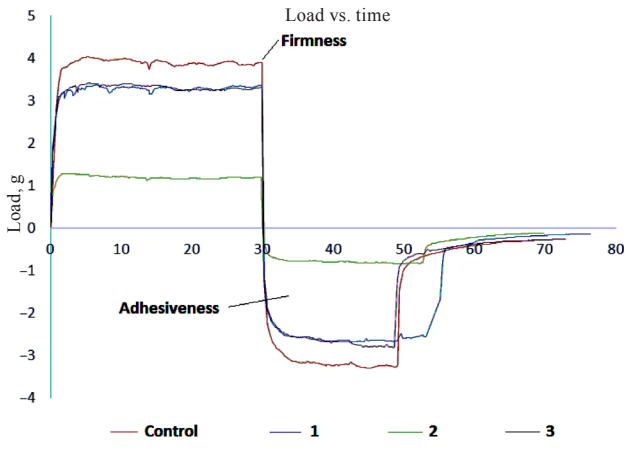


Figure 1 Production process of mayonnaise samples

using a variety of ingredients. Some studies featured the possibility of using pectin to reduce oil content [8, 5]. Pectin proved to be able to reduce the level of oil in mayonnaise from 80 to 40%, while the finished product possessed textural and sensory properties comparable to the high-fat control sample. Other studies [5, 17] tested beta-glucan for low-fat mayonnaise production. The finished product appeared more stable than low-fat mayonnaise. Replacing up to 50% of the initial oil content (from 82 to 41%) had no effect on the sensory properties of mayonnaise. 4α-GTASE-modified starch with xanthan gum also appeared effective in reducing oil content [10, 13]. The resulting product with 37.5% of oil, 5.6% of starch, and 0.1% of xanthan was similar in its appearance and rheological properties to the high-fat samples. Combined with citrus fiber and guar, xanthan was able to reduce oil from 73% to 36.5% [2, 16]. The mayonnaise sample with 1% of citrus fiber, 0.5% of guar, and 1.5% of xanthan gum had almost the same yield stress, viscosity, and flow behavior index as the control sample. In [5, 6, 14], scientists studied barley dextrin, maltodextrin with xanthan gum, and pre-gelatinized wheat starch in low-fat mayonnaise production, respectively.



control – 3.5% oil stabilizer + 0.3% egg stabilizer; 1 – 1.75% oil stabilizer + 0.3% whey protein concentrate + 0.3% Tragacanth gum; 2 – 1.75% oil stabilizer + 0.4% whey protein concentrate + 0.5% Tragacanth gum; 3 – 1.75% oil stabilizer + 0.5% whey protein concentrate + 1.0% Tragacanth gum.

Figure 2 TPA curves of mayonnaise samples obtained from the textural analysis device

Given the adverse effects of oil on human health, low concentrations of hydrocolloids, such as starches and gums, seem quite promising for mayonnaise production. As they have a remarkable effect on the properties of food systems, they can be used to modify food properties and formulate new ones. In addition, they often possess economic benefits [7, 9]. Most sauces contain gum. By increasing the continuous phase concentration, these polysaccharides decrease the adhesiveness and compress the fat droplets. They decrease the collision and attachment of these droplets to each other by reducing release and movement of dispersed emulsion droplets, which stabilizes the emulsion [9, 17].

This study aimed to produce mayonnaise with whey protein concentrate as a substitute for eggs and Tragacanth gum as a substitute for fat. We also examined the textural attributes of the product obtained.

STUDY OBJECTS AND METHODS

The mayonnaise formulation included sunflower oil, sugar, salt, oil stabilizer[I], egg stabilizer[II], citric acid, vinegar, mustard powder, sodium benzoate, potassium sorbate, drinking water, Tragacanth gum, and whey protein concentrate.

Preparation of mayonnaise. According to the Iranian standards, a mayonnaise with 50% oil is a low-fat mayonnaise. The research featured three mayonnaise samples. A control sample contained 3.5% of oil stabilizer and 0.3% of egg stabilizer. The three experimental samples included a reduced oil stabilizer (1.75%), no egg stabilizer, whey protein concentrate (0.3, 0.4, and 0.5%), and Tragacanth gum (0.3, 0.5, and 1.0%). The mayonnaises were made according to the formulation in Fig. 1.

Textural properties examination. Textural analysis device (QTS 25 Faranel CNS, UK) was used to examine

[I] Modified Starch Hydroxypropyl distarch phosphate (E1442)
[II] Modified Starch Sodium Actinyl Succinate (E1450)

Table 1 Raw materials used to produce different samples of mayonnaise, %

Samples	Control	Sample No. 1	Sample No. 2	Sample No. 3
Sunflower oil	20	20	20	20
Egg stabilizer	–	–	–	0.3
Oil stabilizer	1.75	1.75	1.75	1.75
Tragacanth gum	–	0.3	0.5	1.0
Whey protein concentrate	–	0.3	0.4	0.5
Vinegar	5.2	5.2	5.2	5.2
Salt	1.5	1.5	1.5	1.5
Sugar	5	5	5	5
Citric acid	0.13	0.13	0.13	0.13
Mustard	0.3	0.3	0.3	0.3
Sodium benzoate	0.07	0.07	0.07	0.07
Potassium sorbate	0.07	0.07	0.07	0.07

the mayonnaise samples for such textural properties as firmness, consistency, adhesive load, and adhesiveness. The measurement was done by back-estrogen method using a measuring vessel with a height of 58 mm and 50 mm in diameter, as well as a probe with a diameter of 45 mm. The penetration depth of the probe into the sample was 40 mm. The probe and other parameters were selected according to the manufacturer's instructions. The textural properties, such as firmness, cohesiveness, and adhesiveness, were plotted by the device in the form of load–time curves (Fig. 2) [8]. As a result, the textural properties were defined as follows:

Firmness: Maximum load during first compression rotation.

Cohesiveness: area ratio of level 2 to level 1.

Adhesiveness: the area of negative load resulting from the first plunge and indicating the work required to pull the probe out of the sample.

Statistical analysis. The experiments were conducted in a completely randomized design with three replications. The mean comparison of treatments was performed by Duncan test at 95% confidence level. SPSS 16.0 and Excel 2007 were used for data analysis and drawing charts, respectively. For this purpose, appropriate equations were plotted by the abovementioned software to show the relationship of each of the dependent variables in the regression model with the independent variables. The R^2 values were determined to evaluate the accuracy.

RESULTS AND DISCUSSION

Compounds known as hydrocolloids with a high ability to absorb water and to develop texture are widely used in the production of low-fat products. This research featured the physical performance of whey protein concentrate and Tragacanth gum in the texture formation. Both whey protein concentrate and Tragacanth gum proved to possess water-absorbing capacity and improved textural properties.

Both compounds revealed a good potential as egg and fat substitutes to obtain desirable texture. They provided the same texture that high-fat mayonnaise owes to large quantities of oil. In addition to its fat-replacement role, Tragacanth appeared capable of enhancing the consistency of low-fat mayonnaise samples and reducing their caloric value. Since Tragacanth gum can bond with water in the continuous phase, it improved the stability of the emulsion. Therefore, it can be used as a fat substitute in the low-fat mayonnaise formulation. In spite of the fact that the use of Tragacanth gum in mayonnaise production requires further research, the present study was an effective step towards self-sufficiency and localization of this product.

Textural properties. Table 2 shows the textural properties of the mayonnaise samples under study. Firmness is one of the most important factors affecting mayonnaise as it is believed to affect customers' attitude. The control sample (with egg stabilizer) and the sample with 0.4% whey protein concentrate and 0.5% Tragacanth gum demonstrated the highest and the lowest firmness, respectively. Similarly, they had the highest and the lowest consistency, adhesiveness, and firmness, respectively. Regression coefficients of firmness, adhesiveness, and textural cohesiveness showed that the experimental samples fit the model well: the coefficient of determination was 99.96, 99.78, and 98.72, respectively.

The firmness percentage increased when egg stabilizer was replaced with 0.3% whey protein concentrate and 0.3% Tragacanth gum (Table 2). 0.4% whey protein concentrate and 0.5% Tragacanth gum decreased firmness, while 0.5% whey protein concentrate and 1.0% Tragacanth gum increased it again. The results of this study were consistent with [17].

Table 2 Effect of whey protein concentrate and Tragacanth gum on textural properties of mayonnaise

Samples	Adhesive force, N	Adhesiveness, $N \cdot s^{-1}$	Consistency, $N \cdot s^{-1}$	Firmness, N
Control (no WPC and TG)	70.92	3.37	117.30	4.14
0.3% WPC + 0.3% TG	65.32	2.73	98.93	3.45
0.4% WPC + 0.5% TG	22.10	0.85	36.22	1.32
0.5% WPC + 1.0% TG	61.63	2.88	100.10	3.50

WPC – whey protein concentrate

TG – Tragacanth gum

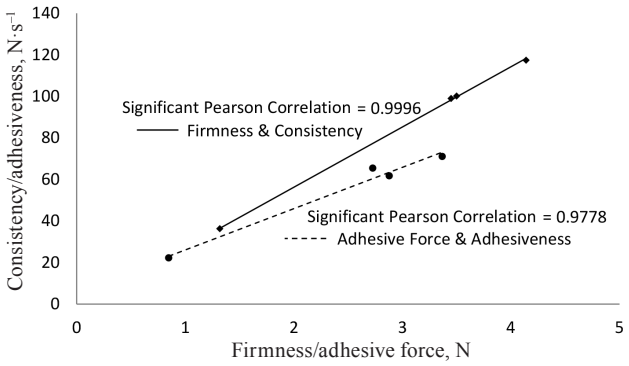


Figure 3 Firmness vs. consistency and adhesive force vs. adhesiveness correlation

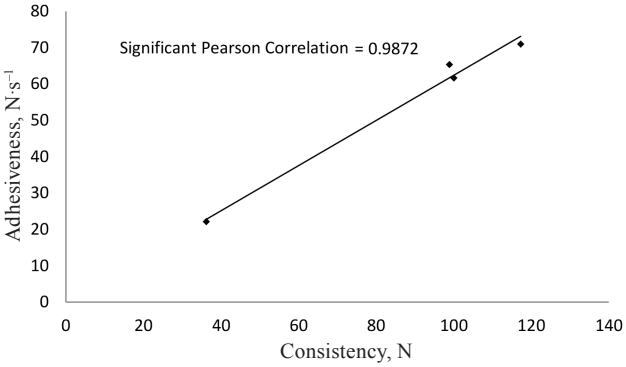


Figure 5. Correlation between adhesive force and consistency

In other studies, 50% and 75% beta-glucan increased the firmness of low-fat mayonnaise [16]. The highest consistency was observed in the control sample (177.30 $N \cdot s^{-1}$) and sample with 0.5% whey protein concentrate and 1.0% Tragacanth gum (100.10 $N \cdot s^{-1}$). The lowest consistency was observed in the sample with 0.4% whey protein concentrate and 0.5% Tragacanth gum (36.22 $N \cdot s^{-1}$). The highest (3.37 $N \cdot s^{-1}$) and the lowest (0.85 $N \cdot s^{-1}$) adhesiveness was observed in the control sample and the sample with 0.4% whey protein concentrate and 0.5% Tragacanth gum, respectively.

The presence of hydrocolloids proved to played a significant part in developing the texture properties of mayonnaise. Accordingly, the maximum load recorded in histometry test, known as the sample tolerance for flow initiation, was obtained for the control sample and then the sample with 0.5% whey protein concentrate and 1.0% Tragacanth gum. The sample with 0.4% whey protein concentrate and 0.5% Tragacanth gum showed the lowest value of this parameter. Based on the obtained results, the sample with the highest levels of whey protein concantrate (0.5%) and Tragacanth gum (1.0%) demonstrated the closest texture behavior to the control sample containing egg stabilizer (Figs. 3–5). Other studies had similar results: gum reduced the elasticity due to the formation of a strong gel-like structure in the continuous phase [7, 9, 11]. The gum made the structure firmer and more complex while affecting the formation of oil droplets with smaller diameter in the emulsion, thus improving the texture properties of low-fat mayonnaise.

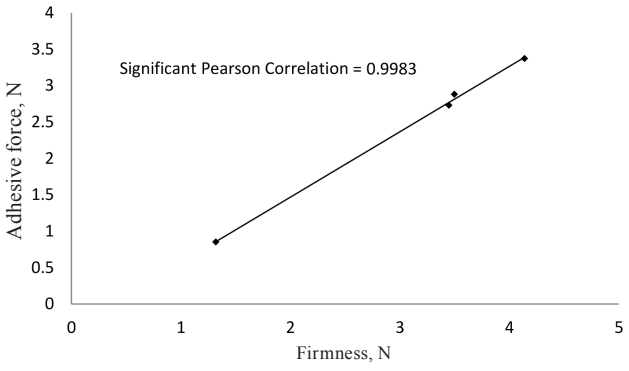


Figure 4 Correlation between adhesive force and firmness

CONCLUSION

Given the importance of such food emulsions as mayonnaise, the qualitative properties of this high-fat seasoning require careful scientific attention. Texture is one of the factors that affect the quality of this product. Gum is one of the most important compounds used to achieve a proper texture. Considering the effect this irreplaceable component has on the quality properties, localization of this product is impossible without introducing the native Tragacanth gum into the formulation and studying its functionality.

The research objective was to measure the effect of Tragacanth gum as a partial substitute for fat on the textural properties of low-fat mayonnaise. All the samples proved acceptable in terms of pH and acidity according to Iranian National Standard. However, different levels of whey protein concentrate and Tragacanth gum changed the texture properties of mayonnaise. The synergistic effect of whey protein concentrate and Tragacanth gum at amounts of 0.5% and 1.0 %, respectively, improved the product's texture.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for any possible plagiarism.

CONFLICT OF INTEREST

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

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Antagonistic effects of raffia sap with probiotics against pathogenic microorganisms

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Abstract:
Introduction. Probiotics are known for their beneficial properties. Numerous studies have been conducted to find advantages that probiotics can provide. This study aimed to evaluate the functional properties of raffia sap, a Cameroonian drink, fermented with probiotics by investigating its antagonistic activity against pathogenic bacteria.
Study objects and methods. The study objective was raffia sap fermented by *Lactobacillus fermentum* and *Bifidobacterium bifidum*. Box-Behnken design with four factors (seeding rates of *L. fermentum* and *B. bifidum*, temperature, and incubation time) was used to generate mathematical models. The disc diffusion method was used to evaluate an antagonistic effect of the probiotics against four pathogenic bacteria (*Escherichia coli*, *Listeria monocytogenes*, *Salmonella* sp., and *Bacillus cereus*). An optimization of mathematical models of the inhibition diameters allowed to determine the optimal conditions of antagonistic effect.
Results and discussion. The experimental data showed that zones of inhibition were 0–21 mm for *Salmonella* sp., 0–23 mm for *E. coli*, 0–20 mm for *L. monocytogenes*, and 0–22 mm for *B. cereus*. ANOVA results and the mathematical models obtained showed that *L. fermentum* was effective against *B. cereus* and *B. bifidum* against *Salmonella* sp., *E. coli*, and *B. cereus*. The optimization of the models revealed maximum zones of inhibition at the seeding rates of *L. fermentum* and *B. bifidum* of 2 and 10%, respectively, incubation time of 48 h, and temperature of 37°C.
Conclusion. Raffia sap fermented by *L. fermentum* and *B. bifidum* demonstrated antagonistic effect against pathogenic bacteria such as *E. coli*, *L. monocytogenes*, *Salmonella* sp., and *B. cereus*.

Keywords: Probiotics, antagonistic activity, pathogenic bacteria, response surface methodology, mathematical model

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INTRODUCTION

Probiotics are defined as microorganisms that, when ingested in sufficient quantity, effect beneficially the host [1, 2]. The beneficial effects resulting from the consumption of foods enriched with probiotics have been known for millennia [3]. At the beginning of the 20th century, Mechnikov, a winner of the Nobel Prize, suggested replacing the dangerous germs by useful bacteria [4]. Additionally, *Bifidobacterium* spp. was recommended against infantile diarrhea [3, 5]. Despite the scientists' research, the idea of eating certain bacteria to improve the health of the digestive system was ignored. Taking into account the different technical issues related to the production of foods with probiotics, attention must be focused on their beneficial effects on health [6].

The latest studies in this area have shown that probiotic bacteria are able to stimulate the immune system and inhibit the adhesion and multiplication of pathogenic bacteria [7, 8]. Since pathogenic microorganisms are becoming resistant to antibiotics, probiotics are a new alternative to be studied in the search for new molecules and/or antibacterial organisms [9].

Such an antimicrobial or antibacterial effect is generally called an antagonistic effect. Factors responsible for the antagonistic effect of one microorganism against another one are: production

of organic acids or hydrogen peroxide that lower pH, competitive exclusion, immune system modulation, stimulation of defence systems, as well as production of antimicrobials such as bacteriocins and antioxidants [10]. Lactic, acetic, benzoic and other organic acids are the antimicrobial substances generally produced by beneficial microorganisms. The most produced bacteriocins are plantaricin, enterolysin, lacticin, lactocin, reuterin, pisciolin, enterocin, and pediocin [11].

Many probiotics have a broad spectrum of action and can be effective against diseases caused by food contaminated with certain pathogenic strains such as *Listeria monocytogenes*, *Escherichia coli*, *Bacillus cereus* and *Salmonella*. These four bacteria are the most common pathogens causing food-borne diseases [12]. Generally, there are difficulties in selecting an appropriate strain, substrate, as well as in determining optimal conditions for probiotic effectiveness.

In this context, researchers use local Cameroonian raw materials, including raffia sap. Raffia sap is a widespread drink in sub-Saharan Africa and particularly in Cameroon. Raffia sap undergoes wild fermentation and produces raffia wine that is difficult to keep. In 10 h after the harvest, alcohol produced during the primary fermentation transforms into acid, which seriously compromises the organoleptic characteristics appreciated by consumers.

In our previous research, we developed a probiotic beverage with raffia sap fermented by *Lactobacillus fermentum* and *Bifidobacterium bifidum* [13]. In the current research we studied an antagonistic potential of raffia sap inoculated by probiotics. The study was aimed to use Response Surface Methodology (RSM) to evaluate and optimize the effectiveness of *L. fermentum* and *B. bifidum* against *E. coli*, *L. monocytogenes*, *Salmonella* sp., and *B. cereus*.

STUDY OBJECTS AND METHODS

Raffia sap harvesting. The fresh sap of less than eight hours was harvested in a 25 L container and transferred to the laboratory. The sap then was immediately dispensed into 1 L bottles and sterilized in a water bath at 65°C for 30 min. The bottles were cooled and stored at 4°C.

Bacteria and probiotics. Pathogenic bacteria (*Escherichia coli*, *Listeria monocytogenes*, *Salmonella* sp. and *Bacillus cereus*) were provided by the Food Microbiology Laboratory of Ngaoundere University. Probiotics (*Lactobacillus fermentum* and *Bifidobacterium bifidum*) were prepared using KwikStik™ lyophilized microorganism.

Revitalization and multiplication of probiotics. To revitalize and multiply probiotic cells contained in the freeze-dried products, 1 g of lyophilisate of each strain was rehydrated as recommended by the manufacturer. First, the powder was rehydrated in 10 mL of dilute saline solution (DS)consisting of 0.85% NaCl and 0.1% peptone in distilled water and stirred for 10 min until maximum recovery was reached. The solution was then transferred into 1 L of MRS broth previously prepared and sterilized. After incubation at 42°C for 48 h, MRS broth with probiotics was centrifuged at 6500 g and 4°C for 15 min.

The supernatant was removed, the pellet was washed in the saline solution without being resuspended and then recentrifuged as above. The supernatant was discarded and the pellet was finally resuspended in 10 mL of DS first and then transferred into 250 mL of DS. The concentration of probiotics in this solution was obtained by serial dilutions. The dilutions were spread on MRS petri dishes and incubated at 42°C for 24 h, then the colonies were counted [14].

Antagonistic effect of raffia sap fermented. To evaluate the antagonistic effect of the fermented raffia sap, we used the disc method described by Tadesse *et al*., with some modifications [5]. Mueller-Hinton agar was seeded with pathogenic bacteria (*L. monocytogenes*, *B. cereus*, *E. coli* and *Salmonella* sp.) and incubated at 37°C for 30 min. Sterile discs (5 mm) then were placed on the agar surface incubated at 37°C for 24 h. Each disk was impregnated with 100 µL of raffia sap fermented by probiotics according to the experimental design (Table 1). The inhibition of pathogenic bacteria resulted in the formation of clear zones around the discs. The zone of these inhibition zones was measured, which was used as the main response of the trial.

Experimental design for sap fermentation process and data analysis. Fermentation was done following a four factor Box-Behnken design. The factors were seeding rates of *L. fermentum* (X_1) and *B. bifidum* (X_2), temperature (X_3), and incubation time (X_4). The levels of each factor were chosen after prior testing (Table 1).

The Box-Benhken experimental matrix in coded variables (–1; 0; +1) was generated with the Minitab 18 software. This coded variable matrix consisted of 28 trials, four of which enabled a better evaluation of the experimental error; each trial was repeated three times. The experimental matrix applicable to the laboratory was obtained by transforming the matrix into the coded variables with the EXCEL software using the following formula:

Table 1 Range of variation and factor levels

Variable		Level of factor		
		–1	0	+1
Seeding rate of *Lactobacillus fermentum*, % (v/v)	X_1	0	5	10
Seeding rate of *Bifidobacterium bifidum*, % (v/v)	X_2	0	5	10
Temperature, °C	X_3	37.0	39.5	42.0
Incubation time, h	X_4	2	25	48

$$X_j = \frac{U_j - U_j0}{\Delta U_j} \quad (1)$$

where X_j is a value of the coded variable j; U_j is a value of the real variable j; U_j0 is a value of real variable j at the center, and ΔU_j is called a "step" of variation.

Modelling and optimization. The zones of inhibition zones obtained after the application of the various tests of the experimental matrix were analysed on Minitab 18. The obtained models were in the form of:

$$y = \beta_0 + \sum_{j=1}^{k} \beta_j x_j + \sum_{j=1}^{k} \beta_{jj} x_j^2 + \sum\sum_{i<j} \beta_{ij} x_i x_j \quad (2)$$

where y is the model of the inhibition zones of the strain concerned, $\beta_{(i,j)}$ are model coefficients and $x_{(i,j)}$ are the factors. The data was analysed at the level of 10%, including the maximum of significant factors on each model. Response Surface Methodology was used for the three-dimensional graphical representation of the models of each inhibition zone after setting temperature and incubation time at constant values. Sigmaplot 12 software was used to plot the curves. Optimization was done on Minitab with the specifications for maximizing the inhibition zones of each pathogen.

RESULTS AND DISCUSSION

The results of the measurements of the inhibition zones of pathogenic bacteria (*Salmonella* sp., *Escherichia coli*, *Listeria monocytogenes*, and *Bacillus cereus*) obtained after the implementation of the four factor Box-Behnken experimental matrix showed that the zones of inhibition ranged from 0 to 21 mm for *Salmonella* sp., 0 to 23 mm for *E. coli*, 0 to 20 mm for *L. monocytogenes*, and 0 to 22 mm for *B. cereus*.

Raffia sap without probiotics did not demonstrated an inhibitory activity against pathogenic bacteria (inhibition zone = 0). However, the study conducted by Ojo and Agboola displayed different results [15]. The authors evaluated the antagonistic activity of bacteria isolated from Palm wine (*Raphia vinifera* L.) towards *Salmonella typhi*. The study revealed that raffia sap, due to its own microbial flora, was antagonistic against several pathogenic bacteria, including *Salmonella* sp. This also could be explained by pasteurization of fresh sap to avoid any interaction between the natural microflora of the sap and added probiotics, as well as wild fermentation.

Thus, seeding rates of *Lactobacillus fermentum* and *Bifidobacterium bifidum* played an important role in the antagonistic effect of the drink against the pathogenic bacteria tested, but statistical analysis was performed for a better demonstration of these effects (Table 2).

Effect of factors on microbial inhibition. According the data in Table 2, *B. bifidum* did not show a strong antagonistic effect on *E. coli*, *L. monocytogenes*, and *Salmonella* sp., but it was effective against *B. cereus* ($P \leq 0.1$). *L. fermentum* had a significant antagonistic effect on *Salmonella* sp., *E. coli*, and *B. cereus* with probabilities of 0.060, 0.040 and 0.072, respectively. Moreover, the incubation time significantly increased all the zones of inhibition ($P = 0.000$).

Effect of incubation time on inhibition of pathogenic bacteria. The curves of inhibition zone of *E. coli*, *L. monocytogenes*, *B. cereus*, and *Salmonella* sp. as a function of time were obtained after fixing seeding rates of *B. bifidum* and *L. fermentum* at 0 in coded variables (5% in real variables) and the temperature at 0 in coded variable (39.5°C in real variable). Under these conditions, these curves (Fig. 1) showed that the inhibition zones of *E. coli* ranged from 8 mm (2 h of incubation) to 20 mm (48 h).

Table 2 ANOVA results and coefficients of mathematical model of inhibition zones for *Lactobacillus fermentum* and *Bifidobacterium bifidum* on *Escherichia coli*, *Listeria monocytogenes*, *Bacillus cereus,* and *Salmonella* sp.

Terms	*Escherichia coli*		*Listeria monocytogenes*		*Bacillus cereus*		*Salomonella sp.*	
	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*
Constant	13.750	0	15.750	0	14.250	0	15.000	0
X_1	0.917	0.233	0.333	0.722	1.500	0.058	1.167	0.173
X_2	1.667	0.041	1.083	0.259	1.417	0.072	1.667	0.060
X_3	–0.500	0.507	0.333	0.722	0	1.000	0.417	0.615
X_4	6.417	0	5.750	0	6.083	0	6.583	0
X_1+X_1	–1.290	0.235	–1.580	0.244	–1.750	0.110	–1.040	0.379
X_2+X_2	–1.170	0.281	–3.460	0.019	–0.630	0.551	–2.540	0.045
X_3+X_3	–0.170	0.875	0.170	0.900	0.750	0.476	1.080	0.361
X_4+X_4	0.710	0.506	–0.710	0.594	0.370	0.719	–0.920	0.438
X_1+X_2	–3.750	0.011	–2.750	0.107	–4.000	0.007	–2.750	0.072
X_1+X_3	–0.250	0.847	–1.000	0.540	0.250	0.845	–1.000	0.488
X_1+X_4	0.250	0.847	0.250	0.877	–0.250	0.845	–0.250	0.861
X_2+X_3	0	1.000	0.250	0.877	1.750	0.185	2.250	0.133
X_2+X_4	0.250	0.847	–1.250	0.446	–0.500	0.696	0.500	0.727
X_3+X_4	–0.750	0.565	–0.750	0.645	–1.000	0.438	–1.000	0.488

where X_1 and X_2 are seeding rates of *L. fermentum* and *B. bifidum*, respectively, X_3 is temperature, and X_4 is incubation time

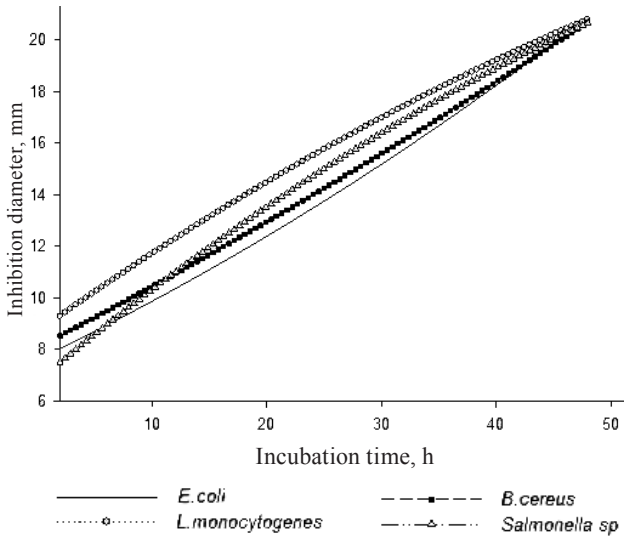

Figure 1 Effect of incubation time on inhibition of pathogenic bacteria

The inhibition zones of *B. cereus*, *L. monocytogenes*, and *Salomonella* sp. varied from 8.5, 9.0 and 7.3 mm in 2 h of incubation, respectively. In 48 h, the zones reached 20 mm in all the samples. The inhibition zones measured for each pathogenic strain as a function of the incubation time demonstrated that time is an essential factor to assess the antagonistic effect of probiotic drink based on raffia sap fermented with *L. fermentum* and *B. bifidum*. In fact, *B. bifidum* and *L. fermentum* need time to synthesize acids and other antimicrobial compounds contributing to antagonist effect against pathogenic bacteria [16, 17].

Individual effect of *B. bifidum* on *E. coli*, *B. cereus* and *Salmonella* sp. To obtain the inhibition curves of *E. coli*, *B. cereus*, and *Salmonella* sp. (Fig. 2) as a function of the seeding rate of *B. bifidum*, the seeding rate, incubation temperature, and incubation time of *L. fermentum* were set at 0 in coded variable – 5%, 39.5°C, and 25 h in real variables, respectively.

The inhibition curve of *Salmonella* sp. as a function of the seeding rate of *B. bifidum* showed that the maximum zone of inhibition of *Salmonella* sp. (15 mm) was obtained when the seeding rate of *B. bifidum* was 6%. The curve of the inhibition zone of *B. cereus* demonstrated that the inhibition zone depended directly on the seeding rate of *B. bifidum*. The inhibition zones of *B. cereus* ranged from 12.1 to 14.2 mm for the seeding rates of 0 and 10%. As for *E. coli*, its curve of the inhibition increased and then decreased, with a peak of 13.3 mm when the seeding rate of *B. bifidum* was 6.6 %.

According to Luquet and Corrieu, bifidobacteria promote better absorption of milk lactose in adults with intestinal lactase deficiency [18]. In our study, these probiotics (in particular *B. bifidum*) in raffia sap also played an important antagonistic role against *E. coli*, *B. cereus*, and *Salmonella* sp. In addition, some invitro studies showed that bifidobacteria and their metabolites stimulated IgA production, phagocytic activity, and growth [19]. These metabolites produced in raffia sap as well as the *B. bifidum* strain itself can therefore be a natural way to stimulate the immune system, to inhibit pathogenic strains such as *E. coli*, *B. cereus* and *Salmonella* sp., and to balance intestinal flora.

Individual effect of *L. fermentum* on *B. cereus*. Figure 3 shows the curve of the inhibition zone of *B. cereus* as a function of the seeding rate of *L. fermentum*. This curve increased then decreased, with the inhibition zone peak of 14.3 mm at the seeding rate of 6.5%. This curve was obtained by setting the seeding rate of *B. bifidum*, incubation temperature, and incubation time at 0 in coded variables – 5%, 39.5°C, and 25 h in real variables, respectively.

Thus, if it were necessary to optimize the antagonistic properties of our probiotic drink by referring only to an ability to inhibit the *B. cereus*

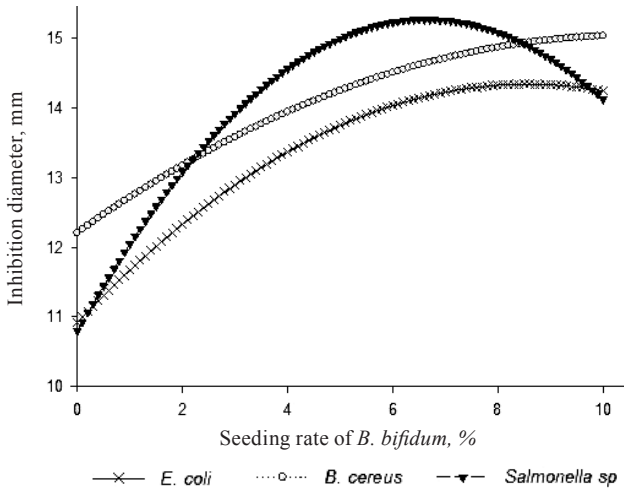

Figure 2 Individual effect of *Bifidobacterium bifidum* on *Escherichia coli*, *Bacillus cereus*, and *Salmonella* sp.

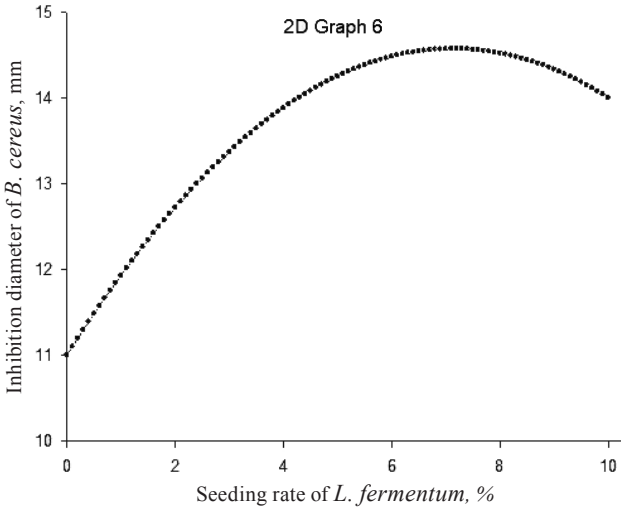

Figure 3 Individual effect of *Lactobacillus fermentum* on *Bacillus cereus*

strain, the seeding rates of *L. fermentum* and *B. bifidum* would be 5% and 5%, respectively, with an incubation temperature of 39.5°C and an incubation time of 25 h. Under these conditions, this probiotic drink could eventually be used as a means of combating infectious diseases which can be caused by *B. cereus*. *B. cereus* is a group of bacteria that can be pathogenic for humans. The infections they can cause are generally infrequent and not serious. However, ingestion of these bacteria, and their toxins in particular, can lead to infections characterized by vomiting or diarrhea [20].

In spite of the fact that our results were obtained *in vitro*, it is clear that *L. fermentum* introduced into raffia sap had a significant antagonistic effect on *B. cereus*. However, further research should be carried out *in vivo* to take into account factors that could affect the drink properties such as its passage through the intestinal tract, the survival of strains and the bioavailability of antibacterial compounds, as well as their direct or indirect effect on the body.

Effects produced by combination of *L. fermentum* and *B. bifidum* in raffia sap on the pathogens tested. The response surface methodology was applied to represent the mathematical models obtained by holding temperature and incubation time at 0 in coded variables –39.5°C and 25 h in real values, respectively.

Figure 4 presents the response surface of the mathematical model of inhibition zone of *L. fermentum* and *B. bifidum* against *Salmonella* sp. An increase in the seeding rate of *B. bifidum* and a simultaneous increase in the seeding rate of *L. fermentum* and *B. bifidum* considerably increased the antagonistic effect, with the inhibition zone of 16 mm.

However, only *B. bifidum* had a significant antagonistic effect on *Salmonella* sp. (P = 0.060, Table 2) at a 10% probability level. Indeed, lactic acid produced by *B. bifidum* lowers the pH by creating an unfavorable conditions for pathogenic microorganisms such as *Salmonella* sp. [21, 22]. Garcia *et al.* and Callaway *et al.* reported that bifidobacteria can prevent or reduce diseases caused by pathogens, protecting thus consumers' health [16, 23]. Based on our study results, raffia sap fermented by *B. bifidum* can be effective against salmonellosis due to *Salmonella* proliferation.

Figure 5 demonstrates the response surface of *L. fermentum* and *B. bifidum* against *E. coli*. As in the case with *Salmonella* sp., only *B. bifidum* showed a significant antagonistic effect on *E. coli* (P = 0.041, Table 2) at a 10% probability level. An increase in the seeding rate of *B. bifidum* considerably increased the antagonistic effect, with the maximum inhibition zone of 18 mm.

Indeed, lactic acid bacteria exert a strong antagonistic activity against several microorganisms, including those causing the deterioration of food and pathogenic microbes such as *E. coli* [4, 24]. In addition, the antimicrobial effect of some probiotic extends the shelf life of food [25]. This effect is mainly due to the production of organic acids (lactic acid) and also the production of antimicrobial compounds such as hydrogen peroxide, diacetyl, acetaldehyde, amino acid isomers and bacteriocins [19, 26].

It is important to remember that *E. coli* is a Gram-negative mammalian intestinal bacterium that makes up about 80% of the aerobic intestinal flora in humans [27, 28]. However, some strains of *E. coli* can be pathogenic, resulting in gastroenteritis, urinary tract infections, meningitis, or sepsis. Therefore, consumption of raffia

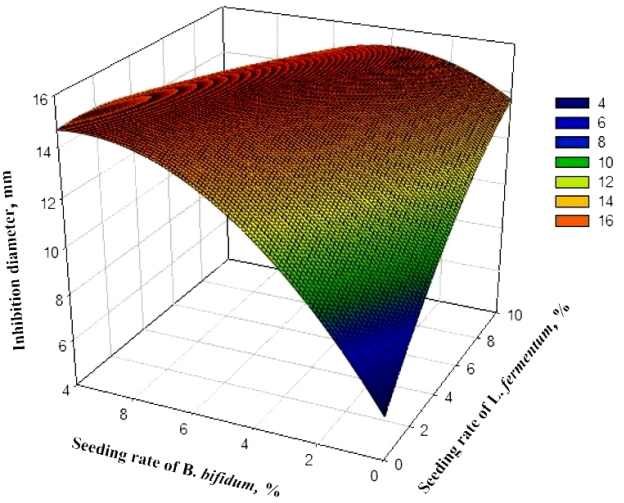

Figure 4 Response surface model of inhibition zone of *Lactobacillus fermentum* and *Bifidobacterium bifidum* against *Salmonella* sp. with seeding rates of *Lactobacillus fermentum* (X_1) and *Bifidobacterium bifidum* (X_2), incubation temperature (X_3), and incubation time (X_4) at temperature 0 (39.5°C) and time 0 (24 h)

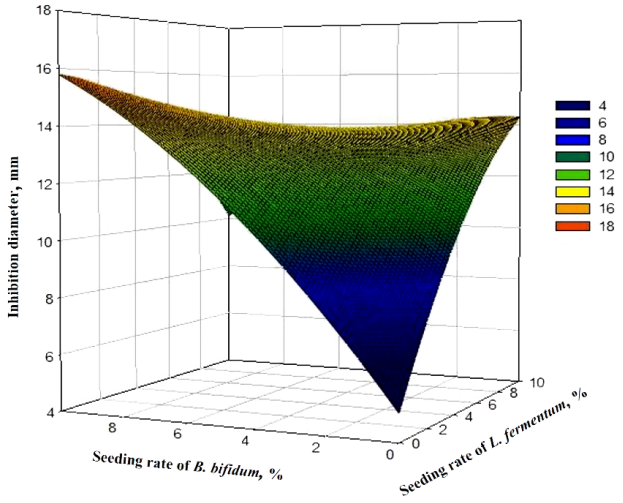

Figure 5 Response surface model of inhibition zone of *Lactobacillus fermentum* and *Bifidobacterium bifidum* against *Escherichia coli* with seeding rates of *Lactobacillus fermentum* (X_1) and *Bifidobacterium bifidum* (X_2), incubation temperature (X_3), and incubation time (X_4) at temperature 0 (39.5°C) and time 0 (24 h)

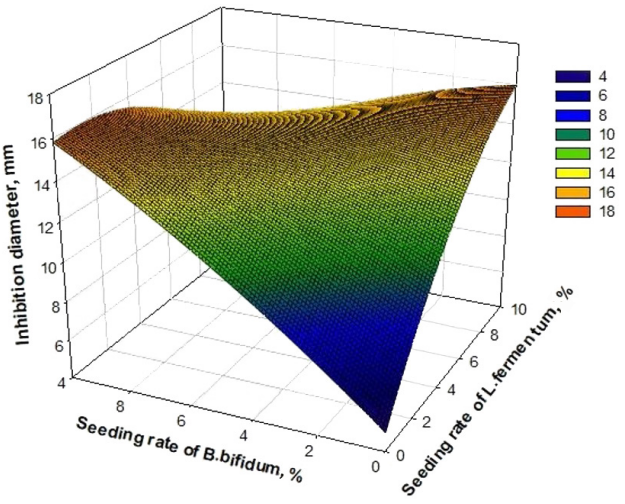

Figure 6 Response surface model of inhibition zone of *Lactobacillus fermentum* and *Bifidobacterium bifidum* against *Bacillus cereus* with seeding rates of *Lactobacillus fermentum* (X_1) and *Bifidobacterium bifidum* (X_2), incubation temperature (X_3), and incubation time (X_4) at temperature 0 (39.5°C) and time 0 (24 h)

sap fermented by *B. bifidum* can prevent and control the pathogenicity of *E. coli*.

Figure 6 presents the response surface of the mathematical model of inhibition zone of *L. fermentum* and *B. bifidum* against *B. cereus*. Both *L. fermentum* and *B. bifidum* individually had a significant antagonistic effect (P = 0.058 and 0.072, respectively, Table 2), whereas their combination was a highly effective (P = 0.007). *B. cereus* had similar sensitivities to both probiotics in raffia sap (Fig. 6). Inhibition zones reached 18 mm when the seeding rates of *L. fermentum* and *B. bifidum* were maximum. The acids and antimicrobial compounds secreted by *L. fermentum* and *B. bifidum* in raffia sap are thus a pathway to be exploited to treat diseases, although rare, due to consumption of *B. cereus*-infected foods. *B. cereus* is a well-known food-borne pathogen that is ubiquitously distributed in nature and is frequently responsible for food poisoning [20].

Effect of *L. fermentum* and *B. bifidum* on *L. monocytogenes* and optimization of the antagonistic effect. In the case of *L. monocytogenes*, response surface curves were not required because neither of the probiotic bacteria in raffia sap had a significant antagonistic effect (P = 0.722 for *L. fermentum* and P = 0.259 for *B. bifidum*, Table 2). This can be explained by the greater resistance of this bacterium to acidity [29]. Probably, the fermentation time should be increased to enhance the antagonistic properties of the raffia sap drink, but it would make the drink more acidic and hence undrinkable. It would be better to exploit this hypothesis in the context of the synthesis, isolation and production of biologically active compounds from raffia sap fermented by *L. fermentum* and *B. bifidum*.

In conclusion, the optimization of the antagonistic effect was done on the basis of specifications that aimed to maximize the inhibition zones. Thus, an optimal antagonistic effect would be given by seeding rates of *L. fermentum* and *B. bifidum* of 2 and 10%, respectively, incubation time of 48 h, and temperature of 37°C.

CONCLUSION

The results obtained in this study revealed that raffia sap fermented by probiotics (*Lactobacillus fermentum* and *Bifidobacterium bifidum*) had antibacterial properties against bacteria such as *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* sp., and *Bacillus cereus* which can sometimes be pathogenic. However, further studies should be carry out to determine the mechanism of action of this finding and to confirm its beneficial effect in animal models.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism. The idea and analysis belongs to S.C.Z. Desobgo. M.J.A. Mbarga and L.N. Tatsadjieu collected the data, performed the analysis and wrote the paper. L. Kalisa and N. Kavhiza translated and edited the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

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

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Electrochemical activation as a fat rendering technology

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Abstract:
Introduction. The existing methods of animal fat obtaining have certain disadvantages, hence fat extraction study highly is relevant. Electrochemically activated solutions are known to have a great potential for animal fat extraction. The present paper introduced a new advanced fat obtaining technology based on the principle of electrochemical activation.
Study objects and methods. The research featured ostrich fat obtained by wet rendering in water and in an electrochemically activated solution (catholyte) using various processing methods and technological parameters. Standard methods helped define the physical and chemical parameters of the obtained fat samples.
Results and discussion. The paper introduced a technological and hardware setup of an ostrich fat production line with the necessary equipment specifications. The research made it possible to define the optimal parameters for fat extraction: the salt concentration for the catholyte = 4 g/100 cm^3, voltage = 40–42 V, pH = 11, and redox potential of the catholyte = between –600 and –700 mV. During the fat processing, cell membranes in the electrolyte were destroyed, which inactivated the enzyme system. The obtained combination of physical and chemical factors resulted in ostrich fat of high quality. Fat extraction in an electrochemically activated solution (catholyte) catalyzed the process and increased the fat yield, regardless of the processing temperature. The fat yield exceeded 58% at 55°C and catholyte pH of 11.0. At 95–100°C and pH of 9.5–10.6, it exceeded 95%.
Conclusion. The new technology increased the fat yield, maintained its high quality, and reduced the processing cost. Therefore, the developed production line could be recommended for fat extraction of farm animals, depending on the intended use.

Keywords: Fatty acid, fats, raw material, electrolysis, catholyte, food production, water, solutions

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INTRODUCTION

Fat rendering technology should take into account the specific character of the technical solutions. Wet rendering produces a three-phase system, which includes fat, broth, and greaves. However, the process is slow and renders no fat at low processing temperatures. Dry rendering involves high temperatures and results in a two-phase system of greaves and fat. Chemical rendering is fast and highly productive, but acid or alkali treatment limits the intended use of the final product. Hydrolytic rendering destroys the protein component of fat with hot alkali. Unfortunately, the resulting fat is instable, and the greaves cannot be used for feed production. Hydromechanical and electric pulse methods are most effective for glue and gelatin production, as they destroy the connective tissue of the fat cell of tubular bones. Extraction methods based on organic solvents are extremely rare in the meat industry: not only are they fire-hazardous and labor-consuming, but they also require sophisticated equipment. It is fat rendering with electrochemically activated solutions that gives the most positive results [1]. The last decades have seen a growing scientific interest in the use of water as a potential source of unconventional chemical reactions [2–5].

Electrochemically activated solutions make various production processes less energy-, time-, and material-consuming [6]. Electrolysis of water has a number of advantages over mechanochemical, magnetic, and thermal activation methods [7]. Even at low

concentrations, electrochemically activated solutions demonstrate properties similar to concentrated acid and alkaline solutions [8, 9]. Domestic and foreign research confirmed their effectiveness in various fields and resulted in a number of patents [10–18]. Some studies featured advanced production methods for electrolyzers and activated water with improved properties [19–22]. An efficient electrocatalyst possesses a number of certain properties. For instance, it has low hydrogen overpotential at industrial current densities with a constant potential value that remains stable over time. It has a good chemical and electrochemical resistance and is capable of a long-time service. In addition, an effective electrocatalyst does not emit harmful products during electrolysis [23–27].

The present analysis of modern scientific research in the field of animal fat extraction indicated the relevance of new technical solutions in this sphere.

The research objective was to create an ecologically safe fat rendering production line based on the principle of electrochemical activation. The study focused on intensifying the process of fat extraction and increasing the fat-water separation.

STUDY OBJECTS AND METHODS

Ostrich fat became a research object as a novel raw material resource. In addition, ostrich fat is rich in unsaturated fatty acids, easy to separate into fractions, and similar with traditional poultry fats in terms of physical and chemical properties. Ostrich fat has low melting and chilling points. For subcutaneous fat, the melting point is 33–35°C, and the chilling point is 25–27°C. For internal fat, the melting and chilling points are 30–32 and 21–23°C, respectively. As for the iodine value, ostrich fat surpasses that of other farm animals and is close to that of chicken: J_2 of subcutaneous fat is 56.6 ± 0.5 g/100 g; J_2 of internal fat is 61.7 ± 0.5 g/100 g. Table 1 illustrates a comparative analysis of the fatty acid composition of subcutaneous and internal ostrich fat.

The process of in-lab ostrich fat rendering started with preliminary grinding of the refined raw fat to a particle size of 0.5 mm. The wet rendering in water (control sample) and in an electrochemically activated solution, namely catholyte (experimental sample), was performed in an HM-220VM vacuum reactor (Japan – Taiwan). The equipment provided uniform heating and temperature control, as well as vacuum and fat mixing. The water was used at a ratio of 8:1 to the mass of raw materials and met the requirements of State Standard R 51232-98[1]. Extraction was carried out at 55, 75, and 100°C for 60 min. The stepwise thermal processing time was 20 min per stage.

Filtration was used to separate the raw materials into solid and liquid fractions. Vacuum under the filtering surface intensified the process. The fat was separated from the water-protein phase by centrifugation at 6.000 rpm/min for 5 min. The resulting fat with different melting points was dehydrated in a PE-8910 rotary evaporator (Russia) at a residual pressure of 2666.44 Pa. The remaining solid phase underwent subsequent melting at higher temperatures. The new technical approach appeared to have a number of advantages over the existing ones (Patent No. 2382072 RU, February 20, 2010). It proved to be less water- and time-consuming, produced less waste, and rendered fat with a controlled melting point.

Table 1 Fatty acid composition of ostrich fat, % of the total fatty acids

Fatty acids	Symbol	Subcutaneous fat	Internal fat
Myristic	C14:0	0.80	0.7
Myristooleic	C14:1	0.10	0.1
Pentodecanoic	C15:0	0.10	0.1
Palmitic	C16:0	28.70	28.3
Palmitoleic	C16:1	5.20	5.2
Margaric	C17:0	0.10	0.1
Cis-10-heptadecene	C17:1	0.10	0.1
Stearic	C18:0	7.15	8.7
Trans-elaidic	C18:1	0.40	0.1
Oleic	C18:1	37.00	38.5
Linoleic	C18:2	17.95	16.3
Arachidonic	C20:0	0.10	0.1
γ-Linolenic	C18:3	1.40	0.4
Eicosenoic	C20:1	0.60	0.6
Heneicosanoic	C21:0	–	–
Linolenic	C18:3	0.20	0.2
Eicosatrienoic	C20:3	0.10	0.1
Total saturated:		36.95	38.0
Total unsaturated, including:		63.05	62.0
monosaturated		43.40	44.8
polysaturated		19.65	17.2

The electrochemically activated solution was obtained in an electrolyzer by electrolyzing a 10% aqueous NaCl solution at the following modes: DC power = 0.5–0.6A, voltage = 40–42V, pH = 9–11, redox potential = –(400–700) mV, mass fraction of sodium chloride = 4 g/100 cm^3.

Table 2 describes the methods and modes of the fat rendering process.

The conditions and parameters of fat rendering were established both experimentally and by calculation using the Box-Wilson orthogonal central composite design, where each factor was fixed at five levels (–α, –1, 0, 1, +α). The number of design points equaled:

$$N = N_1 + 2n + N_0 \quad (1)$$

where: N_1 – number of points in complete factorial experiment $2n$; $2n$ – number of paired points on the coordinate axes; N_0 – number of experiments in the central design.

[1] State Standard R 51232-98. Drinking water. General requirements for organization and quality control methods. Moscow: Standartinform; 2010. 21 p.

Table 2 Methods and modes of ostrich fat rendering

Fat	Rendering method	Processing details
Internal	Wet rendering in water, pH = 7–8 at various processing temperatures	The thermal treatment included several stages: at the first stage, the heating temperature reached 39°C, at the second – 55°C, at the third – 75°C, at the fourth – 100°C. The melting time was 20 min at each stage. Before each stage, the fat mass was mixed, and the fat was separated stepwise. The resulting fat fractions were pooled into one sample.
Subcutaneous		
Internal	Wet rendering in water, pH = 7–8	The raw fat was processed at 55°C with constant stirring.
Subcutaneous		
Internal	wet rendering in water, pH = 7–8	The raw fat was processed at 75°C with constant stirring.
Subcutaneous		
Internal	Wet rendering in water, pH = 7–8	The raw fat was processed at 100°C with constant stirring.
Subcutaneous		
Internal	Wet rendering in electrochemically activated solution (catholyte), pH = 9–11 at various processing temperatures	The thermal treatment included several stages: at the first stage, the heating temperature reached 39°C, at the second – 55°C, at the third – 75°C, at the fourth – 100°C. The melting time at each stage was 20 min. Before each stage, the fat mass was mixed, and the fat was separated stepwise. The resulting fat fractions were pooled into one sample.
Subcutaneous		
Internal	Wet rendering in electrochemically activated solution (catholyte), pH = 9–11	The raw fat was processed at 55°C with constant stirring.
Subcutaneous		
Internal	wet rendering in electrochemically activated solution (catholyte), pH = 9–11	The raw fat was processed at 75°C with constant stirring.
Subcutaneous		
Internal	Wet rendering in electrochemically activated solution (catholyte), pH = 9–11	The raw fat was processed at 100°C with constant stirring.
Subcutaneous		

The matrix rendered the following equation:

$$\hat{y} = b_0 + b_1\chi_1 + b_2\chi_2 + \ldots + b_k\chi_k + b_{12}\chi_1\chi_2 + b_{13}\chi_1\chi_3 + \ldots + b_{k-1}\chi_{k-1}\chi_k + b_{11}\chi_1^2 + \ldots + b_{kk}\chi_k^2 \quad (2)$$

where b_0 – absolute term; x_1, $x_2 \ldots x_n$ – factors that determine the level of the performance parameter; b_1, $b_2 \ldots b_n$ – regression coefficients for factor indicators that describe the effect of each factor on the performance parameter in absolute terms.

The melting time $X_1(Z_1)$, the pH of the electroactivated medium $X_2(Z_2)$, and the processing temperature of the raw fat $X_3(Z_3)$ were selected as primary factors. The response function was the output of rendered ostrich fat (Y_3). The significance of the coefficients of the mathematical model was established based on the Student's criteria. The tabulated value of the Student's test $t_p(f)$ for $P = 0.05$ and the number of degrees of freedom $f = 3$ was 3.18. The adequacy of the regression equation was controlled by calculating the error square and the error mean square. The Fisher criterion was determined using the Table as $F_{1-p} = (f_1, f_2)$. The equation was adequate if the resulting F-ratio was less than the tabular $F < F_{1-p}$. (f_1, f_2) for the selected significance level ($P = 0.05$) and the numbers of degrees of freedom.

Gas chromatography helped define the fatty acid composition of ostrich fat. The methyl ester extraction and chromatography were based on State Standard 32150-2013[II] under the following conditions: gas chromatograph – Kristall 5000.2, chromatographic column – Omegawax-320 (30 m×0.32 mm×0.50 μm), carrier gas – nitrogen, evaporator gas pressure – 85 kPa, split ratio – 1:40, and evaporator temperature – 250°C. The temperature of the column thermostat was programmed as follows: it was maintained at 120°C for 9 min, and then it was brought up to 210°C at 3°C/min, where it stayed the same for 30–40 min.

The data processing was conducted as follows. The mass fraction of fatty acids was calculated as the ratio of the chromatographic peak area of a particular fatty acid to the sum of all peak areas in the chromatogram. The experiment did not take into account the corrections for the detector sensitivity to the particular fatty acid. It also left out the difference in the molecular weight of the methyl ester and that of the fatty acid itself. The reason was that these corrections are significant only for short-chain fatty acids with the number of carbon atoms between C4 and C10.

The mass fraction of water and volatile substances was determined according to the methods described in State Standard R 50456-92 (ISO 662-80)[III]. The iodine

[II] State Standard 32150-2013. Food eggs and foodstuffs of processed poultry eggs. Method for determination of fatty acid composition. Moscow: Standartinform; 2019. 13 p.

[III] State Standard R 50456-92 (ISO 662-80). Animal and vegetable fats and oils. Determination of moisture and volatile matter content. Moscow: Standartinform; 2008. 6 p.

Table 3 Physicochemical indicators of ostrich fat obtained by electrochemical activation

Rendering type	Processing temperature, °C	Fat	Acid number, mg KOH/g	Peroxide number, mmol of active oxygen per kg	Anisidine number	Viscosity at melting point, kPA×s
In tap water, pH = 7–8	39, 55, 75, 100	Internal	3.00	8.0	4.9	1.03*
		Subcutaneous	2.70	7.7	4.1	1.53*
	55	Internal	2.50	5.4	4.0	0.82
		Subcutaneous	2.40	6.9	4.1	0.85
	75	Internal	2.60	6.9	5.5	0.75
		Subcutaneous	2.30	7.0	4.2	0.78
	100	Internal	2.20	5.5	5.0	0.55
		Subcutaneous	1.30	5.3	4.0	0.60
In electrochemically activated solution (catholyte), pH = 11	39, 55, 75, 100	Internal	3.00	8.0	4.9	1.25*
		Subcutaneous	2.70	7.7	4.1	1.75*
	55	Internal	0.20	1.8	2.0	0.73
		Subcutaneous	0.21	2.0	2.1	0.80
	75	Internal	0.20	2.9	2.2	0.75
		Subcutaneous	0.19	2.2	2.0	0.81
	100	Internal	1.05	4.8	2.9	0.45
		Subcutaneous	0.90	4.6	2.5	0.56

* The viscosity of the combined ostrich fat fraction was determined at 39°C

number was calculated according to State Standard R ISO 3961-2010[IV]. The melting and chilling points were based on State Standard 32189-2013[V]. The control of acid and peroxide numbers was based on State Standard R ISO 27107-2010[VI]. The peroxide number was determined using the potentiometric method according to the end point. The density was measured according to State Standard 18329-2014 (ISO 1675:1985)[VII]. The anisidine number was measured by the optical density of the test solution after its reaction with an acetic acid solution of para-anisidine according to State Standard 31756-2012 (ISO 6885:2006)[VIII]. A rotary digital viscometer was used to determine fat viscosity as specified in State Standard 1929-87[IX]. The experiments were done in triplicates and processed statistically.

RESULTS AND DISCUSSION

The safety and intended use of the product depend on the physicochemical indicators of the rendered fats that are regulated by technical documentation and standards.

The physicochemical indicators of the quality of ostrich fat clearly showed the advantage of the proposed method (Table 3). The fat rendered using the experimental technology had low acid, peroxide, and anisidine values. Processing raw materials in an electrochemically activated solution inactivated the enzyme system (lipase, phospholipase) even at low temperatures (39 and 55°C). The catholyte mechanism proved to be effective, as it lowered and, in some cases, prevented the hydrolytic processes of triacylglycerols. Wet rendering in tap water demonstrated the opposite tendency. The acid numbers revealed a significant amount of free fatty acids and peroxide compounds in the rendered fat. Unlike the experimental technology, water rendering resulted in a characteristic smell.

Fat viscosity is known to affect the rate of heat transfer, separation, and settling. Therefore, this indicator was considered significant for the new technology and equipment. Table 3 shows that internal fat had a lower viscosity than subcutaneous fat, which was due to its unsaturation degree and an increase in the number of double bonds. According to A.V. Gorbatov, liquid products have no ultimate shear stress, and the flow occurs at any minimum shear stress [28]. After cooling, the ostrich fat did not lose all its viscosity. In contrast, pork fat develops a solid crystallization framework at temperatures below 27°C and loses its viscosity.

Table 4 demonstrates that the effectiveness of the experimental technology was fully confirmed by the yield.

[IV] State Standard R ISO 3961-2010. Animal and vegetable fats and oils. Determination of iodine value. Moscow: Standartinform; 2019. 12 p.

[V] State Standard 32189-2013. Margarines, cooking fats, fats for confectionery, baking and dairy industry. Sampling rules and methods of control. Moscow: Standartinform; 2014. 36 p.

[VI] State Standard R ISO 27107-2010. Animal and vegetable fats and oils. Determination of peroxide value by potentiometric end-point determination. Moscow: Standartinform; 2011. 16 p.

[VII] State Standard 18329-2014 (ISO 1675:1985). Liquid resins and plasticizers. Methods for determination of density. Moscow: Standartinform; 2015. 12 p.

[VIII] State Standard 31756-2012 (ISO 6885:2006). Animal and vegetable fats and oils. Determination of anisidine value. Moscow: Standartinform; 2014. 13 p.

[IX] State Standard 1929-87. Petroleum products. Methods of test for determination of dynamic viscosity by rotary viscosimeter. Mooscow: Izdatel′stvo standartov; 2002. 7 p.

Table 4 Fat yield at various processing methods

Rendering method	Processing temperature, °C	Fat	Fat yield, %
In tap water, pH = 7–8	39, 55, 75, 100	Internal	45.0–48.0*
		Subcutaneous	
	55	Internal	50.0–55.0
		Subcutaneous	
	75	Internal	65.0–70.0
		Subcutaneous	
	100	Internal	82.0–87.0
		Subcutaneous	
In electrochemically activated solution (catholyte), pH = 11	39, 55, 75, 100	Internal	55.0–58.0*
		Subcutaneous	
	55	Internal	68.0–72.0
		Subcutaneous	
	75	Internal	80.0–85.0
		Subcutaneous	
	100	Internal	91.0–95.0
		Subcutaneous	

* The viscosity of the combined ostrich fat fraction was determined at 39°C

Electrochemical activation method produced a significantly higher yield than wet rendering in water (Table 4). Second-order orthogonal designs helped establish the effect of technological parameters on fat yield. Table 5 shows the levels of fat extraction factors, while Table 6 demonstrates the matrix of the experiment. Mathematical model 2^k took into account both the number of star points on the coordinate axes of the factor space and points in the center design. Star shoulder α indicated the distance from the center design to the star point.

Table 5 Variation of factors for fat rendering in electrochemically activated liquid (catholyte)

Factors	–α (–1.21)	Z_j^{min}	Z_{j0}	Z_j^{max}	+α (+1.21)
Time, min (Z_1)	25	30	45	60	65
pH (Z_2)	7.5	8	9	10	10.5
Temperature, °C (Z_3)	50	55	75	95	100

The dependence of the output on the set parameters is given as a regression equation (Eqs. (3) and (4)).

$$Y = 81.96 + 3.481\chi_1 + 2.89\chi_2 + 3.17\chi_3 + \\ + 0.67\chi_1\chi_2 + 0.613\chi_1\chi_3 + 0.27\chi_2\chi_3 - \\ - 0.32\chi_1\chi_2\chi_3 - 0.985X_1^2 - 1.26\chi_2^2 - 0.728\chi_3^2 \quad (3)$$

After checking the coefficients of the equation, only nine proved significant. Therefore, the final equation was as follows:

$$Y = 81.96 + 3.481\chi_1 + 2.89\chi_2 + \\ + 3.17\chi_3 + 0.67\chi_1\chi_2 + 0.613\chi_1\chi_3 - \\ - 0.985X_1^2 - 1.26\chi_2^2 - 0.728\chi_3^2 \quad (4)$$

The Fisher's criterion calculated $F = (f_1, f_2) = (6; 3) = 7.39$ proved the obtained Eq. (4) to be adequate for experiment ($S_{reprod.} = 0.207$, $S_{resid.} = 1.53$), as condition $F < F_{1-p}. (f_1, f_2)$; $7.39 < 8.94$ holds at $P = 0.05$.

The obtained equation made it possible to establish an equidirectional positive effect of temperature, rendering time, and catholyte pH on fat yield. In addition, the cross impact of the hydrogen index and the processing temperature was insignificant, while the joint action mechanism of "processing time – catholyte pH" and "processing time – melting temperature" proved

Table 6 Second-order orthogonal design for ostrich fat rendering (k = 3, n_0 = 1)

Experiment number	Factors in a dimensionless coordinate system			Full-scale factors			Y_1, %	Y_{calc}, %	$(Y_1–Y_{calc})^2$
	X_1	X_2	X_3	Z_1, min	Z_2	Z_3, °C			
1	+1.00	+1.00	+1.00	60	10.0	95	88.9	89.80	0.830
2	–1.00	+1.00	+1.00	30	10.0	95	80.3	80.30	0
3	–1.00	–1.00	+1.00	30	8.0	95	74.2	75.80	2.690
4	–1.00	–1.00	–1.00	30	8.0	55	70.0	70.70	0.530
5	+1.00	–1.00	+1.00	60	8.0	95	81.5	82.70	1.420
6	+1.00	–1.00	–1.00	60	8.0	55	74.3	75.10	0.680
7	+1.00	+1.00	–1.00	60	10.0	55	83.1	82.20	0.730
8	–1.00	+1.00	–1.00	30	10.0	55	75.6	75.20	0.160
9	+1.21	0	0	65	9.0	75	84.1	84.70	0.360
10	–1.21	0	0	25	9.0	75	75.5	76.30	0.640
11	0	+1.21	0	45	10.5	75	83.6	83.60	0
12	0	–1.21	0	45	7.5	75	75.7	76.60	0.810
13	0	0	+1.21	45	9.0	100	85.3	84.70	0.360
14	0	0	–1.21	45	9.0	50	76.5	77.10	0.360
15	0	0	0	45	9.0	95	82.0	81.96	0.002

Extra experiments in the center design $Y_1 = 81.0$; $Y_2 = 81.9$; $Y_3 = 81.5$; Z_1 – rendering time, min.; Z_2 – pH value (pH), Z_3 – temperature, °C

to be insignificant. Nevertheless, a significant increase in the abovementioned factors would have a negative effect. Thus, simulating the technological conditions of fat extraction in an electrochemically activated liquid could control the properties of the raw material and its productivity. The identified dependencies were taken into account in order to improve the technological conditions of fat extraction (Fig. 2).

The experimental data made it possible to build a technological line for ostrich fat rendering [29]. It owed its innovative character to a higher fat yield and a more efficient fat extraction process based on electrochemical activation.

Figures 1 and 2 illustrate the sequence of technological operations and hardware design of the fat production line. Table 7 contains the necessary equipment specification.

The ostrich fat production line with the specified properties operated as follows [29]. Raw fat was loaded into the receiving funnel of the AVZh-400 centrifugal machine for primary grinding to 5–6 mm. Simultaneously, hot water (70°C) was added info the grinder in the amount of 20% of the raw material mass (Table 7, *1*). After that, the raw material was fed into the IN-20 worm inactivator (*2*), where it was treated with both indirect and direct heating steam under a pressure of ≤ 0.3 MPa. The electrochemically activated solution (catholyte) flowed in a ratio of 4:1 to the raw material mass through the pipeline that connected the inner working area of the inactivator with the catholyte tank (*19*). Unlike the traditional wet rendering, the fat was watered in an electrolyte (catholyte). A prefabricated 10% aqueous solution of sodium chloride (*25*) was poured into the electrolyzer (*21*), which was connected

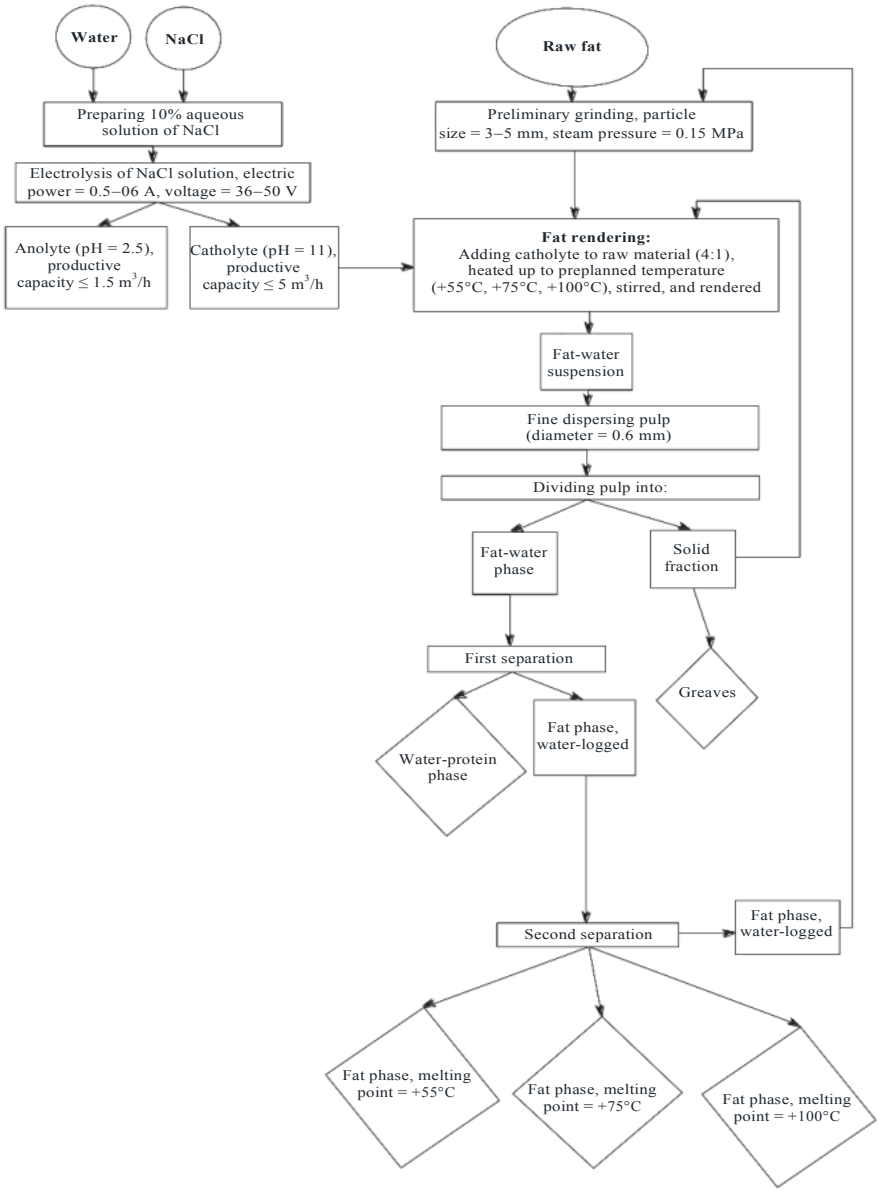

Figure 1 Technological scheme of ostrich fat production

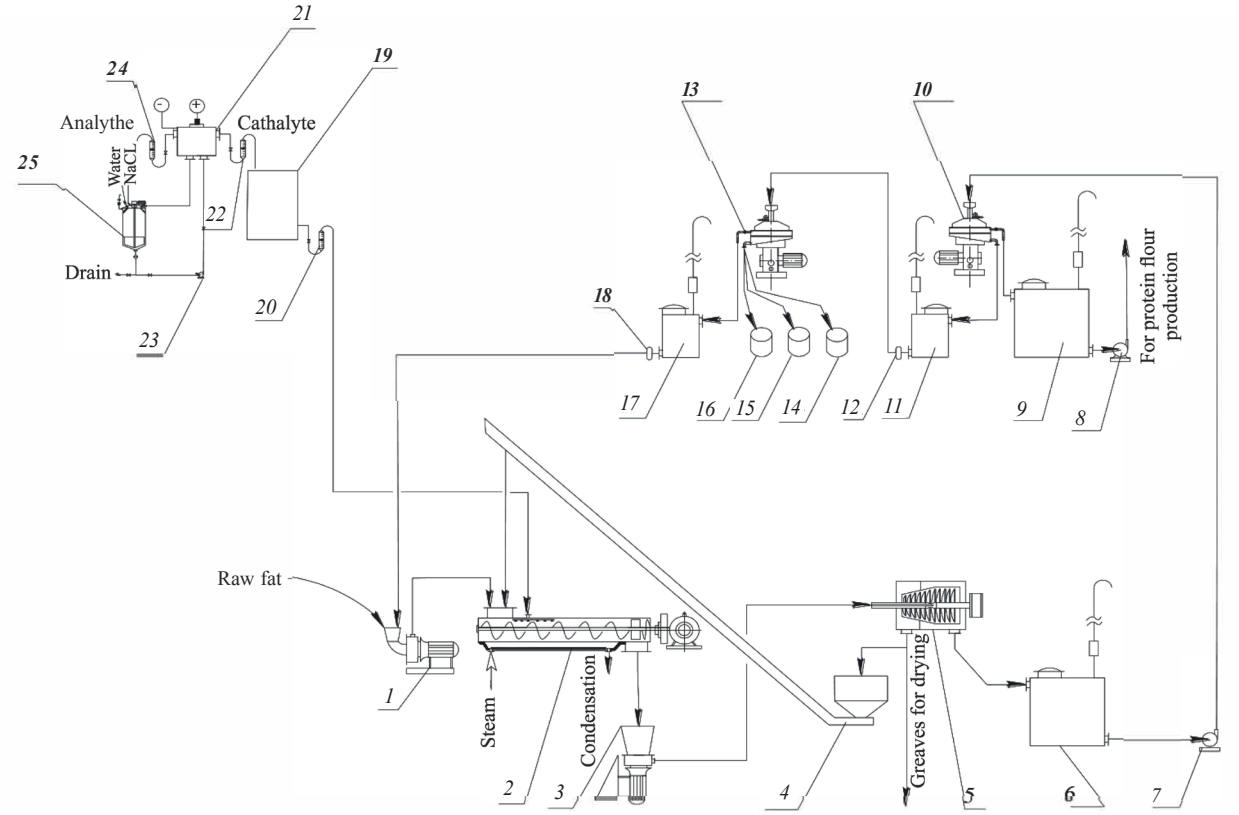

Figure 2 Ostrich fat production line [29]. *1* – device for primary grinding of raw fat; *2* – worm inactivator IN-20; *3* – disintegrator; *4* – hopper with a helix lift; *5* – pulp separation device; *6*, *17* – vertical free-flow tank for collecting fat-water fraction; *7*, *8*, *12*, *18*, *23* – pump; *9* – vertical free-flow tank for collecting water-protein fraction; *10* – the first separator of the fat-water fraction; *13* – the second separator; *14*, *15*, *16* – tanks for collecting fat fractions with a predetermined melting point; *19* – tank for electrochemically activated solution; *20*, *22*, *24* – flowrator; *21* – device for obtaining electrochemically activated solution (electrolyzer); *25* – tank for NaCL solution

to the catholyte tank (*19*). To increase the rendering, the medium for electrochemical activation was applied precisely during the relaxation period. At that stage, its activity and technological properties were at their maximum. The screw rotation frequency was 60 rpm. To sum it up, the raw material was ground and heated with direct heating steam to a predetermined temperature, depending on the melting point of fat rendering.

The obtained combination of mechanical and physicochemical factors made it possible to intensify fat extraction without losing in the yield and quality of fat. The essence of this phenomenon lied in the unipolar processing of sodium chloride solution in the cathode chamber of the electrolyzer. As a result, the solution acquired a metastable state with unique relaxing physicochemical properties. The structural components of the interionic energy interaction underwent a significant change. The active concentration of ions changed because the activity coefficients had changed, not the concentration. The electrolyte is known to reach stable values over a period of time that ranges from minutes to hundreds of hours [27].

The technology made it possible to increase the fat yield without high temperatures or chemicals. Cell membranes were destroyed despite the low processing temperatures.

After that, the processed raw fat was fed to an extra disintegrator (*3*) to obtain a finely ground pulp suspension (particle size = 0.6 mm). The centrifugal force of the ABZh-245 machine pumped the ground fat to the height of 3 m.

At pressure of 0.3 atm, the suspension was fed by gravity to an extra device (Table 7, *5*) in order to separate the pulp into a fat-water fraction and greaves. The fat-water fraction entered the first FCA fat separator (*10*) via the first collection vessel (*6*) through the pipeline and pump (*7*). There it was continuously separated into water-protein glue and fat-water fraction.

The first separation concentrated and purified the fat-water suspension. It entered the rotating drum at the rotation speed of 5,000 min^{-1} and dispersed into thin layers between the conical trays. Under the centrifugal force, protein and other solid particles moved along the bottom surface of the trays and were thrown into the sludge space. Partial sludge discharge occurred at specified time intervals in the operating mode. The processing time depended on the concentration of solid

Table 7 List and technical sepcifications of ostrich fat production equipment

Item	Equipment	Number	Specifications
1	Centrifugal machine AVZh-400	1	Q = 1000 kg/h; N = 15 kW, steam pressure = 0.15 MPa, hole diameter = 30 mm, number of holes = 6, rotor speed = 24.3 s^{-1}, rotor diameter = 310/400 mm. Overall dimensions: 1300 × 400 × 900 mm
2	Worm inactivator IN-20	1	Q = 30 t/s, indirect steam temperature = 120–1,40°C, heating steam consumption = 100 kg/h, steam pressure = ≤ 0.3 MPa, number of nozzles = 12, installed power = 0.75 kW. Overall dimensions: 400 × 600 × 6400 mm
3	ABZh – 245 machine	1	Q = 1,500 kg/h, N = 14 kW, steam pressure = 0.15 MPa, hole diameter = 6 mm, number of holes = 152, rotor speed = 24.3 s^{-1}, rotor diameter = 230/245 mm. Overall dimensions: 1300 × 400 × 900 mm
4	Hopper with a helix lift VK-160 4286– TL –HC–75/187	1	Hopper volume V = 0.25 m^3, length = 4286 mm, lift height = 7000 mm, N = 2.5 kW
5	Centrifuge NOGSh-325	2	Fat-water suspension production = 2.5 t/h, N = 7 kW; inner rotor diameter = 325 mm, inner rotor length = 540 mm, discharge radius = 140 mm, revolutions per minute (max.) = 3500, relative number of screw revolutions per minute = 23.5, power consumption per 1 ton of raw fat = 2.22 kW. Overall dimensions: 520 × 1456 × 1600 mm
6, 9	Vertical free-flow tank BT(B)-0.1-0.0-V	1	V = 0.25 m^3; diameter D = 600 mm, height H = 900 mm, weight = 80 kg
7, 8	Pump X 50-32-125a	2	Q = 10.5 m^3/h, H = 14 m, N = 4.0 kW. Chemical; horizontal overhang pump, with casing support; diameter of the inlet branch pipe = 50 mm, diameter of the outlet branch pipe = 32 mm; nominal wheel diameter = 125 mm
10	Fat separator FTsA	1	Productivity = 1500 cm^3/h; power = 15 kW. Overall dimensions: 1800 × 990 × 1320 mm
11, 17	Vertical free-flow tank BT(B)-0.1-0.0-V	1	V = 0.125 m^3; diameter = 400 mm, height = 1000 mm; weight = 61 kg
12, 18	Pump NSh 10-M-3 L U1	2	Productivity = 0.8 m^3/h, N = 1.1 kW, temperature t = 80°C, pressure = 125 atm
13	Fat separator FSTSP-1	1	Productivity = 1000 cm^3/h, power = 7.5 kW. Overall dimensions: 1440 × 1080 × 1210 mm
14, 15, 16	Vertical free-flow tank BT(B)-0.1-0.0-V	3	V = 0.10 m^3, diameter D = 400 mm, height = 800 mm, mass = 56 kg
19	Tank for electrochemically activated solution (catholyte)		Stainless steel – AISI 304 (08X18H10); volume = 2.5 m^3
20, 22, 24	Electrocontact flowrator SWK	3	Q = 0.05–0.1 dm^3/min, 13–24 dm^3/min; t_{max} = 100°C, p_{max} = 250 bar, material – stainless steel, accuracy ± 4%
21	Electrolyzer for obtaining electrochemically activated solution	1	Volume V = 1.25 m^3, power intake N = 0.75 kVt; current strength = 40 Vt, catholyte productivity (pH = 11) = ≤ 5 m^3/h; anolyte productivity (pH = 2.5) = 1.5 m^3
23	Pump TsNS 4/30	1	Feed rate = 1.6 m^3/h, flow pressure = 20 m, power = 1.1 kVt, temperature ≤ 150°C, for corrosive liquid pumping
25	Tank for NaCL solution	1	Stainless steel – AISI 304 (08X18H10). Number of mixers – 1; rotation = 60 rpm; N = 1.75 kVt. Overall dimensions: 1650 × 1650 × 2820 mm

particles in the raw material. The average processing temperature in the separators directly depended on the preset melting temperature in the inactivator; the processing time was 5 sec. The water-protein fraction entered the tank (*9*) via the first outlet of the separator with the help of a pressure disc along the pipeline under a pressure of 0.3 MPa. The mass fraction of the waste water from the first separator was about 70%.

Through the second outlet, the remaining fat-water fraction was fed into the second tank (*11*). After that, it went through the second separator (FSTsP-1) with a one-millimeter inter-plate gap for further separation (*13*), resulting in the target product with a preset melting point (*14*, *15*, *16*).

To reduce fat loss, the remaining fat-water phase returned into the machine for primary grinding of raw fat after the second separation (*1*). From there, it went into the IN-20 worm inactivator for processing (*2*), together with a new batch of raw fat.

The designed line was sustainable and provided not only complete fat rendering, but also its fractionation depending on the melting temperature. A stepwise approach to the processing of raw materials with a gradual increase in temperature made it possible to

Table 8 Quality indicators of the ostrich subcutaneous fat obtained according to the developed technology

Indicator	Fractions of ostrich fat with melting point of:		
	≤ 55°C	≤ 75°C	100°C
Color	Light yellow	Light yellow	Opaque white with a yellow tint
Smell	Characteristic, typical of this type of fat	Characteristic, typical of this type of fat	Characteristic, typical of this type of fat with no signs of greasing or rancidity
Texture	Liquid	Liquid	Ointment-like, with allowable heterogeneous structure (creaming)
Moisture content, %, ≤	0.8	0.8	0.8
Acid number, mg KOH/g, ≤	3.5	3.5	3.5
Peroxide number, mole of active oxygen/kg, ≤	7.0	7.0	7.0
Viscosity, at 24°C, kPa·s	≤ 0.7	0.70–0.85	1.25–1.75
Density ρ_{20}^{24}, kg/m^3	861–865	881–892	898–910

control the process and process the various types of animal and poultry fats. The new technical solution returned the fat-water phase and suspension through a helical conveyor (*4*), which was connected to the centrifuge (*5*), back to the inactivator (Fig. 2).

The new production line provided a closed technological cycle that minimized the loss of valuable fat product low temperature rendering.

Table 8 demonstrates the technical result of the production line.

CONCLUSION

Electrochemically activated solution introduced in the process of fat rendering made it possible to adjust its properties depending on the intended use of the final product. The developed method of fat rendering and the new sustainable fat production line produced high quality fat and increased the yield of the product compared to the traditional wet rendering in water. The method was based on an inexpensive and safe sodium chloride reagent, which reduced costs and losses in the technological cycle. As a result, the new technology increased the productivity of fat extraction and the degree of fat-water fraction separation. The developed fat production line based on electrochemical activation can be recommended for farm animal fat rendering depending on the intended use.

CONTRIBUTION

All the authors are equally responsible for the obtained research results and the manuscript.

CONFLICT OF INTEREST

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

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Effects of variety and maturity stage of coconut on physicochemical and sensory characteristics of powdered coconut drink

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Abstract:
Introduction. Coconut water is rich in nutrients and biologically-active compounds. However, it has a short shelf life that can be prolonged by freeze drying. The purpose of this study was to analyze the physicochemical and sensory characteristics of fresh and powdered coconut drinks.
Study objects and methods. The experiments included eight samples, namely fresh and powdered coconut drinks obtained from coconuts of different varieties (tall and hybrid) and maturity stages (4 and 6 m.o.). The samples were analyzed for nutrient content (ash, protein, fat, total carbohydrate, and fiber), physicochemical properties (titratable acidity, pH, viscosity, total soluble solids, turbidity, water activity, and browning index), and sensory characteristics (color, aroma, taste, texture, and overall acceptance).
Results and discussion. The results obtained showed that there were significant differences among the coconut drinks of different varieties and maturity stages. They differed in nutrient content, pH value, titratable acidity, viscosity, and water activity. Meanwhile, the aroma, taste, and overall acceptance scores of all the samples were not significantly different. The powdered drink from 6 m.o. hybrid coconut was selected as the optimal sample due to its good sensory and physicochemical attributes. These attributes were similar to those of the fresh coconut drink.
Conclusion. The powdered drink from 6 m.o. hybrid coconut obtained by freeze drying could be considered as an alternative healthy drink with good quality characteristics.

Keywords: Coconut, coconut water, powder, freeze drying, physicochemical properties, descriptive analysis

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INTRODUCTION

Coconut water has various health benefits, namely it prevents oxidative damage due to its antioxidant content, helps rehydration and supports physical performance, as well as prevents and lowers a high blood pressure [1–3, 5]. Coconut water also has antiobesity and antiinflammatory, antibacterial, antitumour, and antidiabetic effects [5–8]. Young coconut water can reduce insulin levels and plasma glucose in rats fed on fructose [4]. Until now, fresh young coconut water is often consumed to relieve thirst and improve general health. However, coconut water is not easily stored because of its short shelf life. Coconut water is quickly damaged when exposed to the air and heat, so it can lose most of its nutritional and health benefits. In addition, the composition and physicochemical properties of coconut water, namely pH of 5 and water activity of 0.9, makes coconut water a highly preferred medium for microbial growth [9, 10].

There are various kinds of coconut water treatment techniques. At present, the dominant technology is thermal pasteurization, but high temperatures can damage the nutritional content of coconut water and change taste. Such technologies as ultrafiltration and ultraviolet radiation retain nutrients better than pasteurization but reduce the shelf life of products to 15 days at 25°C [11–14].

Freeze drying allows for obtaining dry products with maintained typical properties of raw materials.

Freeze drying has high standards for reliability and control [15]. Furthermore, freeze dried products are easily distributed, stored and consumed. The nutrient content and taste of coconut water powdered by freeze drying are almost similar to those of fresh coconut water [16].

Freeze drying process includes addition of filler or components that increase the stability of the final product and/or improve processing [17]. Coconut meat or flesh after water removal can be converted into value-added products such as a natural filler in coconut drinks. Coconut meat comes from young coconut water that thickens progressively and becomes hard as a result of intense cellular multiplication. The aluminous endosperm or flesh or meat of young coconut is soft, edible, white, and more jelly-like. The flesh is sweet and relatively high in minerals such as iron, zinc, and phosphorus [18].

There has been no published papers on comparing characteristics of fresh coconut drinks and powdered drinks obtained by freeze drying. Therefore, the objective of this study was to analyze effects of the variety and maturity stages on the physicochemical and sensory characteristics of fresh coconut drink and rehydrated coconut drink powdered by freeze drying.

STUDY OBJECTS AND METHODS

The samples of the study were two different varieties of coconut (tall coconut and hybrid coconut) of two maturity ages (4 and 6 m.o.) were selected in PT Perkebunan Nusantara (PTPN) VIII, Citepus Village, Pelabuhan Ratu Sub-district, Sukabumi District, West Java Province, Indonesia. The four-month after-pollination coconuts contained a soft thin layer of coconut flesh, and the six-month, a soft thick layer of coconut flesh. All the chemical reagents used for the chemical analyses were of analytical grade.

On day 3 after purchase, coconut water was manually extracted from the coconut fruit and filtered through a strainer. The coconut meat was scraped off and pooled in a container. The coconut drink consisted of water and meat at a ratio of 2:1. It was then homogenised with a Waring blender at room temperature. The mixture prepared was taken into rectangular plastic containers and kept in a freezer (RSA, Indonesia) at –19°C for 24 h until freezing.

The frozen coconut mixture was dried using a vacuum freeze dryer (LABCONCO) at ≤ 50°C under vacuum pressure below 1.0 mbar for 90 ± 2 h. The dried product had a water activity value of < 0.6, as reported in [19]. The dried mixture was then powdered and stored in a desiccator using zip-lock aluminium pouch until further analysis. It aimed to protect the samples from air contamination and sunlight [9]. Eight coconut drink samples, namely fresh and powdered coconut drinks obtained from four- and six-month tall and hybrid coconut, were examined. The experiments were performed in three replications.

Physicochemical characteristics. Ash content was measured gravimetrically by incinerating the samples in a muffle furnace at 550°C [20].

Fat content was determined by the Soxhlet method with steps following protocol set by a semiautomatic Soxhlet extraction instrument (FOSS, ST 243 Soxtec with CU 2046 Control Unit 230V, China). A slight modification was used, namely fresh coconut drink should be dried with a rotary evaporator (IKA, HB 10) at 60°C and 40 rpm for 72 min.

Nitrogen content was determined by the micro-Kjeldahl method using steps developed by a semiautomatic Kjeldahl analysis set of instruments including the digestor (FOSS, Labtec™ DT208 Digestor, China) and destillator (FOSS, KT 200 Kjeltec™ 230V, China). The distillate was then titrated with 0.1 N HCl, then the percentage of crude protein was taken as N×6.25.

Crude fiber were determined by acid base hydrolysis method according to the protocol set by semiautomatic crude fiber extraction system including cold extraction (Tecator, Fibretec System 1021 Cold Extractor) unit and hot extraction (Tecator, Fibretec System 1020 Hot Extractor) unit. The residue left from acid base hydrolysis was then calculated using this formula:

$$\%\ Crude\ Fiber = \frac{W_2 - W_3}{W_1} \times 100 \quad (1)$$

Total carbohydrate was determined as by the difference between the whole sample weight and the sum of ash, fat, protein, and crude fiber contents in the sample. The yield of sample was about 10% (w/v). The powdered sample was then rehydrated with distilled water with the ratio 1:10 to determine titratable acidity, turbidity, color, water activity, and sensory attributes of the coconut drink. The proximate composition of drink was assessed in the Department of Nutrition Science, Faculty of Human Ecology, IPB University, Indonesia.

Total soluble solids was measured by using a portable refractometer with autocorrection at 20°C and expressed in °Brix.

The pH value of the coconut drink was determined using a digital pH meter (Ohaus Starter 3100, USA).

Titratable acidity was determined by titrimetry according to titratable acidity determination in wine or juice with slight modifications [21]. Ten milliliters of coconut drink was transferred into a 250 mL Erlenmeyer flask, followed by 100 mL of neutralized deionized water. The mixture was dripped with 2–3 drops of 1% phenolphthalein and then titrated with standardized 0.1 N NaOH until a distinct but faint pink was reached (maintained for 30 s). Titratable acidity was expressed as percentage of malic acid (w/v):

$$\mathrm{TA} = \frac{N \times M \times V \times 100}{Vc} \quad (2)$$

where TA is titratable acidity, N is the normality of NaO, V is the volume of NaOH, Vc is the volume of coconut water added, and M is the malic acid factor (67.05).

Turbidity was determined using a Shimadzu spectrophotometer (UV-1800, Nakagyo-ku Kyoto, Japan) at wavelength of 610 nm [22]. Absorbance of the sample was measured in relation to distilled water, and the transmittance and respective turbidity were calculated according to equations:

$$T = 100 \times (10^{-Abs}) \quad (3a)$$

$$Turbidity = 100 - T \quad (3b)$$

where *Abs* is absorbance at wavelength of 610 nm, and *T* is the transmittance at wavelength of 610 nm.

Color (browning index). Ten milliliters of coconut drink was mixed with 10 mL of distilled water and 30 mL of ethanol. The ingredients were then mixed on a magnetic stirring plate (Daihan Science MSH-20D, Korea) at 200 rpm and room temperature for 3 min and then filtered through Whatman paper No. 1. A blank sample with distilled water instead of coconut water was also prepared. Color intensity of the samples was quantified in a spectrophotometer (Shimadzu, UV-1800, Nakagyo-ku Kyoto, Japan) by measuring the filtrate absorbance at 420 nm [23].

Viscosity in fresh coconut drink and rehidrated powdered sample was measured using a viscometer (Brookfield Viscometer model RVT Serial DY300, Stoughton, Ma, USA). All measurements were expressed in centipoises (cP) by using a look-up table known as "the Brookfield Factor Finder" to convert torque reading:

$$Viscosity\ in\ cP(mPa \cdot s) = Dial\ reading \times factor \quad (4)$$

The water activity of coconut drinks was measured at 26°C by using a manual water activity meter (Rotronic-Hygrolab) that was calibrated using standard solutions provided by the manufacturer.

Sensory analysis. About 40 semi-trained panelists were involved in sensory analysis as suggested in [24], but only 36 panelists completed it. The tests were applied in individual booths with daylight fluorescent lamps. The coconut drink sample (25 g per sample) was served in plastic cups codified with random three-digit numbers at normal consumption temperature (16–18°C). Water was provided as a palate cleanser. Color, aroma, texture (mouthfeel), taste, and overall acceptance were evaluated by using a nine-point structured hedonic scale from 1 ("disliked extremely") to 9 ("liked extremely") [24, 25].

Descriptive analysis with consensus method according to [26] was also performed with nine trained panelists aged between 24 and 27. The panelists were asked to identify aroma and taste descriptors, as well as their references. A total of 10 mL of the sample was served in a closed plastic cups codified with random three-digit numbers and kept at room temperature. In addition, water, tissue, palate cleanser (plain bread), and a container for spitting were provided. Each panelist assessed the reference and sample intensity on a scale of 0 to 15 across aroma and taste descriptors.

Statistical analysis. Data were reported as the mean ± SD. Analysis of variance (ANOVA) followed by Tukey LSD's test was done using SPSS 16.0 program, with significance established at the $P < 0.05$ level.

RESULTS AND DISCUSSION

Proximate analysis. Contents of fat, ash, protein, fiber, and total carbohydrates are presented in Table 1. They differed significantly in the experimental coconut drinks ($P < 0.05$). The fat content in fresh drinks was 0.57–21.82% (the highest value in six-month hybrid) and 4.85–15.11% in powdered drinks (the highest value in six-month hybrid). Freeze drying process did not affect fat content of the samples. On the other hand, fat content of samples increased following maturity. The fat content in coconut drinks did not depend on the coconut variety. Fat contents in our study were lower than those of coconut milk in [27]. However, they were within the range pointed out by Mahayothee *et al.*, namely, 4.70–24.22% [28]. This difference could be due to the variety and maturity of coconut. The dependence of the fat content on maturity in this study was in line with Mahayothee *et al.* in case of some varieties of coconut in Thailand [28]. The thickness and fat content of coconut meat increased significantly with the maturity ages [28].

The ash content in fresh and powered coconut samples was 6.61–9.62% and 7.33–9.43%, respectively. The 4-month hybrid coconut drinks had the highest ash content. There was no dependence of ash contents

Table 1 The proximate analysis offresh and powdered coconut drinks (mean ± SD)

Sample	Fat, %	Ash, %	Protein, %	Crude fibre, %	Total carbohydrates, %
Tall, 4 mo, fresh	0.57 ± 0.01^{d}	6.61 ± 0.79^{c}	$7.33 \pm 0.10a^{b}$	3.91 ± 0.26^{a}	84.75 ± 0.93^{a}
Tall, 6 mo, fresh	15.29 ± 0.77^{bc}	7.20 ± 0.08^{bc}	8.52 ± 0.16^{bc}	2.06 ± 0.07^{b}	67.79 ± 0.79^{cd}
Hybrid, 4 mo, fresh	1.57 ± 0.00^{d}	9.62 ± 0.10^{a}	12.11 ± 0.08^{a}	3.69 ± 0.65^{a}	76.29 ± 0.18^{b}
Hybrid, 6 mo, fresh	21.82 ± 0.76^{a}	8.38 ± 0.35^{ab}	10.09 ± 0.11^{ab}	4.25 ± 0.21^{a}	61.26 ± 2.05^{e}
Tall, 4 mo, powdered	6.09 ± 1.04^{cd}	7.33 ± 0.51^{bc}	7.43 ± 1.41^{b}	0.81 ± 0.09^{bc}	75.24 ± 2.76^{b}
Tall, 6 mo, powdered	10.33 ± 0.30^{bc}	7.75 ± 0.03^{bc}	6.29 ± 0.02^{b}	1.19 ± 0.15^{bc}	73.56 ± 0.35^{bc}
Hybrid, 4 mo, powdered	4.85 ± 0.87^{cd}	9.43 ± 0.40^{a}	8.89 ± 0.77^{ab}	0.69 ± 0.02^{c}	73.13 ± 0.36^{bc}
Hybrid, 6 mo, powdered	15.11 ± 2.25^{b}	7.65 ± 0.21^{bc}	7.98 ± 0.39^{ab}	1.23 ± 0.23^{bc}	65.01 ± 2.27^{cde}

^{a,b,c,d,e,f} = significantly different ($P < 0.05$)

among samples on freeze drying process and maturity, while the variety made a difference. We found that the hybrid variety had a higher ash content compared to the tall variety. The stable content of ash in this study was within the range mentioned by Santana *et al.*, namely, 0.75–10.72%, and it was by 1.60%, 1.43–2.23%, and 1.05% higher than ash in coconut milk, coconut meat, and coconut haustorium, respectively [27–30]. The differences of the results might be due to the differences of maturity stages and compositions of the samples. There was a tendency to increasing ash contents with maturity in the tall variety. This data was in accordance with Mahayothee *et al.*, who stated that ash in coconut meat increased with maturity stages until 190 d of pollination (DAP) [28].

The protein content of in the fresh and powdered samples accounted for 7.33–12.11% and 6.29–8.89%, respectively, with the highest content in fresh and powdered four-month hybrid. The protein content was not influenced by the freeze drying process, maturity stages, and variety. Protein amounts in coconut drinks in this study were higher than those in fresh coconut milk, coconut meat, and coconut grating by 6.10%, 2.71–4.49%, and 5.59%, respectively [27, 28, 31]. However, it was lower than the protein content in coconut meat (16.0%) according to [32]. This difference could be due to the dominant use of coconut water in this study. The stability of protein content with maturity stages in this study confirmed Mahayothee *et al.* data, who found that the protein content of coconut meat did not increase significantly in coconut aged 180 DAP (6 m.o.) and 190 DAP (6+ m.o.), but it would increase significantly in the samples aged 225 DAP (7+ m.o.) (2.71–4.49%) [28].

The crude fiber in the coconut drinks ranged between 2.06 and 4.25% in the fresh samples and between 0.69 and 1.23% in the powdered samples. The six-month hybrid samples in fresh and powdered forms demonstrated the highest values. The crude fiber of the samples was not influenced by maturity stages and the variety of coconut. Our results were close to fiber contents in coconut milk and coconut meat, which accounted for 3.10% and 0.92–6.51%, respectively [27, 28].

The total carbohydrate content accounted for 61.26–84.75% in the fresh coconut drinks and 65.01–75.24% in the powdered samples, with the highest value in fresh and powdered four-month tall variety. Freeze drying was not proved to decrease the carbohydrate content, while maturity stage decrease it. Additionally, tall coconut demonstrated a higher total carbohydrates than hybrid coconut. The total carbohydrates in our research were lower than those by Santoso *et al.*, who found that carbohydrates in young coconut were 81.8 and 54.9% in coconut water and in coconut meat, respectively [32]. However, our results conformed with those found by Mahayothee *et al.*, who reported that carbohydrate content of coconut meat increased insignificantly from 180 DAP (6 m.o.) until 190 DAP (6+ m.o.) then decreased significantly until 225 DAP (7+ m.o.) [28]. The difference in carbohydrate content might be due to the difference in varieties and land areas of planting. The decreased carbohydrate content might be due to the increase in fat content that occurred in hybrid coconut as well as in tall coconut with time.

Titratable acidity and pH value. The titratable acidity and pH values of fresh and powdered coconut drinks obtained from different coconut varieties were in the range of 0.63–0.91% and 5.56–6.37, respectively (Table 2). They differed among the samples ($P < 0.05$). Younger coconut age showed a tendency to decrease titratable acidity and increase the pH value.

Titratable acidity values in this study were higher than those found by Rattanaburee *et al.*, who reported that the titratable acidity of fresh freeze-dried young coconut juice was 0.016–0.018% [9]. The value of 0.18% (citric acid) was due to the presence of ascorbic acid in tender coconut water [33]. Decreasing titratable acidity with maturity stages was in agreement with Mahayothee *et al.* and Tan *et al.* [28, 34]. This might be due to the decrease of organic acids in coconut drinks with maturity.

The pH value in spray-dried and freeze-dried coconut juice was around 4.87–5.04 and 5.00–5.14, respectively [9, 10]. In this study, pH was slightly higher, which could be due to the addition of coconut meat into the coconut water. The pH value of osmo-dried coconut slices or coconut meat was in the range of 6.32–6.67 but that in coconut sap was slightly higher (7.47) than in our research [35, 36]. An increase in pH value with maturity stages was in agreement with Mahayothee *et al.* and Tan *et al.* [28, 34]. This was appeared to be due to the decrease in titratable acidity along with the increasing maturity of coconut. The titratable acidity was inversely related to the pH value.

Physical analysis. Physical attributes of the fresh and powdered coconut drinks under study are shown in Table 3. Total soluble solids, turbidity, and browning index in fresh and powdered coconut drinks

Table 2 pH value and titratable acidity of fresh and powdered coconut drinks (mean ± SD)

Sample	Titratable acidity, %	pH
Tall, 4 mo, fresh	0.82 ± 0.00^{ab}	5.56 ± 0.02^{d}
Tall, 6 mo, fresh	0.66 ± 0.02^{c}	6.33 ± 0.03^{a}
Hybrid, 4 mo, fresh	0.86 ± 0.02^{ab}	5.80 ± 0.01^{c}
Hybrid, 6 mo, fresh	0.82 ± 0.03^{ab}	6.29 ± 0.03^{a}
Tall, 4 mo, powdered	0.63 ± 0.02^{c}	5.63 ± 0.07^{d}
Tall, 6 mo, powdered	0.78 ± 0.03^{abc}	6.29 ± 0.02^{a}
Hybrid, 4 mo, powdered	0.91 ± 0.06^{a}	6.02 ± 0.00^{b}
Hybrid, 6 mo, powdered	0.75 ± 0.00^{bc}	6.37 ± 0.01^{a}

^{a,b,c,d,e,f} = significantly different ($P < 0.05$)

Table 3 Physical characteristics of fresh and powdered coconut drinks (mean ± SD)

Sample	Total soluble solid, ° Brix	Turbidity, %	Browning index	Viscosity, cP	Water activity
Tall, 4 mo, fresh	5.00 ± 0.00[a]	99.97 ± 0.03[a]	0.02 ± 0.00[a]	43.50 ± 1.50[cd]	0.978 ± 0.00[a]
Tall, 6 mo, fresh	5.00 ± 0.00[a]	99.78 ± 0.03[a]	0.01 ± 0.00[a]	107.50 ± 2.50[b]	0.977 ± 0.00[a]
Hybrid, 4 mo, fresh	5.00 ± 0.00[a]	99.80 ± 0.05[a]	0.02 ± 0.00[a]	19.75 ± 0.25[e]	0.975 ± 0.00[a]
Hybrid, 6 mo, fresh	6.00 ± 0.00[a]	99.72 ± 0.05[a]	0.02 ± 0.01[a]	275.00 ± 5.00[a]	0.978 ± 0.01[a]
Tall, 4 mo, powdered	5.00 ± 0.00[a]	99.73 ± 0.05[a]	0.02 ± 0.00[a]	36.75 ± 3.25[d]	0.430 ± 0.01[b]
Tall, 6 mo, powdered	5.00 ± 0.00[a]	99.70 ± 0.01[a]	0.02 ± 0.00[a]	98.38 ± 1.62[b]	0.233 ± 0.03[c]
Hybrid, 4 mo, powdered	5.00 ± 0.00[a]	99.66 ± 0.06[a]	0.02 ± 0.00[a]	54.50 ± 0.50[c]	0.416 ± 0.00[b]
Hybrid, 6 mo, powdered	6.00 ± 0.00[a]	99.83 ± 0.04[a]	0.29 ± 0.00[a]	268.00 ± 2.00[a]	0.409 ± 0.01[b]

[a,b,c,d,e,f] = significantly different ($P < 0.05$)

were not significantly different among the samples ($P > 0.05$). There were significant differences in viscosity and water activity among the samples ($P < 0.05$), namely the viscosity increased with increasing maturity stages and in the hybrid variety, and the powdered drinks had a decreased water activity. Maturity stages and the variety of coconut did not influenced the water activity.

Total soluble solids in this study (5.0–6.0°Brix) were slightly lower than those in freeze-dried and spray-dried young coconut juice reported by Rattanaeburee *et al.*, which were in the ranges of 6.25–6.50°Brix and 7.23–7.98°Brix, respectively [9, 10]. The results demonstrated one of the advantages of freeze drying [9]. In addition, total soluble solids did not show any significant changes with increasing coconut maturity, which can be due to young category of the sample used (4 and 6 m.o.). According to data found by Terdwongworakul *et al.*, total soluble solids in young coconut water increased from 5.39 to 6.76°Brix in the immature stage, then increased linearly to 7.45°Brix at the end of the mature stage, and continued rising at a slightly higher rate to 8.24°Brix at the end of the over-mature stage [37]. The solids present in tender coconut water of about 5.3°Brix were mainly soluble solids such as sugars [38].

Turbidity values in fresh and powdered coconut drinks in this study were slightly higher (99.66–99.97%) than those in spray-dried young coconut juice (96.58–97.89%) [10]. Nevertheless, they were within the range of the values in fresh and freeze-dried young coconut juice (97.72–101.61%) [9]. The percentage of flesh or coconut meat in coconut water was assumed to affect adversely the color, which was indicated by the high turbidity in the samples.

The browning index values of the samples were extremely small (0.10–0.29), which showed that freeze drying did not result in browning of the samples. However, samples in other studies experienced browning that might be caused by several factors such as degradation of reducing sugars, Maillard reaction, and degradation of decomposition products of ascorbic acid (hydroxymethylfurfural) during coconut processing [39–41].

Coconut water is categorized as a Newtonian fluid that has the same viscosity at different shear rates. The viscosity value in this study was higher (up to 275.00 cP) than that in tender coconut water at 25°C and 5.3°Brix (62.6 cP) [38]. The tendency of increasing viscosity with maturity stages in the hybrid variety in this study could be due to an increased fiber content in coconut meat, especially in fresh and powdered six-month hybrid coconut, which had the highest total soluble solids. Viscosity in tender coconut water increased significantly with the increase in total soluble solids and decreased with the increase in water activity and temperature. Temperature and total soluble solid concentration were reported to have strong effects on the viscosity of Newtonian fluids [38].

The water activity of the fresh coconut drink under study (0.975–0.978) was within the range of the values in fresh tender coconut water obtained by Manjunatha and Raju, which were beetwen 0.870 and 0.982 [38]. However, the powdered coconut drinks had a slightly higher water activity (0.233–0.430) compared to coconut sugar powder (0.20–0.33), being within the safe limit to prevent damage caused by microorganisms and biochemical reactions (< 0.6) and the critical limit (< 0.7) [42–44].

Just like the water content, there was a tendency of a significant decrease in water activity by freeze drying. Water activity is an important factor affecting the stability of dehydrated products. The reduction in water activity in liquid foods led to better stability, which was a beneficial factor during storage, handling, and transportation of the product [38]. Furthermore, it could reduce costs associated with whole fruit and improve its shelf life. Therefore, water activity is considered as a critical factor that determines the shelf life of food. Water activity in coconut water decreased significantly with the increase in soluble solid content and the increase in viscosity [38].

Sensory analysis. The sensory analysis of the coconut drinks included color, aroma, texture (mouthfeel), taste, and overall acceptance. Subjective organoleptic assessment depends primarily on the sensitivity of the panelist and conditions under which it is carried out [45]. Table 4 demonstrates the mean scores of acceptance sensory analysis of the fresh and powdered coconut drinks.

Table 4 Effect of variety and maturity age of coconut on sensory acceptance of fresh and powdered coconut drinks (mean ± SD)

Sample	Sensory acceptance score				
	Color	Aroma	Texture (mouthfeel)	Taste	Overall aceptance
Tall, 4 mo, fresh	6.31 ± 0.95^{ab}	5.58 ± 1.13^{a}	5.83 ± 1.23^{ab}	5.86 ± 1.44^{a}	6.08 ± 1.16^{a}
Tall, 6 mo, fresh	6.56 ± 0.94^{a}	5.94 ± 1.24^{a}	5.06 ± 1.35^{b}	5.47 ± 1.63^{a}	5.56 ± 1.23^{a}
Hybrid, 4 mo, fresh	5.53 ± 1.30^{bc}	5.67 ± 1.29^{a}	6.11 ± 1.21^{a}	5.89 ± 1.12^{a}	5.94 ± 1.19^{a}
Hybrid, 6 mo, fresh	5.94 ± 1.15^{abc}	5.61 ± 1.34^{a}	5.25 ± 1.73^{ab}	5.39 ± 1.59^{a}	5.72 ± 1.23^{a}
Tall, 4 mo, powdered	5.91 ± 1.08^{abc}	5.26 ± 1.24^{a}	5.71 ± 1.27^{a}	6.03 ± 1.51^{a}	6.12 ± 1.17^{a}
Tall, 6 mo, powdered	6.29 ± 1.06^{ab}	5.59 ± 1.02^{a}	5.18 ± 1.45^{b}	5.62 ± 1.18^{a}	5.82 ± 1.06^{a}
Hybrid, 4 mo, powdered	5.44 ± 1.24^{c}	5.12 ± 1.45^{a}	5.85 ± 1.31^{ab}	5.71 ± 1.73^{a}	5.82 ± 1.51^{a}
Hybrid, 6 mo, powdered	6.03 ± 1.31^{abc}	5.68 ± 1.07^{a}	5.74 ± 1.58^{ab}	6.26 ± 1.33^{a}	6.18 ± 1.17^{a}

^{a,b,c,d,e,f} = significantly different ($P < 0.05$)

The color of a drink is one of the most important characteristics that determines the customer choice. The color of the coconut drink indicated that six-month tall coconut had the highest score both in fresh and powdered forms (6.56 and 6.29), respectively. Color acceptance tended to increase with the increase in coconut maturity. In spite the fact that the samples' browning index values did not change significantly, this did not affect the acceptance of color by the panelists.

Mouthfeel is a parameter related to rheological viscosity and defined as the mixed experience derived from the sensation on the skin of the mouth after ingestion of food or beverage [33]. The texture (mouthfeel) of the coconut drink considerably depended on the ratio of water to meat during the process, while the variety and maturity stages of coconut did not affect the panelists' preferences. The texture (mouthfeel) score was the highest in four-month fresh hybrid coconut (6.11) and four-month powdered tall coconut (5.71). This high score might be due to the fiber content in coconut meat. Staffolo *et al*. found that yoghurt enriched with fiber had a better mouthfeel [46].

The taste acceptance was not significantly different ($P > 0.05$). It might be due to the total soluble solid value as an indicator of sugar which did not change significantly by freeze drying. In addition, the use of coconut meat in the product formulation, in addition to being used as a natural filler, could also help improve the good taste of coconut drinks. Immature coconut water is a delicious and nutritious drink [47]. However, at maturity, coconut water loses its delicious taste to the advantage of the kernel [48]. Coconut meat contains several sugars such as sucrose, glucose, and fructose which along with organic acids, such as citric acid and malic acid, give a good taste to the coconut drink [32].

Also, there were no significant differences in the panelists' opinions on aroma and overall acceptance. It was revealed that there were no significant changes in the overall acceptance of the fresh and powdered coconut drink. The freeze drying method of processing had been reported by Shukla to preserve the aroma and smell of the product [17].

Descriptive analysis in this study showed that coconut had coconut-like, creamy, nutty, rancid, sweet, and ferment aroma (Fig. 1). The various aromas

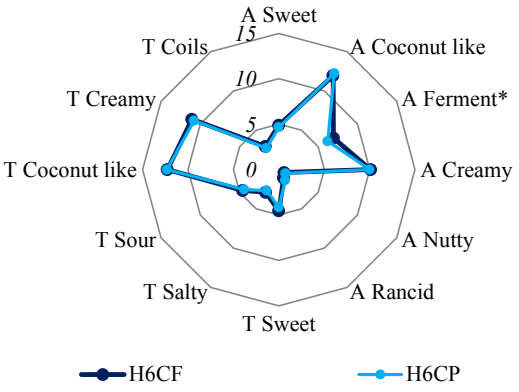

Figure 1 Descriptive analyse of aroma and taste of hybrid six-month coconut drinks

identified are explained by the present of various volatile compounds in coconut water [49]. The tastes identified by the panelists were coconut-like, sweet, sour, acid, salty, and coils. Freeze drying maintained the aroma and taste attributes except ferment aroma and sweet taste ($P < 0.05$).

The results of the descriptive analysis in this study were in line with those in other studies, which revealed that coconuts had fresh coconut, nutty, rancid, and sweet aroma [50, 51]. The decrease in ferment aroma in the coconut drinks after freeze drying was in line with that in coconut water after heat treatment, which could be due to a decrease in acetoin during the processing [52].

The tastes identified by the panelists were in line with the results of other studies, which revealed coconut-like, sweet, acid, and salty tastes [50, 53]. The powdered coconut drinks showed less sweet taste compared to the fresh samples. This could be because of reducing not only water but also sugars in the product by freeze drying. Moreover, rehydrated drinks include mineral water, while fresh coconut drinks contain sweet coconut water. Thus, sweet taste between fresh coconut drinks and powdered ones can be different.

CONCLUSION

There were found significant differences in physicochemical characteristics, such as proximate

composition, pH, titratable acidity, viscosity, and water activity among fresh and powdered coconut drinks of different varieties and maturity stages. Meanwhile, aroma, taste, and overall acceptance of all coconut drinks were similar. In the whole, the nutrient content of the hybrid coconut was higher compared to the tall variety, so the powdered six-month hybrid coconut drink was selected for descriptive test. The test showed that freeze drying did not significantly change the sensory properties of the sample, except for ferment aroma. Consequently, powdered six-month hybrid coconut should be further studied because of its good sensory and physical characteristics, as well as nutrient content.

CONTRIBUTION

J.M. Azra contributed to the conception, collected and analyzed data for the work, and wrote the manuscript. J.M. Azra, Z. Nasution, A. Sulaeman, and B. Setiawan designed the work. Z. Nasution and B. Setiawan revised the manuscript for important intellectual content, and B. Setiawan conducted the final revision of the manuscript.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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Bioconversion of soy under the influence of *Aspergillus oryzae* strains producing hydrolytic enzymes

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Abstract:
Introduction. The fungus *Aspergillus oryzae* is widely used in the production of fermented soy-based products. However, there is little data on how its genetic characteristics affect the biochemical and fractional composition of protein substances during fermentation and the quality of fermented products. This study aimed to investigate the conversion of soy meal under the influence of two *A. oryzae* strains with different morphological and cultural properties during the production of a fermented soy sauce.
Study objects and methods. The study used two *A. oryzae* strains, RCAM 01133 and RCAM 01134, which were isolated from the industrial F-931 strain (Russian Collection of Industrial Microorganisms), a producer of hydrolytic enzymes. Micromycetes were cultivated by a solid-phase method on soy meal, followed by dry fermentation. The results were analyzed with regard to accumulation of amine nitrogen, bound and free amino acids, proteins and carbohydrates.
Results and discussion. The cultivation of micromycetes resulted in a 35–38% increase in protein, a tenfold increase in free amino acids, and a 1.5–1.7 fold decrease in polysaccharides. The contents of essential amino acids in the fermented soy sauce were 1.7 and 1.2 times as high as in the initial medium (soy meal) and in the reference protein, respectively. Fermentation enhanced the biological value of proteins, increasing the amino acid scores of phenylalanine (7.3–7.7 times), phenylalanine (2 times), as well as valine, threonine, tryptophan, and lysine. The contents of protein and essential amino acids were slightly higher in the sauce with the RCAM 01133 strain.
Conclusion. Fermenting soy materials with the RCAM 01133 strain of *A. oryzae* is an alternative way to produce food ingredients with good sensory properties containing carbohydrates and biologically complete protein in easily digestible forms.

Keywords: Micromycetes, soy meal, enzyme, protein, amino acids, amino acid score

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INTRODUCTION

Soy is the most common plant protein source in the world that has a wide range of health benefits. Clinical studies of soy-based products have revealed their antioxidant, antitumor, hypoglycemic, and hypotensive effects [1–5]. Having been cultivated since ancient times in Asian countries, since the 18th century in Europe, and since the 19th century in the USA, soy is now one of the most important agricultural crops worldwide [6, 7]. The USA is currently the leading producer of soybean, accounting for over 30% of world production. The popularity of soy products in North America has been growing over the past decades, especially after the Food and Drug Administration (FDA) linked soy protein with a reduced risk of coronary heart disease [8–10]. Soy protein contains all essential amino acids. Also, it has a higher biological value than plant proteins of cereals and a lower content of saturated fatty acids than animal products [11].

Asian countries mostly use fermented soy. Fermentation increases the solubility of soy polymers and improves absorption in the intestine, which provides soy-based products with high nutritional value and functional properties [12]. High soy consumption in Japan and China correlates with a low incidence of

cardiovascular and oncological diseases. Soy-based products contain a large amount of soluble proteins, including bioactive peptides and amino acids, as well as isoflavones, polyamines, and other bioactive substances resulting from soy bioconversion [13–16].

Aspergillus oryzae is widely used in the production of fermented soy-based foods (soy sauce, soy paste, rice wine, etc.). The FDA declared the strains of this filamentous fungus safe for food production (GRAS), which was supported by the World Health Organization (WHO) [17–19]. The *A. oryzae* strains are industrial producers of enzyme preparations with proteolytic and amylolytic action [20]. The fungus synthesizes proteases as it adapts to a protein-rich environment during soy fermentation, with acidic proteases (carboxyl proteinase, carboxy-peptidase) playing the most important role in protein hydrolysis to form free amino acids [12, 20–23].

In addition, *A. oryzae* produces hydrolytic enzymes that break down polysaccharides into smaller mono- and oligosaccharides, which can also affect the quality of fermented products [20, 24, 25]. Apparently, these enzymes play a regulatory role in the long-term conversion of plant materials, activating subsequent fermentation and supplying metabolites that act as precursors for the formation of the characteristic taste of soy sauce [26–29]. However, literature lacks information on the biosynthesis and catalysis of *A. oryzae* biomass or the influence of genetic and physiological characteristics of its strains on the biochemical and fractional composition of protein substances during microbial conversion of soy materials.

We conducted comparative studies of soy conversion under the influence of two *A. oryzae* strains with different morphological and cultural properties to produce a fermented sauce.

STUDY OBJECTS AND METHODS

Our study objects were two strains of the filamentous fungus *Aspergillus oryzae* isolated as a result of selection from the F-931 industrial strain, a producer of proteolytic and amylolytic enzymes (Russian Collection of Industrial Microorganisms) [29]. The strains were deposited in the Russian Collection of Agricultural Microorganisms (RCAM) and differed in their morphological characteristics and cultural properties. RCAM 01133 and RCAM 01134 *A. oryzae* strains were used for the fermentation of soybean meal.

Micromycetes were cultivated by the solid-phase method in flasks on natural nutrient media containing soybean meal (55% moisture) for 4 days at 30–32°C. We used standard methods to analyze the activity of hydrolytic enzymes in the fungus biomass [30]. Microscopic studies were conducted with a Nikon Eclipse microscope (Japan).

Fungal cultures grown on soy were dry fermented for 48 h at 55–60°C in a TS-1/180 SPU dry-air thermostat. Surface cultures were extracted with a 20% NaCl solution in a ratio of 1:1.5 for 1 hour at 20–25°C. The incubation mixture was kept on a Biosan ES-20 (EU) incubator shaker at 200–210 rpm. Following the extraction, the soy sauce was separated in an OS-6M centrifuge at 5000 rpm for 10 min.

The biocatalytic conversion of fungal biomass polymers was evaluated according to the degree of accumulation of amine nitrogen, free amino acids, and general reducing substances [31][I]. The total protein content was determined by the Kjeldahl method on an automatic Vadopest system (Germany)[II].

The amino acid content in the soy sauce samples was analyzed on a KNAUER EUROCHROM 2000 chromatograph. After that, the components were determined with a spectrophotometric Smartline UV Detector 2500 at λ = 570 nm (Germany). The aminograms were calculated by comparing the areas of the standard and the sample [32].

The amino acid score (AAS) was expressed as a percentage ratio between the amount of a certain essential amino acid in the test protein and that of the same amino acid in the FAO/WHO reference protein [33]:

$$\text{AAS} = \frac{M}{M_{\text{R}}} * 100 \quad (1)$$

where AAS is the amino acid score, %; M is the content of an essential amino acid in the test protein, g/100 g protein; M_{R} is the content of an essential amino acid in the reference protein, g/100 g protein.

The experiments were carried out in at least three replicates. The data were statistically processed in Microsoft Excel using the Student's coefficient (confidence interval 0.95).

RESULTS AND DISCUSSION

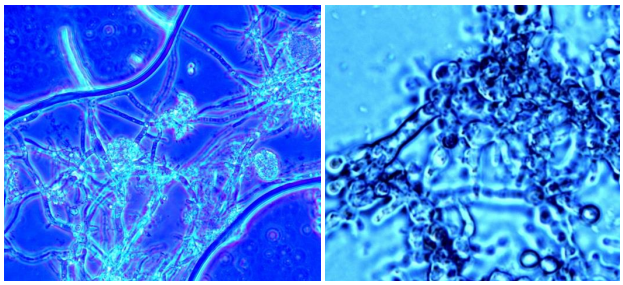
The solid-phase cultivation of *Aspergillus oryzae* RCAM 01133 and RCAM 01134 strains on nutrient media containing soybean meal revealed significant differences in their growth and development. At the first stage of growth (24 h), we observed swelling and germination of spores, as well as the formation of hyphae in both cultures.

At the second stage (42 h), RCAM 01133 showed more active mycelium formation accompanied by the release of metabolites and excess heat (the temperature in the medium layer increased to 34°C). RCAM 01134, however, featured a slightly slower rate of growth and mycelium formation.

By the third stage (72 h), both cultures had formed mycelium, which was airier and whiter in RCAM 01133. We also noticed the beginning of sporulation in

[I] OFS.1.2.3.0022.15 Opredelenie aminnogo azota metodami formol'nogo i yodometricheskogo titrovaniya [GPC.1.2.3.0022.15 Determination of amine nitrogen by formal and iodometric titration methods]. 2015. 3 p.

[II] State Standard 32044.1-2012 (ISO 5983-1:2005). Feeds, mixed feeds and raw material. Determination of mass fraction of nitrogen and calculation of mass fraction of crude protein. Part 1. Kjeldahl method. Moscow: Standartinform; 2014. 15 p.



(a) (b)

Figure 1 *Aspergillus oryzae* mycelium: (a) RCAM 01133; (b) RCAM 01134

RCAM 01134 (Fig. 1). The processes of biosynthesis and accumulation of enzymes took place at the same time, with the temperature of the medium dropping to 28–30°C.

By the end of growth, on day 4, the fungal cultures looked like accreted colonies with 55–57% moisture. RCAM 01134 had more abundant yellow-green conidia, while RCAM 01133 had a white colony and a reduced ability to sporulate.

Then, we analyzed the biosynthetic features of the *A. oryzae* strains cultivated on soy. In particular, we measured the content of protein, polysaccharides, and amine nitrogen in surface cultures (Fig. 2), as well as determined the levels of proteolytic and amylolytic enzymes (Fig. 3).

We found that the cultivation of the *A. oryzae* strains increased the content of crude protein by 35–38% and decreased the concentration of polysaccharides 1.5–1.7 times compared to the initial medium. The contents of protein and polysaccharides were 55.3% and 18.0% for RCAM 01133 and 52.3% and 19.5% for RCAM 01134, respectively. The content of amine nitrogen was 17 times as high in RCAM 01134 (13.24 mg/g) and 1.5 times as high in RCAM 01133 (8.59 mg/g), compared to the initial medium (0.8 mg/g).

The *A. oryzae* strains differed in the level of synthesized enzymes (Fig. 3). RCAM 01133 produced

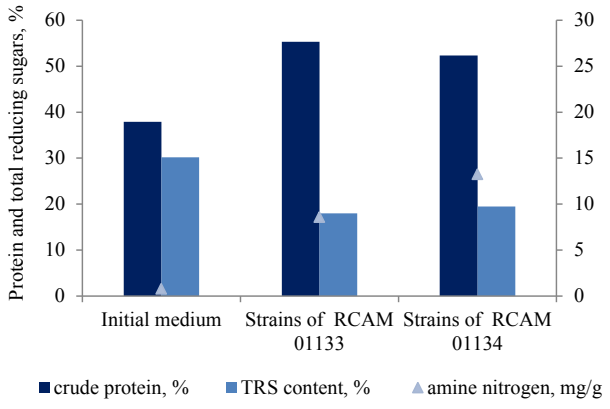


Figure 2 Contents of crude protein, total reducing sugars (TRS), and amine nitrogen in *Aspergillus oryzae* strains of RCAM 01133 and RCAM 01134 during solid-phase cultivation

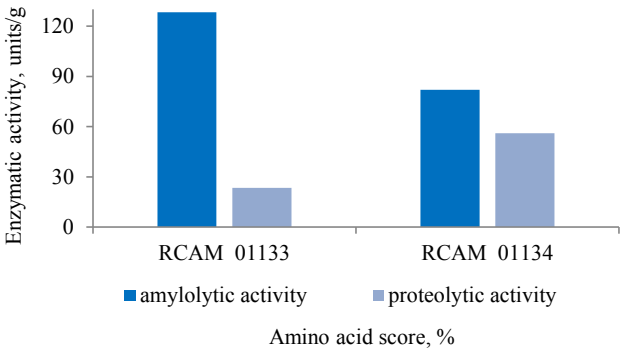


Figure 3 Enzymatic activity of *Aspergillus oryzae* strains, RCAM 01133 and RCAM 01134

more α-amylase. The amylolytic activity of this strain reached 128.3 units/g, which was 1.6 times as high as that of the other strain. However, the proteolytic activity was 2.4 times higher in RCAM 01134 compared to RCAM 01133 (56.0 units/g and 23.5 units/g, respectively).

The grown fungal cultures were subjected to dry fermentation during which protein and carbohydrate polymers converted under the action of intracellular enzymes of micromycetes. After 48 h, the fermented biomass acquired a dark brown color, indicating the end of dry fermentation.

Then, we developed test samples of fermented sauces with the *A. oryzae* strains by extraction with saline. According to their biochemical and sensory analysis, the conversion of soy materials resulted in the accumulation of protein substances in the fermented sauces. The total amounts of amino acids were 23.0 g/100 g and 23.35 g/100 g in the sauces with RCAM 01133 and RCAM 01134, respectively. The percentages of essential amino acids amounted to 44.83% and 42.73%, respectively (Fig. 4.).

In addition to increasing total amino acids in the fermented sauces, caused by the synthesis of protein substances during the solid-phase cultivation of the fungus, the fermentation released amino acids from bound forms into free forms (Figs. 2, 4). Free amino acids accounted for 43.9% and 48.3% in the sauces

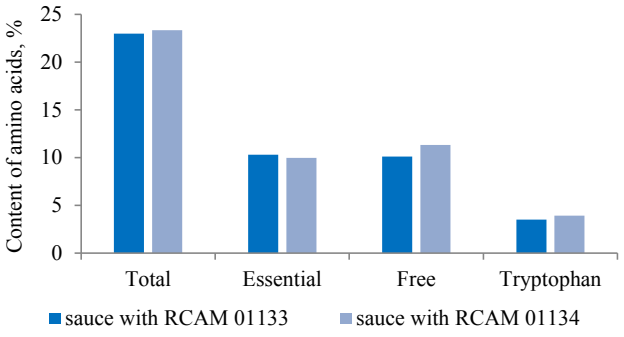


Figure 4 Contents of amino acids in fermented soy sauces with *Aspergillus oryzae*

Table 1 Essential amino acid contents in the initial soy material and fermented sauces vs. reference protein

Amino acid	Content of essential amino acids, g/100 g protein			
	Reference protein	Initial medium	Sauce with RCAM 01134	Sauce with RCAM 01134
Valine	5.0	4.11	5.60	4.74
Isoleucine	4.0	4.16	3.89	3.36
Leucine	7.0	7.03	4.04	3.71
Lysine	5.5	5.28	7.17	6.19
Methionine	3.5	1.17	2.65	2.76
Threonine	4.0	3.26	3.92	3.43
Phenylalanine	6.0	0.28	2.16	2.06
Tryptophan	1.0	1.07	15.40	16.48
TOTAL	36.0	26.36	44.83	42.73

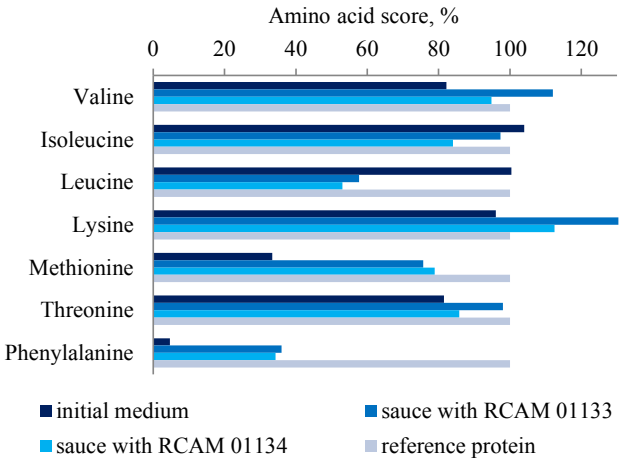


Figure 5 Amino acid scores of initial medium and fermented sources vs. reference protein

with RCAM 01134 and RCAM 01134, respectively. About 50% of them were essential amino acids, which indicated higher medical and biological efficiency of the fermented products since free amino acids are easily absorbed into the blood, activating metabolic processes in the human body (Fig. 4)

The cultivation of *A. oryzae* micromycetes on a plant medium not only increased the content of protein substances but also improved their biological value in the fermented sauces. As we know, the content and composition of essential amino acids in the FAO/WHO reference protein can fully meet the physiological needs of the human body [34–36]. Our studies showed that the test samples of fermented soy sauce contained a full range of essential amino acids. Their amount was 1.7 times as high as in the original plant medium and 1.2 times as high as in the reference protein, mainly due to a high content of tryptophan (Table 1, Fig. 4).

We compared the composition of essential amino acids and their scores in the fermented products (Table 1, Fig. 5). The amino acid score, which is calculated by comparing the content of each amino acid in the proteins of the initial medium (soy meal) and the fermented sauces with its content in the reference protein, shows changes taking place during soy fermentation [37, 38].

We found that phenylalanine, methionine, valine, and threonine were the limiting amino acids in the protein of the initial soy material, with their score under 82.2%. Although the score of the main limiting amino acid, phenylalanine, was only 4.7%, it increased 7.7–7.3 times in the fermented sauces, reaching 36.0–34.3%. The score of the second limiting amino acid, methionine, more than doubled as a result of fermentation. The scores also increased for valine, threonine, tryptophan, and lysine but decreased for leucine (Fig. 5).

The composition of essential amino acids in the fermented sauce proteins did not differ significantly, featuring three limiting amino acids – phenylalanine, leucine, and methionine. The score of the main limiting amino acids was slightly higher in the sauce with the RCAM 01133 strain of *A. oryzae*.

The increased score of individual amino acids (tryptophan, valine, lysine, methionine, and phenylalanine), which resulted from the fermentation of plant materials by micromycetes, indicated a higher biological value of the fermented products. The highest score was recorded for tryptophan, whose content was 15.40 g and 16.48 g per 100 g protein in the sauces with RCAM 01133 and RCAM 01134, respectively (Table 1).

It appears that the predominant amount of tryptophan gave the sauces their major functional properties. Tryptophan is known for its antidepressant, hypoglycemic, and cardioprotective effects. It helps to regulate appetite and synthesize the "joy hormone", serotonin. Also, it stimulates the production of growth hormone and vitamin B3 (niacin). According to recent studies, tryptophan exerts a stress-protective effect on the cardiovascular system and acts not only as a precursor of the main neurotransmitter, serotonin, but also as a component of the body's antioxidant system [39]. Tryptophan deficiency entails disturbances in the psycho-emotional state of a person such as sleep disturbances, digestive problems, and mental disorders. Together with magnesium deficiency, it can cause

Table 2 Sensory and physicochemical indicators of the fermented sauces based on *Aspergillus oryzae*

Indicator	Sauce with RCAM 01133	Sauce with RCAM 01134
Appearance	Opaque liquid	
Color	Dark brown	
Aroma	Strong mush-room-like aroma	Mushroom-like aroma
Dry substances, %	29.7	27.5
Carbohydrates (reducing substances), %	2.3	1.9
Protein, %	27.9	26.1
Amine nitrogen, mg%	574.0	630.0

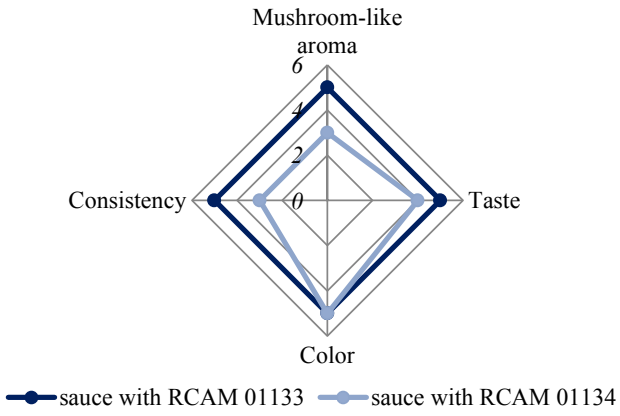


Figure 6 Sensory profilogram for fermented sauces based on *Aspergillus oryzae*

spasms of the coronary artery, cardiovascular pathology, and diabetes mellitus.

Increased amounts of valine, lysine, and methionine add more functional properties to fermented foods. They are among the most important essential amino acids, whose biological role in the human body is primarily associated with protein metabolism. They help with the growth and repair of tissues, accelerate cell regeneration, prevent the formation of lipoproteins, and participate in the regulation of the immune system [40, 41].

Sensory and physicochemical indicators of the fermented sauces are shown in Table 2 and Fig. 6. We found that the RCAM 01133-based sauce had a more pronounced mushroom-like aroma and a uniform consistency.

CONCLUSION

Our study investigated the bioconversion of soy materials under the influence of two strains of the filamentous fungus *Aspergillus oryzae* that produce hydrolytic enzymes. The strains differed in their morphological characteristics and cultural properties.

We found that the cultivation of the RCAM 01133 and RCAM 01134 strains on a nutrient medium with soy meal increased the concentration of protein and amine nitrogen, as well as decreased the content of polysaccharides. The fermented soy sauce contained a full range of essential amino acids whose amount was larger than in the protein of the initial soy material and in the reference protein.

The comparative analysis of the fermented sauce proteins and the reference protein showed that the test samples had a higher biological value compared to the initial medium (soy meal). We found that fermentation increased the score of the main limiting amino acids, phenylalanine and methionine, as well as that of valine, threonine, tryptophan, and lysine. The score of leucine, however, decreased.

As a result, we selected the *A. oryzae* RCAM 01133 strain for use in biotechnology of functional additives based on microbial and biocatalytic conversion of plant materials. This strain had a high growth rate and a low spore-forming ability, which makes it technologically attractive for use in production.

To conclude, the fermented RCAM 01133-based soy sauce contained a full range of essential amino acids in free form and possessed good sensory properties. Thus, we can recommend it for ready meals as a seasoning and a salt substitute enriched with essential amino acids.

CONTRIBUTION

All the authors are equally responsible for the obtained research results and the manuscript.

CONFLICT OF INTEREST

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

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Modelling formation and removal of biofilms in secondary dairy raw materials

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Abstract:
Introduction. Microorganisms of dairy raw materials tend to adhere to the surfaces of processing equipment and form sustainable biofilms, which is a serious issue in the dairy industry. The goal of the present work was to investigate formation of biofilms on a glass surface in static model conditions, and removal of such biofilms by cleaning.
Study objects and methods. The study objects were the permeates of skim milk, sweet whey and acid whey, as well as the biofilms formed and washings from glass slides. Biofilms were removed from the glass with detergents used in the dairy industry. Standard methods of determining microbiological and physicochemical properties were used to characterize the permeates. The biofilm structure and morphology of microorganisms participating in biofilm formation were investigated with light spectroscopy. The efficiency of biofilm removal in a cleaning process was quantified with optical density of washings.
Results and discussion. Biofilms in whey permeates formed slower compared to those in skimmed milk permeate during the first 24 h. Yeasts contributed significantly to the biofilm microflora in acid whey permeate throughout five days of biofilm growth. Well adhered biofilm layers were the most stable in skimmed milk permeate. The highest growth of both well and poorly adhered biofilm layers was observed in sweet whey permeate after three to five days. It was established that the primary attachment of microorganisms to a glass surface occurred within 8 h, mature multicultural biofilms formed within 48 h, and their partial destruction occurred within 72 h.
Conclusion. The research results can be used to improve the cleaning equipment procedures in processing secondary dairy raw materials.

Keywords: Biofilm, whey, formation, removal, filtration, dairy industry, secondary dairy raw materials

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INTRODUCTION

Biofilms are complex microbial ecosystems formed at the interface usually on hard surfaces. Different types of viruses, bacteria and fungi in natural biofilms coexist, immersed in an extracellular matrix of polysaccharides, proteins and DNA. The matrix provides structural and functional benefits for biofilm microorganisms, including hydration, uptake and digestion of nutrients, protection from adverse environmental conditions, and exchange of genetic information [1].

Biofilms is a serious problem in the food industry as they can form on equipment and food surfaces. Biofilm microorganisms release substances that destroy the equipment material and spoil food products. The development of pathogenic microbes in biofilms is most dangerous, as they can acquire resistance to antimicrobial drugs in such communities.

Traditional chemical, physical and mechanical methods of removing contaminants and suppressing microorganisms used in the food industry are not always effective enough against biofilms. Therefore, different approaches are used to control them: proteases enzymatic treatment, glycosidases or DNAs; a steel surface modification by applying silver, copper or zinc nanoparticles, or new antibiofilm polymers with lysozyme or bacteriocins; introduction of biosurfactants into detergents, etc. [2, 3].

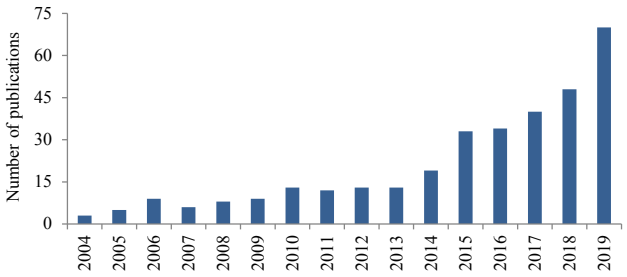

Figure 1 The number of publications with the words "biofilm dairy" in PubMed by year (https://www.ncbi.nlm.nih.gov/pubmed/?term=biofilm+dairy; Date of the application: 02.04.2020)

The biofilm formation in the equipment surface significantly affects the quality and safety of dairy products [4, 5]. The recent increase of biofilm research publications about the dairy industry indicates the relevance of this research (Fig. 1).

A biofilm goes through a number of stages in its development: an initial attachment, an irreversible adhesion, an early development of the structure (microcolonies formation), a growth of biofilm layers, a formation of a complex three-dimensional structure, and its subsequent partial destruction [2, 6]. During processing, the components of dairy raw materials can settle on the surface of the equipment, which creates favorable conditions for the fixation of microorganisms and the development of biofilms. Their structure and properties significantly depend on the type of processed raw material, its bacterial contamination, and processing conditions. The emerging communities of microorganisms surrounded by an exopolymer layer are very resistant to thermal and chemical treatment. Therefore, a biofilm formation reduces the efficiency of washing and disinfection procedures standard for the dairy industry [5, 6].

Different groups of microorganisms can participate in biofilm formation in dairy processing plants. The attention of biofilm researchers is attracted by *Pseudomonas* psychrophiles, which secrete heat-resistant lipolytic and proteolytic enzymes, and *Geobacillus*, *Brevibacillus*, *Paenibacillus*, *Sporosarcina* spore-forming bacteria [7–9]. Lactic acid microorganisms are also actively involved in biofilm formation [10, 11]. Opportunistic and pathogenic bacteria, such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Campylobacter* spp., *Salmonella* spp., and *Enterobacter sakazakii*, are of particular concern, because microbial communities can transfer horizontally genes resistant not only to disinfectants but also to antibiotics [4, 12, 13].

Biofilms are formed during ultrafiltration and reverse osmosis of secondary dairy raw materials. This is due to the fact that proteins and poorly soluble mineral salts are deposited on the membranes, contributing to the consolidation and reproduction of microorganisms. The rate of these processes depends on the properties of the raw material, its microflora composition, as well as on the roughness and hydrophobicity of the membranes. The formation of protein-mineral layers and biofilms embedded in them leads to several problems: from a decrease of equipment productivity to a destruction of membranes and deterioration in the microbiological parameters of raw materials processing products. This is the reason for improving the processes of cleaning equipment, the search for new methods to prevent the development of biofilms in its surface [14].

The type of secondary dairy raw materials significantly affects the structure and rate of biofilm formation. Thus, the number of Bacillus spore-forming rods in 48 h biofilms on reverse osmosis membranes in the UF permeate of skimmed milk was by a factor of 1.6 more than in sweet whey, and by a factor of 1.2 more when these types of raw materials were alternated. At the same time, bacilli biofilms in skimmed milk permeate proved to be more resistant to standard washing procedures than in sweet whey and with alternation of different types of raw materials. Scanning electron microscopy showed that Bacilli were present in the form of multilayer clumps of cells, aggregates or irregular clusters in biofilms permeate, and in whey-permeate alternation. Monolayers of this culture, smoother and flattened in shape, were found in whey biofilms. The atomic microscopy revealed that biofilms of skimmed milk permeate had the highest surface roughness among other biofilms [15].

The type of raw material (milk, whey) and its preliminary processing (pasteurization, whey clarification) largely determine the diversity of bacteria on spiral ultrafiltration membranes [16]. It has been established that the composition of early communities of microorganisms formed on membranes during milk and whey ultrafiltration is influenced not only by the composition of raw material and the temperature of its supply, but also by the microbial environment of the processing plant [17].

Ryabtseva *et al.* investigated the processes of biofilm formation on glass in skimmed milk permeate and main regularities of their removal by standard washing procedures [18]. It was shown that the microflora of skimmed milk permeate forms complex multicultural biofilms on the glass within a few hours and its mature multilayer structure is observed already after 24 h. The authors noted that the imitation of the standard equipment washing procedure did not remove microorganisms from the glass completely. Considering a significant influence of the raw materials composition on biofilm formation, it is relevant to compare the regularities of this process in skimmed milk permeate and other types of secondary dairy raw materials.

The aim of this work was to study the processes of biofilm formation and removal in permeates of skimmed milk, sweet whey and acid whey in the model static conditions on glass.

STUDY OBJECTS AND METHODS

The research objects were ultrafiltration permeate of secondary dairy raw materials (skimmed milk, sweet whey, and acid whey), biofilms formed on glass, and washings from glass slides in water and detergent solutions. The permeates were obtained in industrial ultrafiltration equipment with Koch Dairy-Pro 6438 UF-10K polymer spiral membranes of a spacer thickness of 0.76 and 1.14 mm (MWCO 10 kDa, Koch Membrane Systems, USA). Ultrafiltration was carried out at 10 ± 2°C; the concentration factor was 1.4, 13.0 ± 1, and 27.0 ± 5 for skimmed milk, sweet whey, and acid whey, respectively.

The analysis of microbiological indicators of secondary dairy raw materials was carried out using petrifilms in accordance with State Standard 32901-2014[I], State Standard 33566-2015[II] and MUK Methodical Guidelines 4.2.2884-11[III]. The number of mesophilic aerobic and facultative anaerobic microorganisms (NMAFAnM) was determined using 3M ™ Petrifilm ™ Aerobic Count Plate (AC), the inoculated media were incubated at 30 ± 1°C for 72 ± 3 h. Red colonies were counted on petrifilms with the number of colonies from 15 to 300.

Yeast and molds were determined using 3M™ Petrifilm™ Yeast and Mold. Incubation was carried out at 24 ± 1°C for 72 ± 3 h for preliminary counting, and 120 ± 3 h for a final counting. Analyzing yeast, colonies of various colors (from pink-yellow to blue-green) with smooth edges were counted on petrifilms with the number of colonies from 5 to 150. Analyzing molds, colonies of various colors (black, yellow, green, blue) with a diffuse edge and a clear center were counted on petrifilms with the number of colonies from 5 to 50. Microscopic methods differentiated yeast and mold colonies.

Coliform bacteria were incubated on a 3M™ Petrifilm™ Coliform Count Plate, at 37 ± 1°C for 24 ± 2 h. Red colonies with gas bubbles were counted on petrifilm with the number of colonies from 15 to 150.

The experiments were repeated three to five times. Statistical processing of experimental results and their graphical presentation was performed with Microsoft Office Excel 2010. Analysis of variance (ANOVA) was used to determine the significance of differences.

To simulate the process of biofilm formation in statics, glass slides (26×76 mm) were placed in Petri dishes with 25 cm^3 of the samples under study. The work was carried out in sterile conditions. The closed Petri dishes were incubated at 25 ± 1°C for 120 h. In certain time intervals (4, 8, 24, 48, 72, 96, and 120 h), the slides were removed with tweezers, washed in distilled water, and used to have fixed preparations. The preparations were stained with methylene blue to determine the shape, size and relative position of cells, and according to Gram to differentiate cells into Gram-positive and Gram-negative microorganisms. The preparations were viewed in a binocular microscope with an Axio Imager 2 digital camera (Carl Zeiss, Germany).

To study the patterns of biofilm removal, a standard procedure of washing an electrodialysis unit was simulated. For this purpose, slides with formed biofilms were dipped into 100 cm^3 glass beakers with distilled water or detergent solutions and kept for 5 min, shaking occasionally. The treatment was carried out with Divos detergents (Diversey, USA), which are generally used in the dairy industry, in the following sequence: water, 1% acid solution (Divos 2), water, 0.1–0.2% enzyme solution (Divos 80-2) and 0.5% buffer solution (Divos 95), water, 0.5% alkaline solution (Divos), water. The optical density of the washings was measured with a UNICO 1201 spectrophotometer (USA) at a wavelength of 460 nm.

RESULTS AND DISCUSSION

Biofilms formation in secondary dairy raw materials on glass. Skimmed milk permeate. The studied samples had the following physicochemical and microbiological parameters: mass fraction of dry substances 4.9 ± 0.1%, pH 6.39 ± 0.15, NMAFAnM 5.6 ± 0.9×10^4 CFU/cm^3, CGB (coli-forms) 2.7 ± 0.3×10^2 CFU/cm^3, yeast 4.1 ± 0.5×10^2 CFU/cm^3, mold 0–21 CFU/cm^3.

Figure 2 shows the images of biofilms formed in skimmed milk permeate on glass slides.

It is known that the onset of biofilm formation depends on the surface physical and chemical properties, characteristics of the initially present bacteria, and the process parameters [14, 17]. We found that biofilms in permeate samples formed rather quickly even on a smooth glass surface (Fig. 2). As early as after 4 h of the experiment, in some fields of vision, we could see individual rod-shaped and spherical cells, both purple (Gram-positive) and red (Gram-negative). However, these fields of vision were not typical and cells were not detected in a large area of glass. After 8 h, almost all fields of view featured cells of well-stained large oval (single and paired) Gram-positive cocci, as well as clusters of weakly stained small short Gram-negative rods and larger Gram-positive rods attached to the glass (Fig. 2a).

After 24 h of biofilm formation, significantly more cells remained on the glass after washing. We observed numerous clusters of spherical cells, as well as individual and long-chain sticks in separate fields of vision. Gram-stained bacteria were dark red

[I] State Standard 32901-2014. Milk and milk products. Methods of microbiological analysis. Moscow: Standartinform; 2015. 26 p.

[II] State Standard 33566-2015. Milk and dairy products. Determination of yeast and mold. Moscow: Standartinform; 2019. 14 p.

[III] MUK 4.2.2884-11 Metody mikrobiologicheskogo kontrolya ob″ektov okruzhayushchey sredy i pishchevykh produktov s ispol′zovaniem petrifil′mov [MG 4.2.2884-11. Methods for microbiological control of environmental objects and food products using petrifilm]. Moscow: Federal Center for Hygiene and Epidemiology of Rospotrebnadzor; 2011. 24 p.

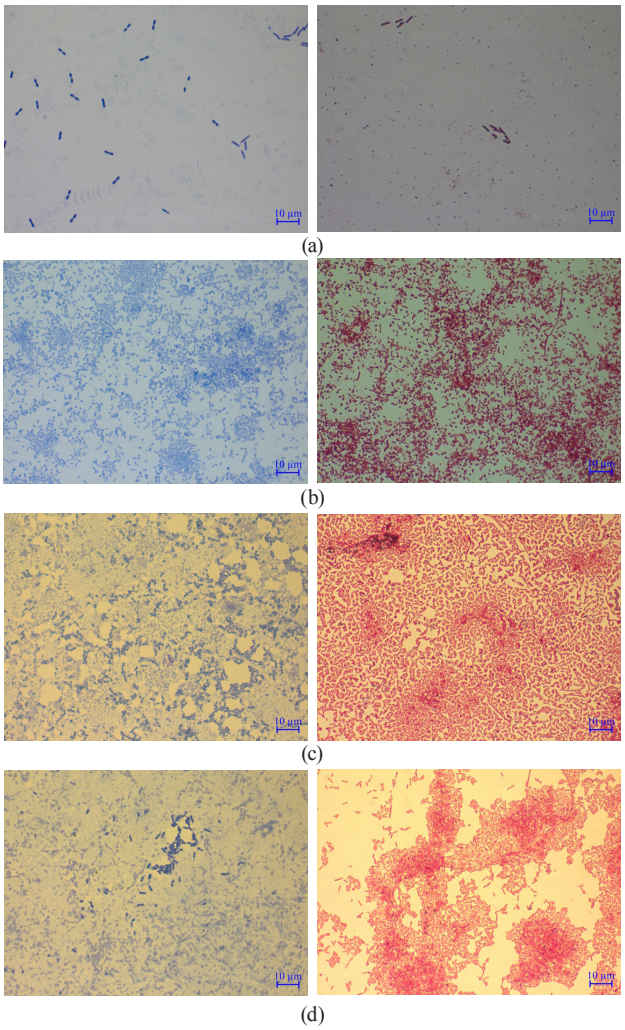

Figure 2 Micrographs of biofilms formed in skimmed milk permeate in: (a) 8 h, (b) 24 h, (c) 48 h, and (d) 72 h. Methylene blue staining (left) and Gram-staining (right). Magnification 1000×

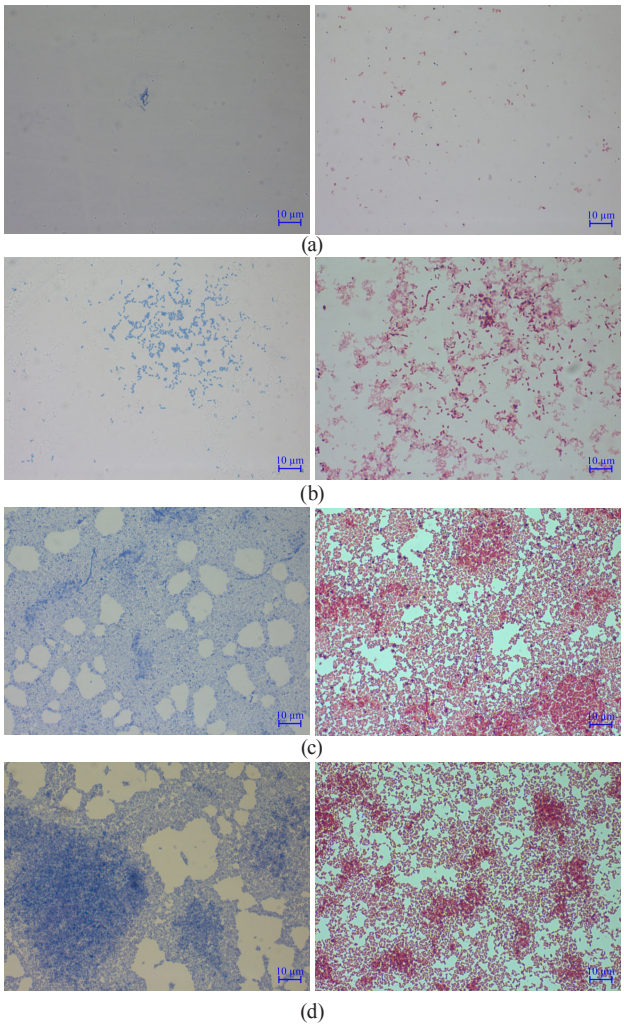

Figure 3 Micrographs of biofilms formed in sweet whey permeate in: (a) 8 h, (b) 24 h, (c) 48 h, and (d) 72 h. Methylene blue staining (left) and Gram-staining (right). Magnification 1000×

and possibly Gram-variable. The background was a network of small bluish or pinkish rods, and their weak coloration, apparently, indicated the formation of a protective exopolymer layer (Fig. 2b). Large oval yeast cells could be seen in some vision fields, though such fields were not typical of biofilms in skimmed milk permeate.

After 48 h, a visible mucous film formed on the glass, partially washed off during the first rinse with water. Microscopic examination on glass revealed an almost continuous layer of small short Gram-negative rod-shaped bacteria with dark red cocci islands. In some fields of vision, under the upper layers of stained cells, there were dense lower layers with individual bacteria that were not visible (Fig. 2c). We can assume that the formation of a mature biofilm well fixed on the glass surface was completed at that stage.

After 72 h, we observed an increase in the mucus thickness on the glass. After rinsing with water, the micropreparations in most fields of vision looked almost the same as they did after 48 h. Large dark blue cocci, mostly paired, were visible on the surface of the biofilm. In some fields of vision, we found significant areas free of cells, which might have formed after large areas of biofilm were detached by glass rinsing (Fig. 2d). With longer incubation (up to five days), we found no significant changes in the structure of biofilms.

Sweet whey permeate. The samples had the following physicochemical and microbiological parameters: mass fraction of dry substances 4.9 ± 0.1%, pH 6.42 ± 0.15, NMAFAnM 1.4 ± 0.5×10^3 CFU/cm^3, CGB (coli-forms) 2.9 ± 0.4×10^2 CFU/cm^3, yeast 2.8 ± 0.1×10^2 CFU/cm^3, mold 17 ± 60 CFU/cm^3.

Figure 3 shows the images of biofilm formed in skimmed milk permeate on glass slides.

After 4 h of incubation, we found no signs of fixation or growth of microorganisms on the glasses. Microscopic examination of stained biofilms formed after 8 h showed individual cells and rare clusters of Gram-positive small cocci and Gram-negative small

rods in some fields of vision (Fig. 3a). After 24 h, separate zones of Gram-positive cocci, forming pairs and short chains, appeared on a thin layer of Gram-negative short rods. We should note that during the first 24 h, biofilms in the sweet whey permeate formed more slowly than those in skimmed milk permeate (Fig. 3b). Another 24 h of incubation resulted in biofilms of approximately the same composition as in the milk samples incubated for 48 h. However, biofilms in the whey samples were less dense than those in the milk permeate (Fig. 3c).

After 72 h, the biofilm became denser, while the unevenness of its distribution increased, and spaces free of cells appeared (Fig. 3d). In many fields of vision, we found thick exopolymer layers with enclosed cells of indefinite shape. These layers were laced with long filaments of rod-shaped cells. In some areas of the biofilm, large yeast cells could be seen. Subsequently, after four and five days of biofilm formation in the sweet whey permeate, there was an increase in the mucous layer on the glass. The microstructure of biofilms after washings was similar to that formed after 72 h.

Acid whey permeate. The permeate samples had the following physicochemical and microbiological indicators: mass fraction of dry substances 4.8 ± 0.1%, pH 4.65 ± 0.05, NMAOAnM 2.9 ± 0.5×10^3 CFU/cm^3, CB (coli-forms) were not detected in 1 cm^3, yeast 2.2 ± 0.6×10^2 CFU/cm^3, mold 11–62×10 CFU/cm^3.

Figure 4 shows the images of biofilm formed in acid whey permeate on glass slides.

After 4 h of the experiment, we found single cells of yeast and Gram-positive cocci in some fields of vision. After 8 h, most fields of vision featured single and paired Gram-positive cocci and small clusters of large oval yeast cells (2–3×4–6 μm). We can assume that these clusters formed as a result of their longitudinal budding, sometimes with the signs of a false mycelium formation (Fig. 4a).

After 24 h, the yeast formed large groups of uniformly colored cells. Staining with methylene blue revealed than they had the clearly delineated elongated shape. We also found clusters of small rods and diplococci weakly stained with methylene blue (Fig. 4b).

After 48 h, they formed a thin broken layer with numerous clusters of yeast cells, and budding became less noticeable. In general, during this period of biofilm formation, its structure (excluding yeast cells) was similar to the structure of biofilms in skimmed milk and sweet whey permeates after 48 h. However, further incubation led to a destruction of the formed layers instead of thickening. After 72 h, we found irregularly spaced individual clusters of rod-shaped and spherical bacteria with single and grouped yeast cells (Fig. 4d). Microorganism-free zones increased. The microstructure of biofilms after four and five days was practically the same.

Some samples contained a mold resembling *Geotrichum lactis* in the morphology of thick hyphae,

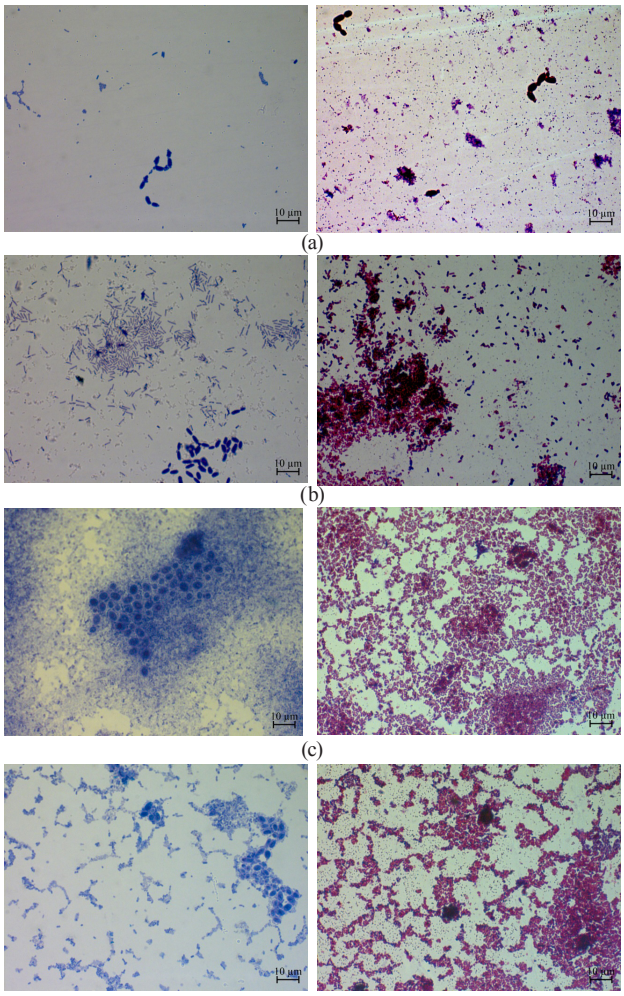

Figure 4 Micrographs of biofilms formed in acid whey permeate in: (a) 8 h, (b) 24 h, (c) 48 h, and (d) 72 h. Methylene blue staining (left) and Gram-staining (right). Magnification 1000×

which easily disintegrated into rectangular oidiospores with rounded ends. After 48 h, a dense whitish film with a pronounced unpleasant (rancid, moldy) odor formed on the surface of the permeate in Petri dishes. This film was tightly attached to the slide and was poorly washed off.

Comparison of results of different types of raw materials and data by other researchers. The analysis of the results showed both common features and differences between the processes of biofilm formation in permeates of different types of secondary dairy raw materials on glass in statics. After 8 h, we observed the attachment of microbial cells to the glass surface and the beginning of their reproduction in all three types of raw materials. Apparently, this was the first stage of reversible attachment of microorganisms when they could be easily removed from the surface but could become the basis for further growth of biofilm [15].

After 24 h, all types of raw materials had a thin, poorly washed off biofilm formed on the glass. This process involved small Gram-negative rods, which

formed a layer tightly adjacent to the glass, and Gram-positive cocci with a predominantly paired arrangement of cells located on the rod layer. Studies have shown that by this time, biofilms can already reach the stage of irreversible attachment to the surface of UF membranes, when their removal with conventional washing and disinfection protocols is difficult [16, 17].

After 48 h, a mature multilayer biofilm formed in all the permeate samples. Unevenly colored layers of biofilms and bridges between them indicated the presence of an extracellular polymeric substance. The exopolymer layer held the cells of microorganisms together, firmly attached them to the surface and protected them from adverse influences. The observed heterogeneous regions in the homogeneous matrix of the biofilm were consistent with the data obtained by Anand *et al*. who found that in multicultural biofilms each species could produce different polymers, which fused later [14].

At this stage, the formation of biofilm layers could occur due to the use of nutrients not only from the environment, but also from the initial film. The secretion of the exopolymer layer continued, and the thickness of the biofilm on the equipment could increase due to a continued deposition and adhesion of dairy components to the layer. This was consistent with previously published data that three-dimensional structures of mature films at a late stage of development commonly had a mushroom shape with an uneven distribution of microorganisms and channels inside. This stage of the biofilm existence might already be characterized by the processes of exchange of signaling molecules and the formation of a "quorum sensing", the exchange of genetic information and the acquisition of inherited resistance to antibiotics and disinfectants [4, 14].

Later (after 72 h or more), rinsing the glasses separated part of the mucus formed on their surface. All the samples showed areas free from microorganisms. Apparently, this occurred due to partial destruction of a biofilm, which corresponded to the last stage of their development [4, 14].

The main difference between biofilms in sweet and acid whey was their slower formation at the first stage (24 h) compared to biofilms in skimmed milk. This might be due to its composition (for example, the higher protein content in milk) and the properties of the raw materials (for example, the lower active acidity of whey compared to milk). Perhaps these differences were due to the influence of starter cultures contained in these types of raw materials, which were based on *Lactococcus lactis* subsp. *lactis* capable of producing nisin.

The obtained data were consistent with the findings of Marka and Adand [15]. The authors found that during the processing of skimmed milk permeate, biofilms on reverse osmosis membranes formed faster than during processing of sweet whey. Apparently, this was due to the fact that the microflora of milk is represented by α-, *β*- and γ-proteobacteria, bacilli, flavobacteria and actinobacteria, while fresh and pasteurized sweet whey mainly contains *Lactococcus spp.* [16].

The structural peculiarity of biofilms in the acid whey permeate was yeast, which took part in their formation at all stages of their development. Probably, due to autolysis and the release of various enzymes into the environment, yeast contributed to the destruction of biofilms after 72 h. We should note that the role of yeast in biofilm formation has been little studied and requires further analysis.

In the later stages of development, the upper layers of biofilms became unstable and were easily detached when the glasses were rinsed. This can cause continuous contamination of raw materials and products. At the same time, the cells separated from a biofilm are more resistant to external influences as compared to ordinary (planktonic) cells.

Studies have shown that the formation of biofilms can lead to biodegradation of materials, equipment malfunctions, a decrease in its performance, increased energy consumption and consumption of detergents, as well as problems with the quality and safety of products [2, 4, 14]. The peculiarities of the composition and properties of biofilms in different types of raw materials must be taken into account when improving the procedures of washing technological equipment with alkalis, acids and enzymes.

Removal of biofilms formed in secondary dairy raw materials on glass. To study the patterns of biofilm removal, we used the standard procedure of washing the electrodialysis unit and the spectrophotometric method for determining the optical density of the washings. When setting up the experiments, we assumed that the upper layers of the biofilm, which were not attached to the glass surface, passed into a wash water after the first rinsing of the glasses. The fixed layers of biofilms were removed with cleaning solutions (acidic, alkaline and enzymatic). After using each type of washing solution, the slides were washed with distilled water.

The washout results are presented in diagrams (Fig. 5). They show the optical density (D) of the wash water after the first rinsing of the glasses (W1), the total D of the acid solution and the subsequent wash water (A + W2) after rinsing glasses; the total D of the enzyme solution and the subsequent wash water (F + W3) after rinsing glasses; the total D of the alkali solution and the subsequent rinsing water (Al + W4) after rinsing the glasses, as well as the total D (Σ) for all washings of the fixed layers of biofilm with washing solutions (A + W2, F + W3 and Al + W4).

Skimmed milk permeate. The analysis of results (Fig. 5a) shows that the amount of structural elements of biofilms formed on glass in the permeate, easily washed off with water, increased significantly after three and four days, and by five days it decreased to the level of the first and second days.

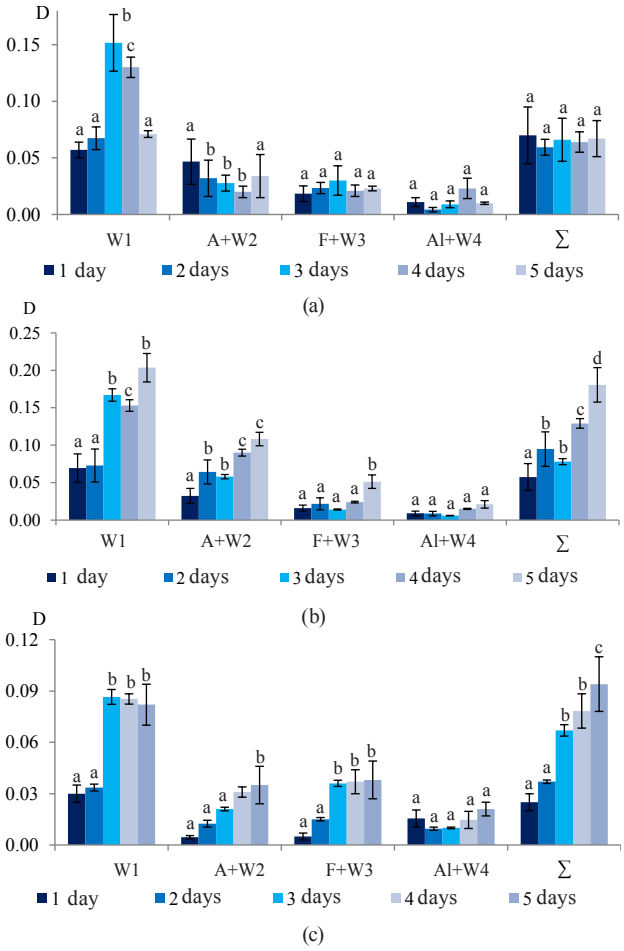

Figure 5 Dependence of the optical density (D) of washings of biofilms formed on glass in permeates of skimmed milk (a), sweet whey (b), and acid whey (c) on processing conditions (W1 – water, A + W2 – acid solution and water; F + W3 – enzyme solution and water; Al + W4 – alkali solution and water; Σ – sum of D for A + W2, F + W3 and AL + W4 washes). Letters a, b, c, d show significant differences ($P \leq 0.05$) between the mean values in the same series of D values for one type of washes

The acidic wash solution was more effective in removing microorganisms from the 24 h biofilm than on the following three days. The efficiency of enzyme solutions was somewhat lower, and the lowest values of optical density were recorded in alkaline solutions. The maximum values of the optical density of washings in enzyme solutions were found after three days, in alkaline solutions – after four days. However, we did not reveal a statistical significance of differences in the optical density of washings in these solutions on different days of the experiments.

The total optical density of washing solutions remained practically constant for five days of the experiment. This indicated that as early as after 24 h, a layer of well-fixed microorganisms with a fairly stable structure and thickness formed on the glass in the skimmed milk permeate. The properties of this layer changed little in the following days, while layers of microorganisms gradually built up on top, which were easily washed off with water. The thickness of these layers reached its maximum after three days.

A microscopic examination of glass slides after all stages of washing in some fields of vision revealed individual cells of cocci and rods.

Sweet whey permeate. We found that in the first two days, the average values of the optical density of the washings after the first rinsing of glasses with water were similar to those obtained in experiments with skimmed milk, but with a wider range of values (Fig. 5b). However, after three and five days of experiments, much more microorganisms were washed off from biofilms in sweet whey permeate than from biofilms in milk permeate. A significant decrease in the optical density values of these washings after four days indicated the instability of the properties of the upper layers of mature biofilms.

Acid treatment was more effective in removing sweet whey biofilms than other detergent solutions. At the same time, there was a gradual increase in the values of optical density of washings in acid solution from 1 to five days, with a slight decrease after three days. Apparently, this was due to the growth of fixed exopolymer layers of biofilms, which were soluble in an acidic medium. The optical density of the washings in enzyme and alkaline solutions was lower than in acidic solutions, and remained at the same level throughout the experiment, except a statistically significant increase in the enzyme solution after five days. Assumingly, few microorganisms remained on the glass after acid washing. However, microscopic examination of the slides after all stages of washing revealed a significant number of microorganisms, including cocci, yeast, and in some fields of view, clusters of rods.

The analysis of the total values of the optical density of washing solutions showed that the fixed layer of biofilms increased significantly after two, four and five days. The 3-day biofilm was more unstable, with part of it removed during the first washing. In general, fixed and non-fixed layers of biofilms in sweet whey permeate tended to grow during the entire experiment, reaching their maximum after five days.

Acid whey permeate. The experiments with acid whey revealed lower values of the optical density of washings after the first rinsing of glasses than in the experiments with other types of secondary dairy raw materials (Fig. 5c). Despite a significant increase in optical density after three and five days, it was approximately 1.5 and 2 times lower than in skimmed milk and in sweet whey, respectively.

The cleaning efficiency with acidic and enzyme solutions gradually increased with the growth of the biofilm. We found a statistically significant increase in the optical density of the washings on day five for acidic solutions and on days three and five for enzyme solutions. Alkaline treatment of a 24 h biofilm was

more effective than in acidic and enzyme solutions. This suggested that the properties of biofilms in acid whey at the initial stage of development differed from those of biofilms in skimmed milk and sweet whey. Subsequently, with an increase of biofilm formation time, the efficiency of acidic and enzymatic treatments increased.

The total optical density of washings from biofilms in the acid whey permeate on the first day was 3 and 2.4 times lower than in similar experiments with skimmed milk and sweet whey, respectively. After five days of biofilm formation in the acid whey permeate, the total optical density reached its maximum values, which was 1.3 times higher than in milk permeate and two times lower than in sweet whey permeate.

Microscopic examination of glass slides after all cleaning procedures revealed cells of microorganisms, including yeast, and in some experiments, milk mold.

Comparison of results of different raw material types and data by other researchers. We found that at the first stages of biofilm formation, all studied types of secondary dairy raw materials showed similar general patterns of biofilm removal during the first washing with water. All the species demonstrated the same level of optical density after one and two days, with its significant increase after three days. At the same time, the highest washout values during research period were found in experiments with sweet whey, the lowest one in experiments with acid whey.

The remaining layers of biofilms were effectively removed in acid solutions, which was characteristic of the experiments with sweet whey permeate. These results were consistent with the data of other researchers who studied the processes of washing ultrafiltration and reverse osmosis membranes used for processing secondary dairy raw materials [14].

Alkaline solutions are known to be widely used in the dairy industry to dissolve and hydrolyze organic substances [4]. In our work, alkaline solutions were ineffective in removing biofilms for all studied samples. Perhaps it was due to the fact that by this time of processing, fewer structural elements of biofilms remained on the glass. Moreover, cells of microorganisms were found on the glasses in all experiments, after all procedures of removing biofilms. Their number gradually increased even when the treated glasses were stored in sterile distilled water. This indicated their viability and a possibility of a new biofilm to form.

Interestingly, fixed biofilm layers formed in a skimmed milk permeate as early as after 24 h, and their thickness and properties hardly changed over the next four days. In contrast to this, fixed biofilm layers in the permeates of sweet and acid whey gradually increased during the entire time of biofilm formation.

The fastest and slowest growth of biofilms, especially after three to five days, was found in sweet whey and in acid whey, respectively. Microorganisms of whey permeates formed the most firmly fixed biofilms, for the removal of which the washing procedures were insufficient. According to Marka and Anand, biofilms of sweet whey were less resistant to standard CIP washing than biofilms of skimmed milk. Noteworthily, these results were obtained in the study on reverse osmosis membranes, with the processing of raw materials lasting 48 hours and the washing conditions being different [15].

We should also note that the conditions of modeling biofilms removal from glass used in this work did not fully correspond to real production conditions. The efficiency of cleaning production equipment does not only depend on the type, dosage and pH of the detergent, but also on pressure, washing time, flow rate during washing, and solution temperature [14]. In this regard, it would be interesting to research biofilms formed in different types of raw materials under industrial conditions.

CONCLUSION

The study revealed general regularities and differences in the formation and removal of biofilms formed on glass in statics in three types of secondary dairy raw materials – skimmed milk permeates, sweet whey and acid whey permeates.

The main general regularities included:

– time needed for primary attachment of microorganisms to glass (about 8 h), the formation of mature unevenly colored multicultural biofilms (about 48 h), and their partial destruction (72 h and more);

– bacteria participating in the formation of biofilm structure, similar in morphological and tinctorial properties, Gram-negative rods and Gram-positive cocci with a predominantly paired arrangement of cells;

– peculiarities of biofilm removal at the first stages of their formation during the first washing of glasses with water; and

– viable cells of microorganisms detected on glasses after all biofilm removal procedures.

The main differences were as follows:

– biofilms formed slower in the permeates of sweet and acid whey at the first stage (24 h) compared to biofilms in skimmed milk permeate;

– yeast participated in the formation of biofilms in the acid whey permeate at all stages of their development compared to the other permeates;

– the fixed layers of biofilms had higher stability in skimmed milk compared to those in whey;

– biofilms in sweet whey demonstrated a more significant growth after 35 days than those in skimmed milk and acid whey.

The revealed features of biofilm composition and properties in different types of secondary dairy raw materials should be taken into account to improve the procedures of washing technological equipment used in their processing.

CONTRIBUTION

S.A. Ryabtseva contributed significantly to the study concept and design, collected, analyzed, and interpreted data, as well as wrote the article. Yu.A. Tabakova collected, analyzed, and interpreted data, as well as wrote the article. A.G. Khramtsov revised substantially the important scientific and intellectual content of the article. G.S. Anisimov and V.A. Kravtsov collected, analyzed, and interpreted data.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest related to this article.

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Using Jerusalem artichoke powder in functional food production

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Abstract:
Introduction. Jerusalem artichoke is a valuable low-maintenance crop whose tubers contain vital nutrients and prebiotics. We propose using Jerusalem artichoke powder as a functional nutrient in the formulation of food products.
Study objects and methods. We studied the influence of vacuum, vibration, and grinding on the kinetics of drying Jerusalem artichoke tubers of a "Skorospelka" variety in the laboratory vacuum vibromixing mill dryer (VVMD).
Results and discussion. The rate of drying in the VVMD was almost 5.5 times as high as that of convective drying. The kinetic curves showed that grinding provided a period of decreasing drying rate until almost complete drying. Vibration drying in the VVMD was twice faster than vacuum drying. The comparison of theoretical and experimental data on moisture and drying time revealed good adequacy. The NMR analysis of changes in the molecular mobility of the samples obtained in the VVMD showed an implicit two-component spectrum, indicative of low moisture. The chemical analysis of the tubers and powders by standard methods confirmed that the proposed gentle technology (fast drying at 30°C) preserved 86% of inulin.
Conclusion. Jerusalem artichoke powder obtained in the VVMD can be used in different branches of the food industry due to its long shelf life, low consolidation, and no caking, with residual moisture of 6.1%.

Keywords: Jerusalem artichoke, tubers, bioactive ingredients, powder, mill dryer, vibration, vacuum drying, NMR, inulin

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INTRODUCTION

The demand for functional foods has grown significantly over the past decades. This trend is primarily due to people's desire to consume food of better quality. Functional foods contain physiologically active substances that produce medical and biological effects on our bodies. These health benefits make the development of functional foods a priority in the modern food industry.

Jerusalem artichoke (*Helianthus tuberosus* L.) is one of the most common ingredients in therapeutic, preventative, and rehabilitation diets. Jerusalem artichoke, also called earth apple (or over 100 other names), is a valuable plant that has several advantages over traditional agricultural crops. For example, it is highly resistant to frost, pests, and diseases. In the non-chernozem zone, its yields can reach 40 t/ha for plants and 65 t/ha for tubers [1, 2]. The commercial production of Jerusalem artichoke in Russia covers an area of only about 3000 ha, compared to 700000 ha in the USA and 500000 ha in France.

Traditionally, Jerusalem artichoke has been eaten raw or used as animal feed. In recent years, however, scientists have discovered some alternative uses. Low cultivation costs make Jerusalem artichoke a promising plant for biofuel production [3, 4]. Pharmacologists use this crop as a source of bioactive compounds [5, 6]. Other studies focus on its potential for obtaining organic acids such as lactic, butyric, and citric [7–9].

Due to the rich chemical composition, Jerusalem artichoke tubers are a valuable ingredient in functional food production [10]. They contain vital nutrients and prebiotics, especially inulin, a polyfructosan with a low glycemic index [11]. Also, the tubers contain pectin substances, vitamins, as well as macro- and microelements (Table 1).

Jerusalem artichoke tubers do not have a long shelf life, even under optimal conditions. This is due to the decomposition of biologically valuable substances caused by their own enzymes [13, 14].

Table 1 Vitamin and mineral composition of Jerusalem artichoke tubers [12]

Component	Content, mg/100 g	Percentage of daily intake, %
Vitamins:		
β-carotene	0.012	0.2
A	0.001	0.1
B1 (thiamine)	0.2	16.7
B2 (riboflavin)	0.1	4.6
B3, PP (niacin, nicotinic acid)	1.3	8.1
B4 (choline)	30	6.0
B5 (pantothenic acid)	0.4	7.9
B6	0.1	5.9
B9 (folic acid)	13	3.3
C	4	4.4
E	0.19	1.3
Minerals:		
Potassium (K)	429	9.1
Sodium (Na)	4	0.3
Calcium (Ca)	14	1.4
Magnesium (Mg)	17	4.3
Phosphorus (P)	78	11.1
Iron (Fe)	3.4	34.0
Zinc (Zn)	0.12	1.1
Copper (Cu)	0.14	15.6
Manganese (Mn)	0.06	2.6
Selenium (Se)	0.7	1.3

Powdering the tubers expands the scope of their uses in the food industry [15–17]. The advantages of food powders include a small volume, low weight, and a high concentration of nutrients. 180–190 g of Jerusalem artichoke powder is nutritionally equivalent to 1 kg of raw tubers. Jerusalem artichoke powder has a rich chemical composition and, therefore, unique properties. It is introduced into food products to improve metabolism and fight diseases such as diabetes, atherosclerosis, and obesity. Such nutrition is also recommended for increased physical and psycho-emotional stress, underperformance, and chronic fatigue syndrome. Finally, it is used to strengthen the immune system, as well as prevent and treat acute and chronic infectious diseases.

The existing technologies for producing powders involve drying the feedstock and its subsequent grinding. Intensifying thermal processes by increasing temperatures has its limitations due to the thermolability of valuable components. High temperatures and long processing times cause significant chemical transformations in the components [18]. These changes lead to losses of bioactive compounds and decrease the quality of finished products. Therefore, we need to find ways to intensify the drying of the feedstock and preserve its useful properties. Among well-known methods are vacuum drying, increasing the particle fineness of the material, as well as non-traditional methods (freeze drying, microwave drying, infrared drying, etc.) [19–21].

In this paper, we propose combining vacuum drying and vibration grinding in one apparatus to produce Jerusalem artichoke powder [22, 23]. Vacuuming helps increase moisture evaporation at low temperatures to preserve the valuable components of the feedstock. Vibromixing helps equalize temperature and humidity in the apparatus. Using grinding media increases the constant drying rate period to almost complete dehydration of the product.

We aimed to study the kinetics of drying Jerusalem artichoke tubers in a vacuum vibromixing mill dryer (VVMD) and to determine the chemical composition of the resulting powder.

To achieve this aim, we set the following objectives:
– study the influence of vacuum, vibration, and grinding on the kinetics of drying Jerusalem artichoke tubers;
– construct experimental drying curves and drying rate curves for Jerusalem artichoke tubers;
– provide a mathematical description of drying in the VVMD;
– analyze changes in the molecular mobility of the feedstock and the powders obtained under different drying conditions;
– determine the chemical composition of the feedstock and the powder obtained in the VVMD;
– assess the effectiveness of the developed technology compared to other methods of drying Jerusalem artichoke tubers; and
– suggest promising directions for the commercial application of the obtained powder.

STUDY OBJECTS AND METHODS

Samples preparation. The study object was Jerusalem artichoke (*Helianthus tuberosus* L.) of a large-fruited "Skorospelka" variety harvested in 2018. Its spherical tubers have smooth white skin (Fig. 1).

For drying and chemical analysis, we used tubers without defects with an average weight of 60 g. After harvesting, the tubers were washed, dried, and sealed in polypropylene bags. They were kept at 4 ± 2°C and relative humidity of over 98% for no longer than a week. 10 ± 0.5 g portions of washed and cleaned Jerusalem artichoke tubers were cut into pieces of 10×10×10 mm.

Chemical analysis. The chemical composition of raw Jerusalem artichoke tubers and the resulting powder was analyzed by standard methods: moisture and ash were determined by the gravimetric method; protein was calculated as nitrogen content multiplied by 6.25 using the micro-Kjeldahl method (Pro-Nitro A, J.P. Selecta, Spain) [14]; fat was measured by the Soxhlet method; and inulin was quantified by the Bertrand-Ofner method. The experiments were carried out in triplicate, and the measurements were recorded as a mean ± standard deviation.

Figure 1 Jerusalem artichoke plants and tubers

Experimental mill dryer. A flow diagram of the laboratory vacuum vibromixing mill dryer (VVMD) is shown in Fig. 2. The apparatus has a cylindrical body which oscillates on eight flexible supports with the help of a vibrator and a drive. Discs with debalances are mounted at the ends of the vibrator shaft, which runs along the central axis of the body. A heating jacket is used to heat the dryer. In the upper part of the body, there is a hopper for feeding raw materials. The feedstock is dosed by volume.

Hot water was used as a heat carrier in the jacket of the apparatus. A Wilo Star-RS 15/2-130 circulation pump (WILO SE, Dortmund, Germany) was used to pump hot water from a boiler with a built-in thermostat (Ariston, Italy). The temperature was controlled by an IT-17-C digital thermometer (Exis, Moscow) with a TSP 100 thermocouple installed inside the body. To create a vacuum, we used a STEGLER 2VP-1 single-stage vacuum pump (PRC); the vacuuming degree was monitored by a DV-05-01 vacuum gauge (Stekloribor, Ukraine). A throttle valve was used to regulate the degree of vacuum in the dryer.

The vibration amplitude that depends on composite debalances was measured with a manual VR-1 vibrograph (Vibropribor, Taganrog, Russia). The oscillation frequency was determined by a number of turnings made by the vibrator shaft and was recorded by a ST-5 stroboscopic tachometer (Analitpribor, Tbilisi, Georgia). Grinding media are balls and rollers sized 10–15 mm. The volumetric ratio of feedstock and grinding media was 1:1 (hereinafter referred to as the "charge").

For comparative analysis, we conducted additional experimental studies using traditional methods of drying Jerusalem artichoke. For convective drying, we used a SNOL 58/350 cabinet for drying under atmospheric pressure (Umega-group, Utena, Lithuania), while vacuum drying (Fig. 3) was carried out in a VTSh-K27-250 vacuum drying cabinet (AKTAN VACUUM, Fryazino, Russia).

Experimental procedure. Our experimental studies of drying Jerusalem artichoke tubers included:
– drying in the VVMD without vibration;
– drying in the VVMD with vibration;
– drying in the VVMD with grinding;
– convective drying under atmospheric pressure; and
– drying in a vacuum cabinet.

In all the experiments, the samples were dried at a constant temperature of the heating wall at 80 ± 1°C. Vacuum drying was carried out at a residual pressure in the drying cabinet within 100 mm of mercury. The samples were periodically weighed on a VIBRA AJH-420CE balance (accuracy class II; Shinko Denshi Co., Ltd, Japan) until a constant weight was achieved.

Based on the experimental data, we calculated the drying parameters as:

$$W = \frac{P_w - P_d}{P_d} \quad (1)$$

where W is moisture content of the material, g moisture/g dry material; P_w is wet weight, g; and P_d is dry weight, g;

$$\Delta W = W_i - W_{i-1} \quad (2)$$

where ΔW is changed moisture content during drying, g moisture/g dry material:

$$N = \frac{\Delta W}{\Delta \tau} \quad (3)$$

where N is the drying rate, h^{-1} and $\Delta \tau$ is the time interval between measurements, h.

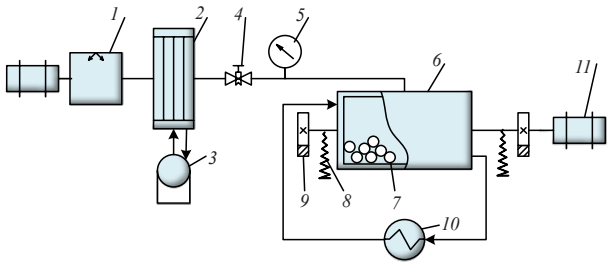


Figure 2 Laboratory vacuum vibromixing mill dryer: *1* – vacuum pump; *2* – condenser; *3* – condensate collector; *4* – throttle valve; *5* – vacuum gauge; *6* – body; *7* – grinding media; *8* – flexible supports; *9* – debalances; *10* – boiler with thermostat; *11* – electric motor

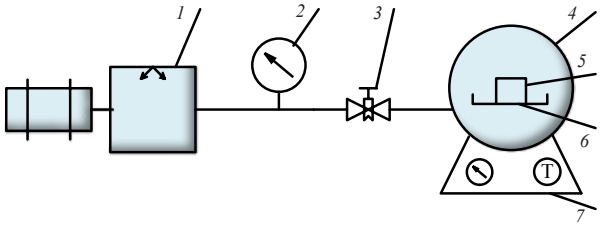


Figure 3 Laboratory vacuum drying unit: *1* – vacuum pump; *2* – vacuum gauge; *3* – throttle valve; *4* – drying cabinet; *5* – material; *6* – shelf; *7* – control panel

Statistical processing of results. The Mathcad software package (Prime 3.0 + Axum S-PLUS Script; PTC, Boston, Massachusetts, USA) was used for the analysis of variance (ANOVA). The significance of differences in the means was analyzed using the Duncan multiple range test (DMRT) at $P < 0.05$.

Mathematical description of drying in the vacuum vibromixing mill dryer. We calculated the kinetics of drying with simultaneous grinding. Due to a continuous renewal of the evaporation surface, most moisture was removed during the constant drying rate period. Therefore, we assumed that the period of the falling rate could be neglected. Vacuum drying in the constant rate period was clearly determined by the pressure in the working chamber.

The heat supplied to the material from the heating wall was used to heat the material and evaporate moisture. The amount of heat Q_{cond} supplied from the source to the material was calculated as:

$$Q_{cond} = \alpha_{cond} F\left(T_{wall} - T_m\right) \quad (4)$$

where α_{cond} is the heat transfer coefficient for conductive heat supply from the heating wall, kJ/(m²·°C); F is the contact surface, m²; and T_{wall}, T_m are the temperatures of the heating wall and the material, respectively, °C.

The period of heating the material is short due to vibration mixing [24]. Therefore, we could exclude the stage of material heating from our calculations. During the constant drying rate period, the heat from the heating wall was spent only on water evaporation. Thus, the heat balance equation was as follows:

$$\alpha_{cond} F\left(T_{wall} - T_m\right) = r\frac{dW}{d\tau} \quad (5)$$

where r is the specific heat of vaporization of moisture, kJ/kg.

The temperature of the material to be dried depends on the pressure in the apparatus, which is kept constant. When a particle contacts the heating wall, it acquires a similar temperature. Due to vibration mixing, the particle moves away from the wall into the chamber, giving off its heat to the entire charge. Then, it acquires the temperature of the material in the chamber until the next contact with the heating wall.

The heat transfer coefficient between the heating wall and the material changes as follows:

$$\alpha_{cond} = \sqrt{\lambda_f \rho_f c_f} / \sqrt{\pi\tau_{cont}} \quad (6)$$

where λ_f is the thermal conductivity of the charge, W/(m·K); ρ_f is the density of the charge, kg/m³; c_f is the heat capacity of the charge, J/(kg·K); and τ_{cont} is the period of contact between the charge and the vibrating heating surface, s.

Moisture content and particle size also change during the drying process. A change in moisture leads to a change in thermophysical characteristics. Grinding and drying reduce the volume of the charge in the chamber. This leads to changes in the ratio between grinding media and drying material. The density and heat capacity of the charge are calculated according to the changed ratio.

The thermal conductivity of the charge is determined as:

$$\lambda_f = \lambda_{air}\left[1 + \frac{(1-\varepsilon)\left(1 - \lambda_m/\lambda_{air}\right)}{\lambda_{air}/\lambda_m + 0.28\varepsilon^{0.63\left(\lambda_m/\lambda_{air}\right)^{0.18}}}\right] \quad (7)$$

where ε is the charge porosity; λ_m, λ_{air} are the thermal conductivities of the material and air, respectively.

Under vibration, the vibrating fluid bed porosity differs from that of the fixed bed. For the charge, it is determined from the criterion equation [26] as:

$$\varepsilon = 0.287\left(\frac{\rho_m d_{eq}^2 \omega}{\mu_m}\right)^{-0.0016}\left(\frac{A}{d_{eq}}\right)^{0.092} \left(\frac{A\omega^2}{g}\right)^{0.144}\left(\frac{m_m}{R_{dr}^3 \rho_m}\right)^{0.176}\left(\frac{r_g}{R_{dr}}\right)^{0.22} \quad (8)$$

where $\rho_m d_{eq}^2 \omega / \mu_m = Re$ is the Reynolds criterion for mixed particles; ρ_m is the density of the material, kg/m³; μ_m is the dynamic viscosity of the material, Pa·s; $m_m / R_{dr}^3 \rho_m$ is the filling degree; R_{dr} is the radius of the dryer body, m; m_m is the weight of the material, kg; $A\omega^2/g = Fr$ is the Froude criterion; ω is the angular speed of rotation of the vibrator shaft, 1/s; A is the vibration amplitude, m; r_g is the radius of the center of gravity of the charge, m; and d_{eq} is the equivalent particle diameter, which changes during drying due to constant grinding, obtained from:

$$\frac{d_{eq}}{d_{initial}} = \exp\left[-10\text{x}2.99^{-9}\left(\frac{A}{D_{dr}}\right)^{-4.21} \left(\frac{A\omega^2}{g}\right)^{1.53}\left(\frac{m_b}{\rho_m A^3}\right)^{1.47}(\omega\tau)\right] \quad (9)$$

where $d_{initial}$ is the initial particle diameter, m; D_{dr} is the diameter of the dryer, m; and m_b is the mass of grinding balls, kg.

As the volume of the material decreases, so does the active surface of its contact with the heating wall. At the beginning of drying, the filling factor is equal to one. Due to vibration mixing, in 1–2 min, the material gets evenly redistributed among the grinding media. At this point, the entire hot surface of the body is in contact with the charge. Then, due to grinding and lower moisture, the material decreases in volume, resulting in a smaller contact surface. When moisture is less than 30%, the charge begins to circulate intensively. The contact surface is calculated from the charge flight and sliding times according to the theory of interaction between the material and the vibrating body [25].

The period of contact between the charge and the heating surface τ_{cont} is determined by the interaction between the charge and the body of the apparatus as the moisture and volume decrease:

$$\tau_{cont} = T - \tau* \quad (10)$$

where T is the time corresponding to one revolution of the vibrator shaft, s; $\tau*$ is the flight of the charge from its separation from, to come into contact with, the body

$$\tau* = \frac{1}{\omega}\arccos\left(-\frac{g}{A\omega^2}\right) + \frac{\pi}{2} \quad (11)$$

To calculate the total drying time in the VVMD, we assume that the vacuum pump removes the entire volume of dry air. In this case, the pressure in the dryer corresponds to the pressure of saturated water vapor. The pressure in the drying chamber is maintained by a continuously operating vacuum pump. Therefore, the kinetics of drying is determined by the rate of evaporation removal, which depends on the characteristics of the pump. To describe the process of evaporation at reduced pressure, we proceed from the following assumption. Changes in the amount of vapor in the chamber over time are equal to the difference between the rates of vapor pumping and moisture evaporation. At constant pressure, the amount of vapor does not change over time. The material balance is calculated as:

$$q_{ev} - \rho Q_{pump} = 0 \quad (12)$$

where q_{ev} is the rate of liquid evaporation, kg/s; ρ is the vapor density, kg/m^3; Q_{pump} is the pump capacity, m^3/s.

The rate of liquid evaporation is calculated as:

$$q_{ev} = -m_d \frac{dW}{d\tau} \quad (13)$$

where m_d is dry weight, kg.

The vapor density is calculated according to the Clapeyron equation:

$$\rho = P_s M / RT_m \quad (14)$$

where P_s is the pressure of saturated vapor, Pa; M is the molar mass of water vapor, kg/kmol; and R is the universal gas constant, J/(mol·K).

Inserting Egs. (13) and (14) into Eg. (12) leads to:

$$\frac{dW}{d\tau} = -\frac{P_s M Q_{pump}(A - \ln P_s)}{RBm_d} \quad (15)$$

where A and B are the coefficients from the Antoine equation.

With Eg. (5) taken into account, the heat and mass transfer is calculated as:

$$\frac{d(\alpha_{cond} F)}{d\tau} = -r\frac{P_s M Q_{pump}(A - \ln P_s)}{RBm_d(T_{wall} - T_m)} \quad (16)$$

Inserting the conditions $\tau = 0 \rightarrow \alpha_{cond} F = \alpha_{initial} F_{initial}$ and $\tau = \tau_{drying} \rightarrow \alpha_{cond} F = \alpha_{final} F_{final}$ into Eg. (16), we calculate the drying time as:

$$\tau = -\frac{(\alpha_{initial} F_{initial} - \alpha_{final} F_{final}) RBm_d(T_{wall} - T_m)}{rP_s MQ_{pump}(A - \ln P_s)} \quad (17)$$

The initial data for calculating the kinetics and drying time in the 0.4 dm^3 laboratory vacuum vibromixing mill dryer were as follows:

– working parameters: D = 0.6 m; T_{wall} = 80°C; P_s = 1333 Pa; ω = 150.72 1/s; $A = 4.5\times10^{-3}$ m; Q_{pump} = 0.003 m^3/s; $F_{initial} = 18.3\times10^{-3}$ m^2; $F_{final} = 7.32\times10^{-3}$ m^2; $m_{initial}$ = 0.9 kg; m_{final} = 0.6 kg;

– thermophysical characteristics of the feedstock: λ_m = 0.6 W/(m·K), ρ_m = 1015 kg/m^3, c_m = 3.60 kJ/(kg·K); and

– thermophysical characteristics of the initial charge (feedstock and grinding media): $\lambda_{f,initial}$ = 24.05 W/(m·K); $\rho_{f,initial}$ = 4433 kg/m^3; $c_{f,initial}$ = 2.05 kJ/(kg·K).

Nuclear Magnetic Resonance (NMR) study. We studied changes in the molecular mobility of original Jerusalem artichoke and its powders obtained by different drying methods. For this, we performed the wide-line (low resolution) NMR on a standard RYa-2301 radio spectrometer (Russia) at 16.2 MHz.

RESULTS AND DISCUSSION

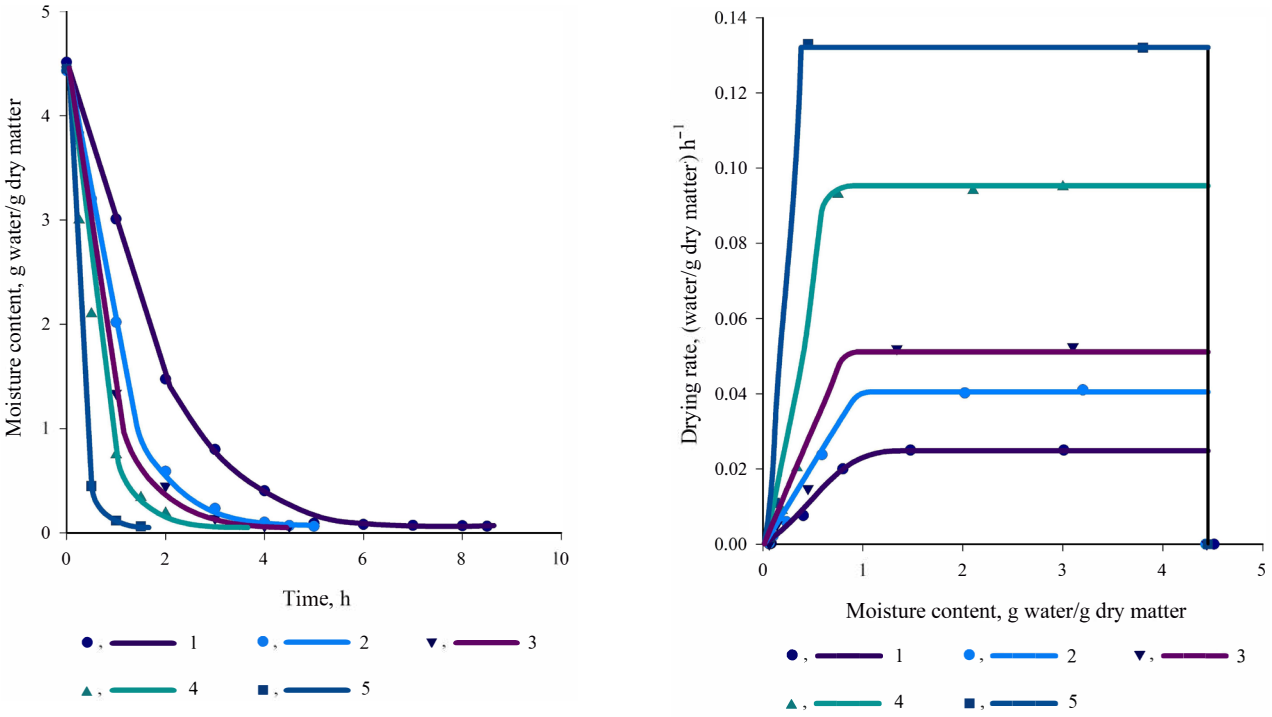
Mathematical description of drying. Visual observations of the drying and grinding of Jerusalem artichoke (*Helianthus tuberosus* L.) in the vacuum vibromixing mill dryer (VVMD) showed the following results. After ten minutes, the pieces of Jerusalem artichoke turned into a wet puree, which proved that the grinding was much faster than drying. The initial particle size was not an important factor for producing powder in this apparatus.

Fig. 4 shows drying curves and drying rate curves for Jerusalem artichoke tubers based on experimental and estimated data.

According to the curves for traditional drying methods, a decrease in pressure in the drying chamber to 100 mm of mercury increased the drying rate by a factor of 1.6–2.1. This was due to the fact that vacuum decreases the boiling point of liquid, making evaporation from the surface of the material more intensive.

Vacuum drying without vibration revealed the effect of the charge volume (mass ratio) on the drying rate. In the vacuum cabinet, the feedstock was laid out evenly in one layer without mutual contact. Therefore, drying in a vacuum oven was 1.30–1.35 times faster than in the VVMD with vacuum, but without vibration.

The rate of vibro-vacuum drying was 3.7–3.8 times higher compared to the analogous process under atmospheric pressure and 1.9–2.0 times higher than under vacuum. Vibromixing intensified the heat and mass transfer between the hot wall and the charge. This was due to the redistribution of heated particles and the equalization of temperature and concentration fields of moisture in the charge.



(a) (b)

Figure 4 Curves of drying (a) and drying rate (b). Points – experimental data; lines – estimated data: 1 – convective drying; 2 – vacuum drying; 3 – drying in the VVMD without vibration; 4 – drying in the VVMD with vibration; 5 – drying in the VVMD with grinding

The highest drying rate was observed when vibration was combined with grinding. The period of constant drying increased due to a continuous renewal of the evaporation surface until complete drying. Thus, our assumption of neglecting the falling rate period when modeling the process in the VVMD was justified. This method increased the drying rate by a factor of 5.4–5.5 compared to drying under atmospheric pressure.

We compared the estimated and experimental data to assess the adequacy of mathematical description. The average statistical error for the moisture content was ± 8% with a sample correlation coefficient of 0.953, while that for the drying time, ±10% with a sample correlation coefficient of 0.951.

NMR results. Figure 5 shows the qualitative analysis of Jerusalem artichoke powder according to the NMR spectra. The arrows indicate implicit narrow components.

We analyzed the shape of the NMR spectra. According to the NMR theory, a complex spectrum

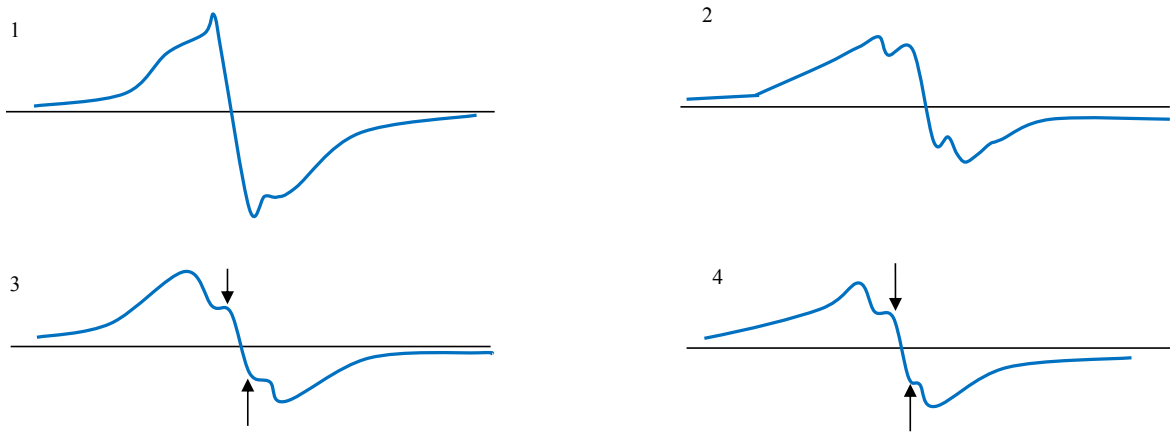


Figure 5 Wide-line NMR spectra for: initial sample of Jerusalem artichoke tubers (1), powder produced under atmospheric pressure (2), powder produced by vacuum drying (3), and powder produced by vacuum vibromixing mill drying (4)

Table 2 NMR parameters for Jerusalem artichoke tubers and powders obtained by different drying methods

Sample	NMR line width, $\delta_н$	Second moment, ΔH_2^2
Initial sample of Jerusalem artichoke tubers	2.4	9.2
Powder produced under atmospheric pressure	3.2	13.4
Powder produced by vacuum drying	3.6	14.6
Powder produced by vacuum vibromixing mill drying	3.8	15.2

is typical for samples containing proton groups, whose molecular mobility differs by 10^3–10^4. The narrow component refers to the proton groups of water contained in the initial Jerusalem artichoke sample. According to the results, the most complex shape of the NMR spectra was for the initial sample and the powder dried under atmospheric pressure. The samples obtained in the vacuum cabinet and in the VVMD featured implicit two-component NMR spectra. Also, the powders obtained under atmospheric pressure contained more moisture than those produced by vacuum drying or in the VVMD.

Taking into account the contribution of the narrow component, we calculated the parameters of the NMR spectra (Table 2). The wide component refers to all other proton groups of the initial Jerusalem artichoke and the dried powders.

Moisture has a plasticizing effect on the system. A decrease in its amount leads to the appearance of local regions with long-range order (analogs of domains). It is these regions that determine the resulting molecular mobility in the system. Changes in the width of the NMR lines are associated with decreased molecular mobility of proton groups in the samples after drying. Structural changes in the Jerusalem artichoke after drying are also evidenced by an increase in the second moments of the NMR absorption line compared to the initial sample. A larger spread of local magnetic fields makes a significant contribution to the second moment of the NMR line, indicating a more rigid structure of the dried samples.

Table 3 Chemical composition of Jerusalem artichoke tubers and powders

Component	Content (per dry weight), %		
	Raw tubers	Powder dried under atmospheric pressure	Powder dried in the VVMD
Protein	9.29 ± 0.14	7.19 ± 0.11[a]	8.72 ± 0.12[a,b]
Fat	0.51 ± 0.01	n.d.	n.d.
Ash	11.87 ± 0.15	11.57 ± 0.14	11.54 ± 0.15
Inulin	27.77 ± 0.27	20.48 ± 0.24[a]	23.88 ± 0.25[a,b]

a – statistically significant differences ($P < 0.05$) from the raw sample; b – statistically significant differences ($P < 0.05$) from the sample obtained under atmospheric pressure

n.d. – not detected

Chemical analysis. The chemical composition of raw Jerusalem artichoke tubers was a basis for a comparative analysis of the powders obtained (Table 3). The moisture content was 80.2% in the initial sample, 12.5% in the sample dried under atmospheric pressure, and only 6.1% in the sample obtained in the vacuum vibromixing mill dryer.

Inulin is the most valuable component of Jerusalem artichoke in functional nutrition. As we can see in Table 3, drying under atmospheric pressure caused a 26% loss of inulin, while drying at 30ºC in the VVMD, about 14%. Thus, a gentle mode of drying in the VVMD ensures good preservation of the target component.

Drying efficiency in the VVMD. We compared our results with those reported in the literature. Golubkovich *et al.* found that microwave drying of Jerusalem artichoke tubers, which lasted 2.75 h, was 2–3 times more intensive than convective drying, with final moisture under 10% [27]. Another study proposed infrared drying as a method that provides intensive and uniform moisture evaporation with the least loss of polysaccharides, including inulin [28]. To preserve inulin, Rubel *et al.* suggested using freeze drying [21]. However, in terms of energy efficiency, microwave drying (2.2–2.5 kW·h/kg removed moisture) and infrared drying (1.8–2.0 kW·h/kg removed moisture) can only be advisable for products with low moisture. Moreover, in both studies, the tubers were dried at 60°C. Freeze drying is also energy-intensive, requiring 2.2–3.7 kW·h/kg of removed moisture. Ermosh *et al.* dried Jerusalem artichoke using convective-vacuum-impulse drying [29]. In their study, the drying lasted 1.5 h (3 times as short as convective drying) at 70°C until the residual moisture reached 7–8%.

The technology of vacuum vibromixing mill drying that we propose for Jerusalem artichoke tubers has the following advantages over existing drying methods:

– high drying rate: 5.5 times as high as traditional (convective) drying, with 6.1% final moisture;

– preservation of bioactive compounds (including inulin) due to a low temperature (under 30°C);

– energy efficiency (1.2–1.3 kW·h/kg removed moisture) due to drying and grinding in one apparatus;

– long shelf life, low consolidation, and no caking due to low residual moisture; and

– no need to buy a additional grinder for the finished product due to drying and grinding in one apparatus.

Prospects for industrial application.

1. Bakery products from grain flour [30, 31]. Adding 2% of Jerusalem artichoke powder to wheat flour during yeast dilution enhances [31]:

– saccharifying and gassing abilities of the dough;

– gluten quality and elasticity;
– hydrophilic properties; and
– nutritional and biological value.

2. Flour confectionery. Adding 3% of Jerusalem artichoke powder to flour mass in the formulation of sugar and hard-dough biscuits promotes [31]:
– a larger volume of finished products;
– a more uniform structure when broken;
– lower density and better wetting;
– reducing sugar by 3%; and
– higher nutritional and biological value.

3. Instant porridges (oatmeal, buckwheat, etc.). Adding 10 to 20% of Jerusalem artichoke powder improves the sensory and functional properties of the grains and lowers their glycemic index [17].

4. Fermented milk products. Adding 2–2.5% of Jerusalem artichoke powder to a normalized mixture provides [32]:
– better functional properties;
– higher rate of acid formation and a shorter fermentation time (up to 6 h); and
– an original taste of the finished product.

5. Substitute for raw meat. Adding 10 to 15% of hydrated Jerusalem artichoke powder to the formulation of meat products contributes to [33]:
– better uniformity and plasticity of minced meat;
– lower adhesive capacity of minced meat;
– higher stability of the product due to inulin; and
– higher content of dietary fiber.

CONCLUSIONS

Our study led us to the following conclusions:

1. The experimental studies of the kinetics of drying Jerusalem artichoke tubers (*Helianthus tuberosus* L.) by various methods proved the effectiveness of vacuum vibromixing mill drying. In this case, the drying rate was almost 5.5 times as high as that of convective drying.

2. According to the kinetic curves, grinding provided a period of decreasing drying rate until almost complete drying. Drying was a time-limiting process.

3. Drying in the vacuum vibromixing mill dryer was approximately twice as fast as thin-layer vacuum drying.

4. The mathematical description of vacuum vibromixing mill drying showed good agreement with the experimental data. The degree of mismatch was less than 8% for the moisture content and 10% for the process time.

5. The wide-line (low resolution) NMR analysis of Jerusalem artichoke powders obtained by vacuum drying and in the vacuum vibromixing mill dryer showed implicit two-component spectra. This indicated a low content of proton groups of water, i.e. low moisture. The calculated widths of the NMR lines and the second moments clearly revealed their rigid structure.

6. Comparing the chemical compositions of powders obtained under atmospheric pressure and in the vacuum vibromixing mill dryer with that of the initial Jerusalem artichoke sample was of great importance for the preservation of nutrients and prebiotics. We found that our technology for powdering the tubers at max. 30°C preserved 86% of the initial amount of inulin.

7. The comparative analysis of various drying methods with the proposed technology confirmed its effectiveness. It provided a lower power consumption (1.2–1.3 kW·h/kg removed moisture) by combining drying and grinding in one apparatus. The low residual moisture (6.1%) ensured a long shelf life, low consolidation, and no caking.

8. Combining drying and grinding in one apparatus saves the capital and operating costs of additional grinding equipment.

9. Jerusalem artichoke powder can be used in the production of bakery, confectionery, dairy, and meat products. It improves the products' technological indicators, sensory profile, and functional properties.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for any possible plagiarism.

CONFLICT OF INTEREST

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

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α-amylase from white pitaya (*Hylocereus undatus* L.) peel: optimization of extraction using full factorial design

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Abstract:

Introduction. Amylase is a significant enzyme with numerous commercial applications, which is largely used to convert starches into oligosaccharides. Extraction of amylase from plant by-products or cheap sources is cost-effective. Annually, pitaya fruit juice industry produces huge amounts of peels that could be utilized as an alternative source in enzyme production industry. The work aimed to examine and optimize extraction process.

Study objects and methods. In this study, we investigated parameters of extraction to optimize the process, as well as activity of α-amylase from white pitaya fruit (*Hylocereus undatus* L.) peel. For this purpose, a two-level full factorial design was applied. Three variables, namely the pH of sodium phosphate buffer (X_1, 4.5–7.5), mixing time (X_2, 1–3 min), and a sample-to-buffer ratio (X_3, 1:3–1:5), were used to identify significant effects and interactions within the samples.

Results and discussion. The results demonstrated that the buffer pH had the most significant ($P \leq 0.05$) effect on total amylase activity. Based on full factorial design analysis, we revealed the optimal conditions for amylase enzyme extraction – pH of 6, mixing time of 2 min, and a sample-to-buffer ratio of 1:4. Lower and higher values influenced adversely on specific activity of amylase.

Conclusion. Optimization increased the enzyme specific activity by a factor of 4.5. Thus, pitaya peel could be used in different industries as a rich natural α-amylase source.

Keywords: Extraction, peel, white pitaya, enzyme, optimization, full factorial design, specific activity

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INTRODUCTION

α-Amylase (EC 3.2.1.1, α-1,4-glucanohydrolase) is an endoacting starch-digesting enzyme that cleaves -1,4 bonds of amylose and amylopectin [1]. Thirty percent of world's enzyme market belongs to amylase, which is widely used in baking, starch liquefaction, textile, as well as detergent and paper industries [2]. Although amylases are extensively used in the food and chemical industries, their production on a large scale is just limited to specific microorganisms emphasizing the need to discover alternative sources to produce them [3]. Amylases are represented in plants, animal tissues, and microorganisms. Nowadays, bacterial α-amylases, especially from *Bacillus* genus, are the most commercially available and industrially-used enzymes [4]. However, their production does not meet the industrial needs worldwide due to their increasing demand. Furthermore, they have caused allergies in 15% of the global population. So, there is a necessity to discover novel resources to produce this valuable enzyme.

It is well documented that plants as abundant sources of α-amylases have higher productivity than bacteria and are the center of focus in developing countries due to their ubiquitous nature [5]. Thus, using plants as an alternative source of the enzyme have great advantages over microbial sources due to their cost-effective production, easy scale-up, and available natural storage organs [6].

Pitaya fruit (*Cactaceae* family) known as dragon fruit, has recently drawn much attention, not only because of their striking color and economic value as food products, but also for their health benefits [7]. Peels account for roughly 33% of the whole pitaya fruit and are the major by-products of fruit juice industry [8, 9]. They are usually discarded in roadsides, causing not only environmental problems but also are a great burden on the industry to treat wastes. So, one of eco-friendly ways to increase resource recovery is to use pitaya peels to extract novel components such as enzymes. Very few reports are accessible about enzymes in pitaya peel [10, 11]. Therefore, the peel of pitaya can be used as an economical and rich source for commercial production of beneficial and natural enzymes.

The most important stage in the recovery of enzymes from plant sources is extraction, in which an inadequate extraction procedure could change the natural structure of the enzyme and result in a reduced enzyme activity. Therefore, it is essential to optimize the extraction process to obtain enzymes with high activity. Two-level factorial design is used to minimize the number of experiments and to find out how the independent variables affect the response variables. This experimental design investigates mathematical relationships between input and output variables of a system.

To date, extraction of amylase enzyme from white pitaya (*Hylocereus undatus* L.) peel and its further optimization using FFD model have not been previously reported elsewhere. This study aimed to primarily examine and develop the optimization level of extraction variables with the minimum total protein and the highest possible total and specific activities. In this study, full factorial design was used to model the potential relationship between the variables of enzyme extraction, such as sodium phosphate buffer (pH 4.5–7.5, X_1), mixing time (1–3 min, X_2), and a sample-to-buffer ratio (1:3–1:5, X_3) which affect the responses variables of amylase derived from white pitaya peel.

STUDY OBJECTS AND METHODS

Plant material. The study objects were white pitaya fruits (*Hylocereus undatus* L.) with the weight range of 400–500 g were obtained from the local market in Selangor, Malaysia. Fruits of the same size, with no visual defects, and under commercial maturity stage were selected for experiments. The fruits (2 kg) were washed with distilled water, drained off, and kept at 4°C prior to extraction.

Chemicals and reagents. 3,5-dinitrosalicylic acid and bovine serum albumin were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Bradford reagent was purchased from Amresco (AMRESCO LLC, Solon, OH, USA). Dibasic sodium phosphate ($Na_2HPO_4{\cdot}2H_2O$), monobasic sodium phosphate ($NaH_2PO_4{\cdot}H_2O$), and maltose were obtained from Merck (Darmstadt, Germany). Sodium potassium tartrate ($NaKC_4H_4O_6{\cdot}4H_2O$) and soluble starch were purchased from Fisher Scientific. All chemicals and reagents were of analytical grade.

Enzyme extraction. The fruits peels were separated, cut into tiny pieces by a stainless steel knife and blended using a Waring blender (32BL 80, Dynamic Corporation of America, New Hartford, Connecticut, USA). Afterwards, 20 g of the mixed sample was weighed and homogenized with a 0.01 M sodium phosphate buffer (pH 4.5–7.5) with a sample-to-buffer ratio of 1:3 to 1:5 for mixing time of 1 to 3 min at 4°C. The homogenized sample was filtered using a cheese cloth and centrifuged at 10 000 rpm for 15 min at 4°C using a refrigerated centrifuge (SIGMA 3-18K, Sartorius, Göttingen, Germany). The obtained supernatant containing the crude enzyme was used to assay amylase activity and protein concentration [12]. The extraction procedures were carried out in triplicate.

Determination of amylase activity. Amylase activity was measured based on the method of Bernfeld with slight modifications [13]. The mixture of enzyme sample containing crude extract (0.5 mL) and 1% (w/v) soluble starch (0.5 mL) was prepared in a 0.1 M phosphate buffer (pH 6). After incubation of the mixture (55°C, 10 min), the reaction was stopped by 1 mL n.d. 3,5-dinitrosalicylic acid. Then, it was heated, cooled, and the volume was adjusted to 12 mL before reading absorbance at 540 nm. A unit of enzyme activity is defined as a quantity of enzyme that releases 1 μmol of maltose per minute.

Determination of protein concentration. A colorimetric protein assay was carried out based on the method of Bradford to measure protein concentration using bovine serum albumin as the standard protein [14].

Determination of specific activity of amylase. Specific activity of amylase was calculated by dividing the enzyme unit per mL (U/mL) to the protein concentration (mg/mL) according to the following formula [15]:

$$\text{Specific activity (U/mg)} = \text{Total activity (U)/total protein (mg)} \quad (1)$$

Experimental design and statistical analysis. In the present work, the effect of three extraction variables, namely the pH of buffer (pH 4.5–7.5), mixing time (1–3 min), and a sample-to-buffer ratio (1:3–1:5) on total protein, total activity, and specific activity of extracted amylase from white pitaya peel was studied using a two-level three-factor (2^3) full factorial design. Twenty-eight experiments were conducted based on the two-level full factorial design with three independent variables and each variable with three levels (Table 1). Analysis of the data was conducted by multiple regressions with the least-square method. The polynomial equation describes the behavior of the present system:

$$Y_r = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{i \neq j=1}^{n} b_{ij} X_i X_j \quad (2)$$

where Y_r represents the measured response variable, while X_i, and X_j are the levels of independent variables; b_0 is coefficient constant for offset term (predicted response at the center); b_i, and b_{ij} are coefficient constants for linear and interactions effects.

R^2 with at least 0.80 demonstrated a good fitness of model [16]. If the absolute F-value became larger and the P-value became smaller, the related variables were found to be more significant ($P \leq 0.05$). The design of experimental data analysis and optimization procedure was carried out with Minitab v.16 statistical package (Minitab Inc., State College, PA, USA).

Optimization and validation procedures. A numerical optimization was used to determine accurate optimal levels of independent parameters that led to obtaining the desired response targets and concurrent multiple optimizations using the response optimizer function in the Minitab software. For validation, a comparison between experimental data and predicted values from the model helps to determine if the regression equation precisely anticipates the values or not [17].

Table 1 The matrix of full factorial design

Treatment runs	Block	pH of buffer (X_1)	Mixing time (X_2)	A sample-to-buffer ratio (X_3)
1	1	4.5	3	5
2	1	7.5	3	3
3[c]	1	6.0	2	4
4[c]	1	6.0	2	4
5[c]	1	6.0	2	4
6	1	7.5	1	5
7	1	4.5	1	3
8	2	4.5	1	5
9	2	4.5	3	3
10	2	7.5	1	3
11	2	7.5	3	5
12[c]	2	6.0	2	4
13[c]	2	6.0	2	4
14[c]	2	6.0	2	4
15[c]	3	6.0	2	4
16	3	7.5	3	3
17[c]	3	6.0	2	4
18	3	6.0	1	3
19[c]	3	6.0	2	4
20	3	7.5	1	5
21	3	4.5	3	5
22[c]	4	6.0	2	4
23	4	6.0	1	5
24[c]	4	6.0	2	4
25	4	4.5	3	3
26	4	7.5	3	5
27[c]	4	6.0	2	4
28	4	7.5	1	3

[c] center point; X_1 – pH of buffer; X_2 – mixing time, min; X_3 – a sample-to-buffer ratio

RESULTS AND DISCUSSION

Fitting the full factorial design. In the preset study, multiple regression analysis was done using full factorial design to fit mathematical models to the experimental data and to attain an optimal region for the response variable. The estimated regression coefficient of the full factorial model, along with the corresponding R^2 and lack-of-fit tests, is presented in Table 2. Significance of the factors studied was confirmed by both P-value and F-ratio as statistical parameters. Results indicated that the R^2 values for total activity, total protein, and specific

Table 2 Regression coefficient R^2, adjusted R^2, probability value, lack of fit, and significance level for extract variables

Source	Total activity (Y_1, U)	Total protein (Y_2, mg)	Specific activity (Y_3, U/mg)
b_0	43.932	16.831	2.5851
b_1	13.408	1.360	0.6706
b_2	3.166	0.902	0.0803
b_3	11.222	3.867	0.0219
b_1b_2	3.084	0.832	–
b_1b_3	2.293	1.916	–0.3178
b_2b_3	–	–	– 0.1879
Regression coefficient R^2	0.992	0.939	0.974
Adjusted regression R^2	0.989	0.909	0.967
P-value	0.000[a]	0.000[a]	0.000[a]
Lack of fit (P-value)	0.073[b]	0.233[b]	0.788[b]

Table 3 Main and interaction effects of pH (X_1), mixing time (X_2), and a sample-to-buffer ratio (X_3) on response variables for extracted amylase

Variables	Main effect			Interaction effect		
	X_1	X_2	X_3	X_1X	X_1X_3	X_2X_3
	Total activity					
P-value	0.000[a]	0.003[a]	0.000[a]	0.004[a]	0.023[a]	–
F-ratio	210.53	11.74	147.49	11.14	6.16	–
	Total protein					
P-value	0.000[a]	0.008[a]	0.000[a]	0.013[a]	0.000[a]	–
F-ratio	20.19	8.89	163.24	7.56	40.05	–
	Specific activity					
P-value	0.000[a]	0.326[b]	0.786[b]	–	0.001[a]	0.028[a]
F-ratio	70.51	1.01	0.08	–	15.83	5.53

b_i – the estimated regression coefficient for the main linear effects. b_{ij} – the estimated regression coefficient for the interaction effects. 1 – pH of buffer; 2 – mixing time; 3 – a sample-to-buffer ratio

X_1, X_2 and X_3 – the main effect of pH of buffer, mixing time, and a sample-to-buffer ratio, respectively; X_1X_2 – the interaction effect of pH of buffer and mixing time; X_1X_3 – the interaction effect of pH of buffer and a sample-to-buffer ratio; and X_2X_3 – the interaction effect of mixing time and a sample-to-buffer

[a] – significant ($P \leq 0.05$)

[b] – not significant ($P > 0.05$)

activity were higher than 0.80, which showed the regression model fits the experimental data.

Table 3 shows the main and interaction effects of pH (X_1), mixing time (X_2) and a sample-to-buffer ratio (X_3) on each response variable for the extracted amylase. The main effect of pH showed the highest significant ($P \leq 0.05$) effects on the total activity of amylase, and the interaction effect of time with ratio had the lowest significant ($P \leq 0.05$) effects on the specific activity of amylase.

Effect of extraction variables on total protein. As shown in Table 2, the total protein value was significantly ($P \leq 0.05$) affected by pH of buffer, mixing time, and a sample-to-buffer ratio, as well as by the interaction effect of pH with a sample-to-buffer ratio and mixing time. As shown in Figs. 1a and 1b, the main and interaction effects of target extraction variables positively affected total protein of amylase. In this study, a sample-to-buffer ratio followed by its interaction effect with pH had the most significant ($P \leq 0.05$) effect on the total protein (Fig. 1c).

The results also indicated that pH 6 of sodium phosphate buffer was the optimum pH for extraction of amylase from white pitaya (*Hylocereus undatus* L.) peel. Indeed, any changes in the pH of buffer can effect on the protein structure by lowering protein solubility and increasing protein hydrophobic interactions, which finally leads to denaturation and precipitation of the enzyme [18, 19].

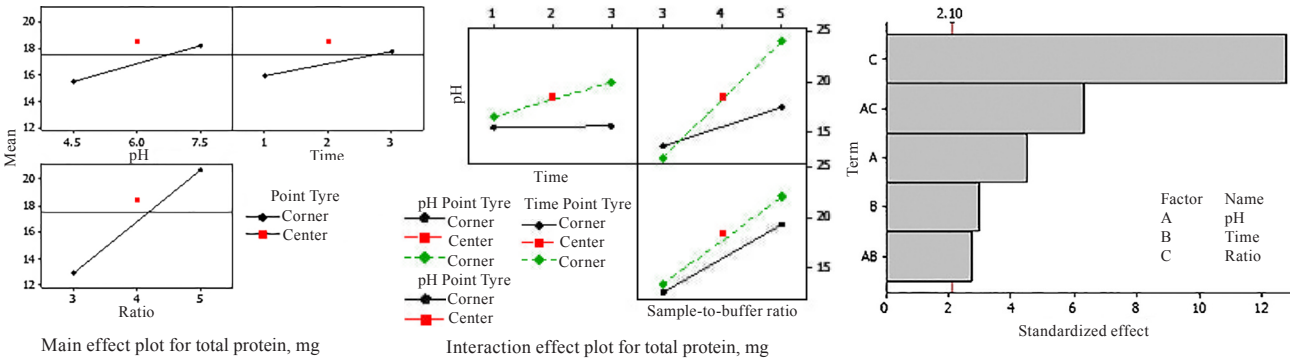
It should be noted that the extraction time also significantly ($P \leq 0.05$) affected the total protein; however, its effect was significantly ($P \leq 0.05$) lower than a sample-to-buffer ratio and pH effects. The most desirable time to mix sodium phosphate buffer with peels was 2 min. Tang *et al.* reported that protein yield increased as the extraction time increased, and decreased with increasing time above certain value [20]. Thus, a prolonged extraction process can lead to protein denaturation, and affect the enzyme function [21].

Figure 1b clearly indicates that interaction between pH and a sample-to-buffer ratio was stronger than the interaction of pH and mixing time. The effect of a sample-to-buffer ratio was more significant ($P \leq 0.05$) at a higher pH, but total protein was lower at a lower pH. The individual optimum region with minimum total protein value (Y_2 = 18.45 mg) was predicted to be achieved at pH 6, mixing time of 2 min, and a 1:4 a sample-to-buffer ratio.

Effect of extraction variables on total amylase activity. As shown in Table 2, the main effect of pH, mixing time, a sample-to-buffer ratio, and the interaction effect of pH with a sample-to-buffer ratio and pH with mixing time had significant ($P \leq 0.05$) effects on the total amylase activity. As given in Figs. 2a and 2b, the main and interaction effect variables positively affected the total activity of amylase enzyme. Main and interaction effects had higher mean total activity at their center point. As seen in Fig. 2c, the main effect of the pH of buffer had the most significant ($P \leq 0.05$) effect on the total activity followed by a sample-to-buffer ratio, time, interaction of pH with mixing time, and interaction of pH with a sample-to-buffer ratio.

Our results revealed that the maximum total activity occurred at extraction pH of 6. Pires *et al.* reported that extraction pH determined the net charge of proteins [22]. The considerable difference in extraction efficiency with nominal changes in aqueous phase pH showed the sensitivity of system to any pH changes. These changes might disrupt the efficiency of enzyme in hydrolyzing the protein, considered as the main cause of the results obtained for pH range. Chen *et al.* showed that fluctuations in the pH of many enzyme reactions normally could result in some reactants changes such as denaturation of protein structure or disturbance of enzyme ionic character related to its active site [21].

We found that the best ratio for amylase extraction was 1:4. A decreased volume of buffer reduced the total activity of amylase, which was due to insufficiency of buffer to infiltrate the solid mass. On the other hand, the increase in the buffer volume beyond the optimum level resulted in an immediate reduction in the total amylase activity due to an excessive dilution of the solution. In



(a) (b) (c)

Figure 1 Main effects (a), interaction effects (b), and Pareto chart of extraction variables (c) on total protein of amylase from white pitaya peel (c)

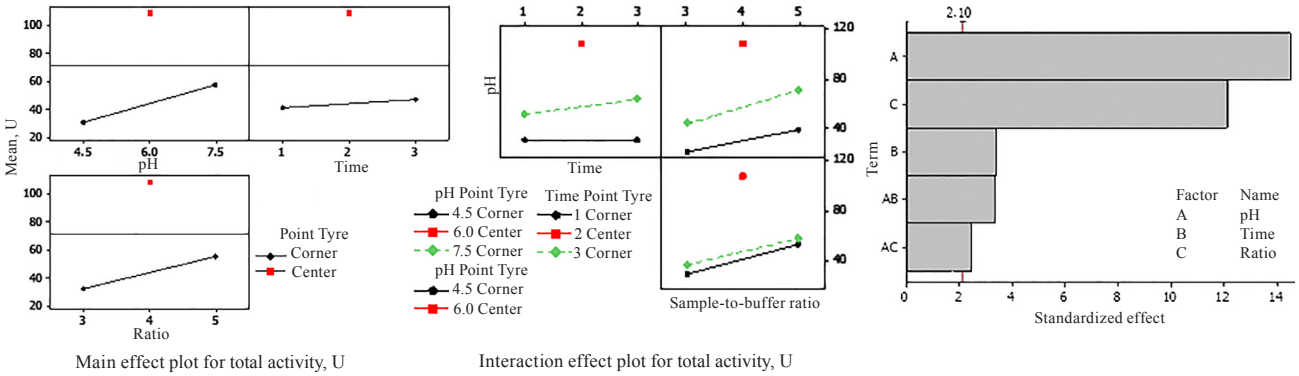


Figure 2 Main effects (a), interaction effects (b), and Pareto chart of extraction variables (c) on total activity of amylase from white pitaya peel (c)

the present study, using more buffers to the center point released more enzymes from the sample, and a lower volume of buffer reduced the enzyme activity. Similarly, other studies found that the amount of extracted enzyme increased with the rise in the buffer volume and vice versa [23–26].

Time is one of the important physical parameters in the enzyme extraction that significantly ($P \leq 0.05$) affected the total activity of amylase. However, its effect was significantly ($P \leq 0.05$) lower than pH and a sample-to-buffer ratio effects. Mixing time of more than 2 min had negative effects on the total activity of amylase. In fact, buffer contact with the enzyme rises when the extraction time increases, although the higher shear forces during mixing with blender could denature and deactivate the enzyme. Our results consist with those of Amid *et al.* who reported that the optimum time required for extraction of amylase from mango peel was 2 min [27].

All the interactions had statistical significance ($P \leq 0.05$) in influencing on the total activity of amylase except for the interaction of mixing time with ratio. The interaction plot illustrates that interaction of pH and mixing time was stronger than that of pH and a sample-to-buffer ratio (Fig. 2b). It was observed that the interaction effect of pH with a sample-to-buffer ratio had the lowest significant ($P \leq 0.05$) effect on the activity of enzyme. The highest total activity (Y_1 = 108.2 U) of amylase was obtained at buffer pH of 6, mixing time of 2 min, and a sample-to-buffer ratio of 1:4.

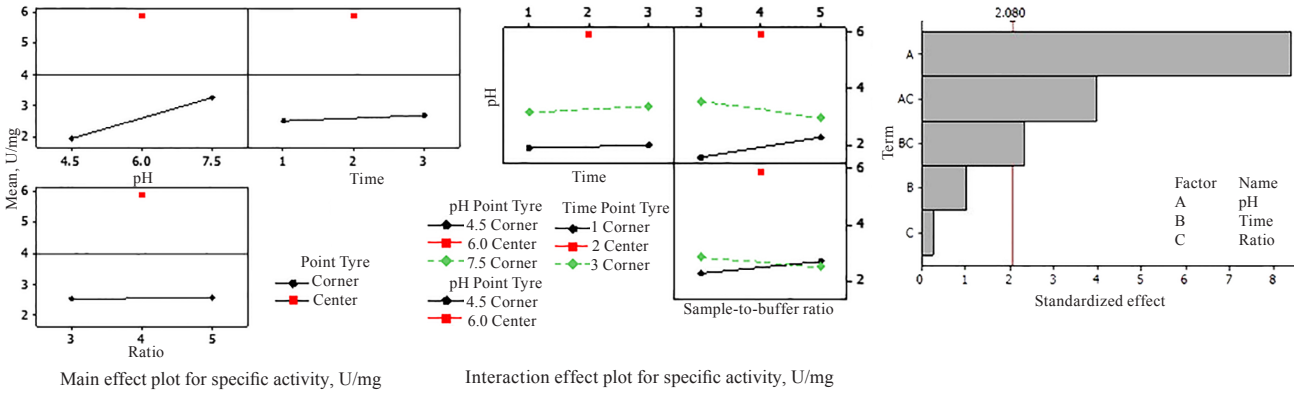
Effect of extraction variables on specific activity of amylase. The specific activity value of the amylase was significantly ($P \leq 0.05$) affected by pH of buffer and interaction effect of a sample-to-buffer ratio with pH and mixing time (Table 2). Figure 3a demonstrates that mixing time and a sample-to-buffer ratio were insignificant ($P > 0.05$), and only pH of buffer exhibited a significant ($P \leq 0.05$) effect on the specific activity.

In this study, the main term of pH indicated the most significant ($P \leq 0.05$) effect followed by interaction of pH with a sample-to-buffer ratio and interaction of mixing time with a sample-to-buffer ratio on the specific activity of amylase (Fig. 3c). The specific activity of amylase significantly ($P \leq 0.05$) decreased at pH buffer of 4.5 and 7.5 due to the changes in enzyme structure at acidic and alkaline pH. The highest specific activity was obtained at pH of 6 that confirmed the enzyme was active at slightly acidic pH, so, extraction efficiency depended on the pH of buffer. Similar observation was made by Amid *et al.*, who investigated the effect of pH on the specific activity of serine protease from Kesinai plant leaves [28]. It was indicated that the activity of enzyme decreased beyond the center point at higher and lower pH of buffer.

In addition, one of the most significant ($P \leq 0.05$) interaction effects was the interaction between pH of buffer and a sample-to-buffer ratio. It was observed that the specific activity increased with increasing the ratio from 1:3 to 1:4, whereas at a higher ratio (1:5) no considerable changes in the specific activity were observed when the pH of the buffer was changed (Fig. 3b). This could be attributed to the excessive dilution of the enzyme extraction solution.

In other words, at the lower ratio (1:5), variation in the pH of buffer had a significant effect on the specific activity possibly due to an adequate volume of buffer required to infiltrate the solid mass. Aikat and Bhattacharyya reported the same observation of the effect of buffer volume on the activity of pectinase enzyme [29]. The interaction effect of mixing time with a sample-to-buffer ratio showed the least significant ($P \leq 0.05$) effect on the specific activity. Overall, the highest amount of specific activity (Y_3 = 5.89 U/mg) was predicted when amylase was extracted under the following conditions: buffer pH of 6, mixing time of 2 min, and a sample-to-buffer ratio of 1:4.

Optimization procedure. The Multiple response optimizations were conducted to evaluate the optimum set level of independent variables to obtain the required results. The findings showed that extraction with buffer of pH 6 for 2 min mixing time in a sample-to-buffer ratio of 1:4 resulted in an optimal condition for total



(a) (b) (c)

Figure 3 Main effect (a), interaction effects (b), and Pareto chart of extraction variables (c) on specific activity of amylase from white pitaya peel (c)

protein, total activity and specific activity of amylase from white pitaya peel. Under optimal conditions, the corresponding expected response variables for total protein, total activity, and specific activity for the extracted amylase were predicted to be 18.45 mg, 108.2 U, and 5.89 U/mg, respectively. The results showed the accuracy of the statistical model for amylase extraction (Fig. 4).

Verification of the fitted model. The acceptability of full factorial design was verified by comparing the experimental and expected values. If these two results are closer, the regression equation can explain the acceptability better. The comparison between experimental values of the responses and the expected values measured employing the models can be done by performing a two-sample *t*-test. There was not a significant ($P > 0.05$) difference between the experimental and predicted values. There was a close correspondence between those values.

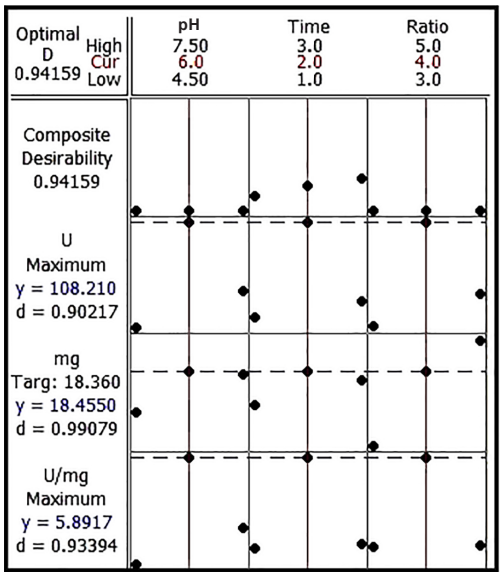


Figure 4 Response optimizer plots for interaction effects of extraction variables on total protein, total activity, and specific activity of amylase

The validation of the models was performed using buffer with pH 6 for 2 min of mixing time in a sample-to-buffer ratio of 1:4. Under optimal extraction conditions, the predicted values for total protein, total activity and specific activity were 18.45 mg, 108.2 U and 5.89 U/mg, respectively. A supplementary experiment under the selected optimal conditions demonstrated the experimental values of total protein, total activity, and specific activity to be 18.82 mg, 105.8 U, and 5.62 U/mg, respectively, which were in close agreement with the expected values indicated the suitability of the corresponding model.

CONCLUSION

The present study revealed that the peel of white pitaya (*Hylocereus undatus* L.) could be a good source of amylase. The results indicated that the activity of the extracted amylase was significantly ($P \leq 0.05$) influenced by main extraction variables. pH had the most significant ($P \leq 0.05$) effect on amylase activity. The analysis using full factorial design demonstrated that the maximum amylase extraction could be achieved at pH of 6. Amylase had a lower enzymatic activity at pH 4.5 and 7, which might be due to denaturation of amylase at such low or high pH.

Among all main extraction variables, time had the least significant ($P \leq 0.05$) effect on the total activity and total protein of amylase. The results exhibited that the highest activity was achieved at the sample-to-buffer of 1:4, but lower or higher ratios would negatively affect the enzymatic activity of amylase. This might be caused by the insufficient buffer volume to infiltrate the sample.

Therefore, the extraction at pH 6 for 2 min using a sample-to-buffer ratio of 1:4 was the optimal extraction condition for amylase. This optimization increased the specific activity of the enzyme by a factor of 4.5 (to a value of 5.89 U/mg). On the basis of such findings, the main effect of pH of buffer, mixing time, and a sample-

to-buffer ratio were the principal factors affecting the extraction of amylase from white pitaya peel. Amylase extracted from white pitaya peel can be used as a potential economical enzyme in different industries and for biotechnological applications.

CONTRIBUTION

Z. Shad, the main author, performed the experiments. H. Mirhosseini and A.S. Meor Hussin designed the work, processed and analyzed the data, and wrote the manuscript. M. Motshakeri managed lab procedures and supervised the project. M.R. Sanjabi performed enzyme experiments, edited the manuscript, and contributed experimental design and data analysis.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Minced products from undersized sea fish: new industrial technology

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Abstract:
Introduction. The ever-growing world population and protein deficiency increase the demand for products of animal origin, especially fish-based. However, canned foods and fillets, which are the most popular types of fish products, are made from medium-sized and large fish. In spite of the fact that undersized fish is cheap, it requires manual processing and remains so time and labour consuming that it is utilized for non-food purposes. The research objective was to develop a new technology for processing undersized sea fish into minced ready-to-eat products.
Study objects and methods. The study featured experimental samples of fish mince with texturing agents and food additives vs. control samples of pure fish mince. The experiment involved block-frozen Peruvian anchovy (*Engraulis ringens* L.). The anchovy was minced without pre-defrosting, gutting, or beheading. The experimental and control samples underwent sensory evaluation and were tested for moisture content, water-binding capacity, and rheological properties using a PNDP-penetrometer.
Results and discussion. Adding 3.6% of wheat fiber, ≤ 15% of pea flour, ≤ 10% of textured soy, and 12% of onion improved the sensory and technological profiles of the finished product. The recommended mass fraction of fish in the finished product did not exceed 55%, as a higher amount deteriorated the sensory quality of the product. The textured soy provided the optimal texture. The fish balls were cooked from the fish mince, which were deep-fried in breadcrumbs, received a high score for sensory properties and could be recommended as basis for various formulations.
Conclusion. Minced undersized fish, traditionally used as fertilizers or crude product, proved to be an advantageous semi-finished and ready-to-eat product. The developed technology is relevant for most undersized block-frozen sea fish.

Keywords: Seafood, anchovy, *Engraulis ringens*, processing, undersized fish, rheological properties, fish mince, semi-finished products, water-binding capacity

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INTRODUCTION

Commercial fishing and fish processing is an important branch of global food industry. Until recently, fish processing mainly involved refrigeration, drying, or salting of freshly-caught fish. Canning and other means of preservation expanded the range of fish products and seafood [23]. However, they changed the sensory properties of fish, reduced its nutritional value, and increased the cost.

Most fish consumers prefer fresh fish and seafood to ready-to-eat semi-finished fish products [2]. As a result, the fishing industry is currently facing a serious challenge: fresh fish has to be delivered from the catch to the point of sale as fast as possible and with as little change in its composition as possible.

However, fast delivery is not always possible:

1. Most often, geographical location makes fast delivery of live or fresh fish impossible without additional measures to prevent spoilage [3].
2. Unreliable refrigeration equipment may cause temperature fluctuations in cooling chambers, thus increasing the risk of product spoilage.
3. Enzymes in the fish entrails are highly active. As a result, chilled fish is impossible to refrigerate if ungutted. However, gutting opens the way for pathogenic and putrefactive microorganisms to the internal tissues, which reduces the shelf life [9].
4. Quick freezing requires expensive modern equipment, which significantly increases the cost of the finished product. Moreover, consumers always prefer fresh fish [5].

5. Slow freezing significantly reduces the biological and nutritional value of fish, as well as its sensory properties. Tissues become pale, and the texture turns loose and watery. Defrosted fish loses in weight; its protein structure is irreversibly damaged. After cooking, defrosted fish is always inferior in quality to fresh or chilled fish [4].

6. Delivery and refrigeration of fish from the place of catch to the point of sale and consumption creates poor added value, few jobs, and insignificant positive impact on the economy. However, uncontrolled delivery and distribution of fish opens the way to various forms of violations [6].

Therefore, fish processing industry remains underdeveloped and requires improvement.

To become profitable, fish processing should follow the path of the meat industry and develop more sustainable processing procedures. For instance, the meat-processing industry cannot afford to concentrate solely on fillet production, while sending the rest of the carcasses away for feed purposes or disposal, and this is exactly what happens now in the fish processing industry. As the deficit of dietary protein in the world keeps growing, all available fish and seafood protein must be used for food purposes. Thus, the fish processing industry has no other option but to develop a wide range of profitable, delicious, and nutritious food products [7, 11].

The research objective was to develop a technology for sustainable and waste-free fish processing for food purposes, namely, processing frozen undersized sea fish into minced food products.

In minced food production, the result depends on the right choice of fish raw material, since the tissues of various fish species vary in composition, nutritional value, quantity, as well as a ratio of edible, inedible, and conditionally edible parts. Moreover, different fish sorts have different ratios of proteins, lipids, and moisture [18, 19].

Nevertheless, undersized fish has good prospects for minced food production:

1. Small schooling fish is usually caught in industrial volumes.
2. By-catch of undersized fish makes up a significant proportion of industrial fishing. As fish producers fail to find profitable and sustainable use for it, undersized fish gets processed into fertilizers, fishmeal, feeds for industrially grown fish, and fish oil [25].
3. Undersized fish usually has a uniform biochemical profile, age, size, and ratio of proteins, lipids, and water content [13].
4. As a rule, undersized fish is much cheaper than large fish; therefore, processing that increases the added value is more profitable for the fishing industry.
5. Undersized fish is easier to process into mince, since it does not have to be divided into muscle tissue and bones; in fact, a proper cutting mode makes it possible not to remove bones at all [12].

The present research featured Peruvian anchovy (*Engraulis ringens L.*) [25]. The choice of anchovy for fish mince production was based on the abovementioned reasons, as well as on the need to expand the range of fish-based ready-to-eat products [10, 11].

Fish mince production presupposes grinding of raw materials either in a fish bone separator Neopress or in a cutter.

A separator is a more traditional option for the fishing industry. However, it requires preprocessing, namely gutting and beheading, which is a significant disadvantage of this technology. Moreover, it produces a large amount of non-food waste, which must be disposed of. The yield of mince is about 55–60%, depending on the Neopress mode and the requirements for the mince texture [13].

Neopress processing requires additional equipment to introduce and distribute food additives, e.g. a mixer, lifters for mince carts, etc. Another disadvantage is the prospective disposal or recycling of industrial waste, e.g. skin, bones, etc.

In contrast to separators, a high-speed cutter ensures complex processing and produces meat of required composition [14].

Industrial fish cutters have the following advantages:

1. They can mince frozen fish without prior defrosting, which saves time and production space required for defrosting. As a result, fish is not exposed to additional microbial contamination as it never comes in direct contact with workers' hands. Also, fish does not lose muscle juice during defrosting. In addition, industrial cutters decrease the risk of heating the product during cutting.

2. Necessary additives can be introduced right during cutting, and their distribution is even. In fact, the entire production cycle takes place inside the cutting bowl. The technology requires no additional equipment, e.g. mixers, and reduces the exposure of the mince to microbial contamination and oxidative spoilage, which is inevitable when the mass is overloaded from the Neopress bowl into the mixer [8].

3. Cutters have a very sensitive control of grinding modes. They are also capable of mixing the introduced components without grinding.

4. Cutters produce no by-products that have to be disposed of. When minced together with its own bones, fish becomes fortified with easily digestible calcium.

5. Cutters can adjust the texture of mince. They even make it possible to obtain fish mince with a pattern on the cut side of the frozen block. Patterning looks especially good when the fish raw material is mixed with various components of contrast color, e.g. spices, herbs, eggs, potato, green pea, etc.

Taking into consideration the obvious advantages of this technology, the experiment involved cutting frozen undersized sea fish entirely, without gutting and beheading.

According to the selected technology, the obtained fish mince is a semi-finished product and cannot be sold directly to retail consumers because of its poor sensory properties. The structure and viscosity of the fish mince vary significantly, depending on the fishing season, age, and size of the fish. Therefore, fish mince must be improved with food additives in order to standardize its properties [16].

STUDY OBJECTS AND METHODS

The research featured samples of minced undersized fish without additives (control) and with flour, wheat fiber, textured soy, onion, food dyer, and spices (experimental samples).

The frozen Peruvian anchovy (*Engraulis ringens L.*) was produced by Colanfish S.A.C. (Peru). It was minced using a Talsa 315 cutter (MP-Technologies, Russia). The experimental samples included the following additives:

– pea flour (Specification 10.61.22-696-37676459-2017. Pea flour[I])
– buckwheat and rice flour (State Standard 31645-2012[II]);
– corn flour (State Standard 14176-69[III]);
– wheat fiber Vitacel WF 200 (Germany);
– textured soy Opttema *M*-03 (Ingredienty. Razvitiye Llc, Russia);
– titanium dioxide E171; and
– spices: paprika powder and turmeric powder (Tsarskaya Priprava Llc, Arta Grupp).

The block-frozen fish was stored at –18°C; the temperature in the center of the block was –16°C at the onset of the experiment. The fish was minced into a fine and pasty homogeneous emulsion.

The physical and chemical properties of the fish mince were determined using standard methods. The mass fraction of moisture and dry matter in the fish mince was registered using several methods: the infrared thermogravimetric method in an Evlas-2M moisture analyzer, the thermogravimetric method in an APS-1 dryer (Analit-Servis), and the arbitration method, which involved drying the sample to constant weight in a drying chamber at 102–105°C.

The rheological properties were measured in a PNDP penetrometer according to State Standard R 50814-95[IV].

The water binding capacity was defined in a Benchmark LC-8 laboratory centrifuge. The control and experimental samples (4 g each) were put in a polyethylene test tube with a perforated insert. The latter was placed in such a way as to allow the liquid to drain. The samples were centrifuged for 20 min at 100 s^{-1}. After centrifugation, the weight of each sample was added to the weight of the substances in the separated liquid, which was determined by drying at 105°C to constant mass.

Percentage of bound moisture was calculated according to the following formula:

$$X = \frac{(m_1+m_3-m_2)\cdot 100}{m_0} \quad (1)$$

where m_1 is the weight of the sample after centrifugation, g; m_3 is the mass of the dry residue of the released liquid, g; m_2 is mass of dry residue in the sample, g; and m_0 is the weight of the sample before centrifugation, g.

The sensory properties of the fish mince were defined using a five-point tasting expert assessment.

RESULTS AND DISCUSSION

Changing the rheological parameters. Minced food industry uses only frozen fish, since undersized fish quickly deteriorates when heated during cutting. Frozen fish is minced to a fine and pasty homogenous emulsion. As a result, the initial and final viscosity values can be used as limit values applicable in practice [14].

Viscosity largely depends on the temperature, which increases with prolonged cutting. Overheating should be avoided by all means: the fish mince must stay below 0–2°C [21]. Figure 1 shows the effect of temperature on viscosity during cutting. The viscosity was measured when the fish mince started to melt and the ice crystals disappeared. The experiment proved that fish has to stay below –4°C during cutting. The present research presupposed no experiments for temperature above +6°C, since temperature this high is unacceptable for microbiological reasons [22].

As rheological properties are concerned, production of mince from whole fish has to pursue two opposing goals. On the one hand, the cutting should be coarse enough to preserve the natural texture, taste, and texture of the fish. However, a coarsely-minced mass maintains fragments of bones and other inclusions. On the other hand, fine grinding reduces the abovementioned risks and produces a fine and uniform paste, but it is quite fluid because muscle fibers and cell walls were destroyed.

In this research, the fish underwent a fine cutting. The mass acquired the desired texture from dietary fiber, various flours, and textured soy protein. These components are cheap, have a reliable technology, and produce a multifactorial effect on the properties of the product [23].

Wheat fiber bound excess water, fortified the product with fiber, and improved the sensory properties of the finished product. Figure 2 shows how the amount of wheat fiber affected the viscosity of the fish mince.

The wheat fiber proved to be a very effective stabilizer of texture in the amounts between 2.5% and 6%. The wheat fiber introduced at the onset of cutting bound the water that resulted from the

[I] TU 10.61.22-696-37676459-2017. Pea flour.
[II] State Standard 31645-2012. Flour for baby's nutrition. Specifications. Moscow: Standartinform; 2019. 11 p.
[III] State Standard 31645-2012. Corn flour. Specifications. Moscow: Standartinform; 2012. 4 p.
[IV] State Standard R 50814-95. Meat products. Methods of penetration determination by means of the cone and the needle indentor. Moscow: Standartinform; 2010. 8 p.

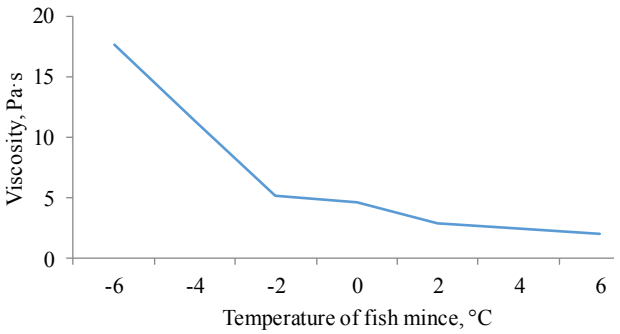

Figure 1 Effect of temperature on viscosity of fish during cutting

destruction of fish tissues. The obtained fish mince was of a medium-dry texture, so that extra water had to be added in a ratio of 2:1 to the fiber weight.

When the fiber was introduced at the final stage of cutting, it was less effective, and the fish mince contained more water. Adding 6% fiber to the fish mass reduced the adhesion of the mince mass to the bowl and to the plastic pads during freezing. However, more than 4.5% of fiber had a negative effect on the taste, adding a blend, "cottony" taste to the finished product.

Wheat fiber products are available in different fiber lengths. Coarse and long fiber proved to be the optimal variant: it was Vitacel WF 200 with the fiber length of 250 μm. Its long fibers served as an additional reinforcing frame for the mince system. The optimal amount was based on economic calculations and technological feasibility: 3.6% of wheat fiber improved the quality of the mince, when introduced at the second stage of cutting, right after table salt (1.3%). A larger amount of fiber impaired the taste of the product and made it crumbly when molded.

Figure 3 shows the results of sensory evaluation of the fish mince samples. The overall sensory quality of the product improved when the amount of fish fell down to 50–55% as it was substituted with onion, pea flour, textured soy, etc. According to the sensory evaluation, the sample with a minimal amount of minced fish tasted better. However, the experts were able to identify the product as fish-based but failed to guess the fish species. The rheological measurements (Table 1) proved that the

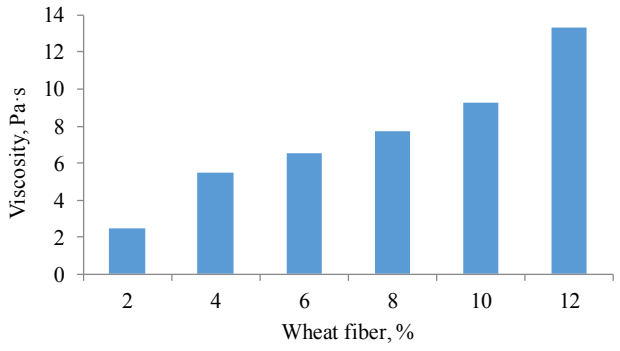

Figure 2 Effect of wheat fiber on viscosity of fish mince

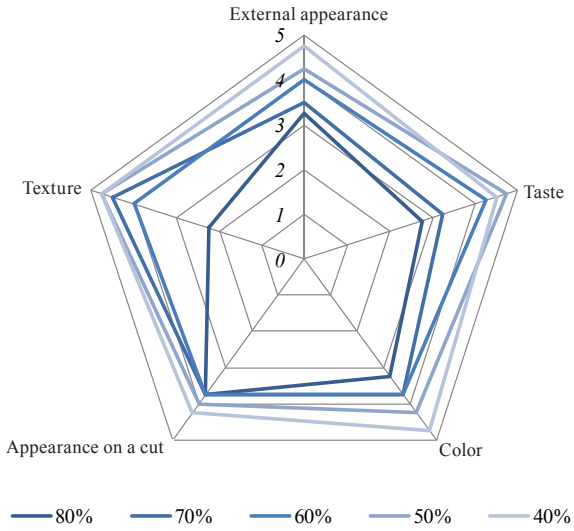

Figure 3 Sensory evaluation of fish mince depending on fish proportion in the product

strength of the sample depended on the mass fraction of the minced fish. A lower mass fraction increased the viscosity and density of the product. Therefore, the mass fraction of fish raw material had to be reduced by introducing various additives. The additives should be cheap and able to improve or, at least, not to spoil the taste of the product. Preference should be given to those additives that have a positive water-binding capacity of at least 1:1 and can improve the nutritional value of the product [16].

The mass fraction of fish could not go below 50%, since such a product would cease to be fish-based.

Additives were introduced at the third stage of cutting, after the texture of the fish mince was stabilized with food fibers. The additives were followed by the same amount of water (1:1) to avoid over-drying. In calculating the mass fraction of the additives, the mass fraction of fish always remained over 55%.

The additives involved rice, corn, buckwheat, and pea flours at the proportion of 5–15% at 2.5 intervals. Figure 4 shows the effect of the mass fraction of the abovementioned plant raw materials on the sensory properties of the samples.

In each case, the plant additives significantly changed the sensory properties, which improved the quality of the semi-finished products. Flour increases the density and texture, as well as the water-retaining

Table 1 Shear stress of fish mince with different mass fraction of fish ($P > 95$, n = 3)

Mass fraction of fish in the sample,%	Max. shear stress, Pa
80	27.8 ± 0.2
70	29.6 ± 0.2
60	35.4 ± 0.3
50	37.2 ± 0.2
40	32.6 ± 0.3

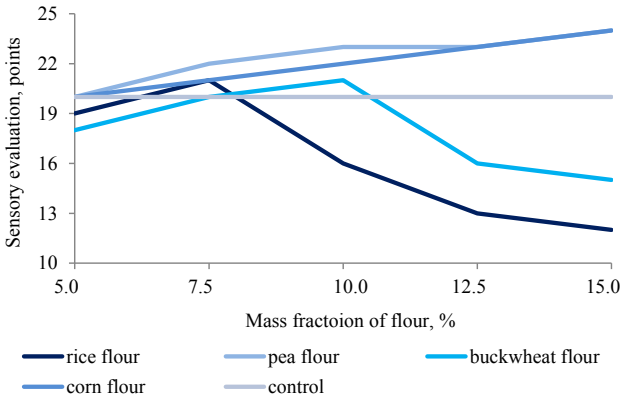

Figure 4 Effect of mass fraction of plant additives on sensory evaluation score of fish mince

and water-binding capacity, and does not affect the typical taste of the product. After the extreme point, the complex sensory index decreased as the properties of the plant component began to prevail. As a result, the product lost its sensory properties. A higher content of flour failed to form a strong structure with the fish component, which decreased the rheological parameters of the semi-finished products at the stage of freezing and subsequent heat treatment.

The effect of the flour mass fraction on the increase in the water-retaining capacity was of complex nature, which could be explained by the high, yet ambiguous water-binding characteristics of the plant ingredients (Table 2). The pea flour demonstrated the most pronounced water-binding capacity, while the rice flour had the lowest index. High water content made the finished product tenderer and increased its economic efficiency.

The rice flour had a low water-retaining capacity (1:0.8). Its amino acid score was also lower than that in the other flours [15]. The buckwheat flour gave the fish product an uncharacteristic color and taste, which makes it unsuitable for such products. The corn flour and the buckwheat flour had equally high water-retaining properties (1:2.2), while the pea flour demonstrated a better amino acid score and proved cheaper [15].

Therefore, the pea flour appeared to be the optimal additive. The amount was calculated for combined use with other ingredients. The sample with a mass fraction of pea flour ≤ 15% showed the best results. The same amount of water was added to hydrate the pea flour. A higher amount resulted in a specific pea taste, which was stronger than the taste of fish.

Flours and dietary fiber made it possible to raise the viscosity of the fish mince to the required level. However, the resulting product had a suspiciously uniform texture without any inclusions on the cut, which became even smoother after heat treatment. As a result, the experts questioned the presence of fish in the product, as nothing in the mince reminded of fish but its taste. The presence of minced fish in semi-finished products, e.g. fish balls, cutlets, fishburgers, etc., is

Table 2 Effect of the mass fraction and type of flour on water content and water-binding capacity, % ($P > 95$, n = 3)

Additive	Water content, %	Water-binding capacity, %
Rice flour:		
5.0	68.3 ± 0.1	18.3 ± 0.1
7.5	70.6 ± 0.1	18.5 ± 0.1
10.0	72.4 ± 0.2	19.2 ± 0.1
12.5	73.1 ± 0.1	19.8 ± 0.2
15.0	74.4 ± 0.2	20.3 ± 0.1
Pea flour:		
5.0	70.1 ± 0.1	19.4 ± 0.1
7.5	72.5 ± 0.1	21.1 ± 0.1
10.0	74.2 ± 0.1	22.3 ± 0.1
12.5	75.7 ± 0.2	23.0 ± 0.1
15.0	76.6 ± 0.1	24.2 ± 0.1
Buckwheat flour:		
5.0	69.1 ± 0.1	18.3 ± 0.2
7.5	72.1 ± 0.1	19.2 ± 0.1
10.0	73.0 ± 0.1	21.6 ± 0.1
12.5	73.7 ± 0.2	22.7 ± 0.1
15.0	74.2 ± 0.1	23.6 ± 0.1
Corn flour:		
5.0	69.5 ± 0.2	18.7 ± 0.2
7.5	71.0 ± 0.1	18.9 ± 0.1
10.0	72.1 ± 0.2	20.1 ± 0.1
12.5	72.8 ± 0.1	21.1 ± 0.1
15.0	73.6 ± 0.1	22.0 ± 0.1
Control	72.2 ± 0.1	18.6 ± 0.2

usually quite obvious and marks the product as natural. Any expert would interpret the homogeneous structure of a semi-finished product as a sign of its artificial nature.

The textured soy proved to be the best ingredient that gave the fish mince a non-uniform texture of fish meatballs. Introducing textured soy into the fish mince had the following specifics:

1. The texturing agent should be added after hydration in a ratio of 1:2.2 to water. A larger hydration ratio dissolved the coarse texture.
2. The texturing agent should be introduced at the very last stage of cutting, and the knives should be turned to mixing mode in order to prevent excessive cutting and loss of texture.
3. The texturing agent should not be too dry, since the fish mince has no free water left at the last stage of cutting, and the next stage is refrigeration.

The amount of textured soy depends on the desired properties of the final product. For fish rolls and fishburgers, the experiment delivered the following optimal parameters: 10% of dry texturing agent to the mass of anchovy and 22% of water for its hydration. A smaller amount of hydrated texturing agent did not make the product heterogeneous, while a higher amount made the experts mistake it for artificial.

Changing the color of the minced products. The color of the fish product depends on the properties of the fish itself. When minced, it darkened even more due to

(a) (b)

Figure 5 Samples of fish mince: a – unbleached, b – bleached (titanium dioxide, 0.8%)

the contact of muscle tissue with oxygen. Food pigments and dyes could change the color of fish mince and finished products. Titanium dioxide (E171) was able to bleach the fish mince. It was chosen for its effectiveness and good safety requirements [17, 20]. Depending on the amount, the fish mince acquired different hues of light gray (Fig. 5). The maximal amount was 0.8–1%; a further increase in concentration was inappropriate in terms of color. Paprika and turmeric powder gave the product different hues (Fig. 6).

Color correction was optimal after preliminary bleaching with titanium dioxide E171; if added before bleaching, a much larger amount of dyes and pigments was required.

Changing the taste of the minced products. The experiment featured undersized fish with a low sensory profile, which was minced together with heads and entrails. As a result, the taste indicators of the fish mince samples were in direct proportion to the percentage of the fish in the finished product. With the concentration of minced fish exceeded 55%, the sensory properties decreased, and the taste, color, and texture deteriorated.

Spices, especially dried fried and fresh onion, improved the taste of the finished product, masking the typical taste of cheap fish. The fresh onion provided the best taste and also improved the texture of the fish mince by changing the degree of fineness. Onion bleached the color as the concentration of fish in the sample was lower, and the antioxidants in the onion juice affected the pigments in the tissues of the minced anchovy. Onion also extended the shelf life of the samples as its phytoncides affected the microflora.

The experiment featured several types of onion: dried onion flakes, dried fried onion granules, and fresh onion. On the one hand, such a variety of dried onion is convenient in production. The flakes were easy to store, hydrate, and apply exactly according to the formulation. On the other hand, dried onion was more expensive and had neither antioxidant nor antimicrobial effects. Moreover, it did not lighten the color of the fish mince.

Finally, fresh onion was considered optimal at ≤ 12% to the mass of the raw fish. A larger amount of onion could water in the mince and make it impractical. Onion was introduced before the textured soy to ensure its uniform cutting and distribution.

Fish cutter technology. To avoid excessive temperature rise and oxidation, the cutting time was as short as possible and included the following stages:

1. High-speed cutting of frozen fish in the shortest time possible, preferably under vacuum.
2. Adding sodium chloride and titanium dioxide E171 to bleach the color of the mince.
3. Introducing dietary fiber to bind the water released from the mince, prevent the mince from sticking to the bottom of the bowl, and avoid its overheating.
4. Adding a precalculated volume of water to hydrate the dietary fiber in a ratio of 2:1.
5. Introducing fresh onion.
6. Introducing additives and textured soy hydrated in a ratio of 1:2.2, as well as extra water to hydrate the additives.
7. Adding spices.
8. Ceasing the cutting process before the mass reaches +2°C to prevent its oxidative and microbial deterioration.

(a) (b) (c)

Figure 6 Effect of spices on the color of fish mince: a – bleached (titanium dioxide, 0.8%), b – with 0.8% of paprika, c – with 1.3% of turmeric

Table 3 Microbiological indicators of finished products before cooking

Product	Microbiological indicators: actual content/standard			
	QMAFAnM, CFU/g, ≤	Coliforms	*S. aureus*	Pathogenic, including *Salmonellae* and *Listeria monocytogenes*
Fish bolls	$0.34\times10^5/1\times10^5$	0.0008/0.001	not detected	not detected

9. Molding the fish mince into cutlets or balls.
10. Breading the balls.
11. Freezing or cooking.

The microbiological control showed acceptable amounts of mesophilic aerobic and facultative anaerobic microorganisms (QMAFAnM) and bacteria of the *Esherichia coli* group (Table 3).

CONCLUSION

Cutting proved to be a reliable and efficient technology. The research featured samples of undersized block-frozen sea fish, which appeared to be a perfect raw material for cutter processing. The best results belonged to samples frozen at –6… –4°C without preliminary defrosting or heating.

The optimal rheological and sensory parameters belonged to the samples with mass fraction of dietary fiber = 3.6%, pea flour = 15%, and soy texturing agent = ≤ 15% (hydration level depended on the desired texture). Titanium dioxide E171 (0.8%) bleached the color of the fish mince. Natural spices, food pigments, and dyes gave the samples attractive yellow-red color range. Onion significantly improved the taste of the product. Samples with 12% of onion received the highest sensory evaluation score.

Although the technology was tested on Peruvian anchovy (*Engraulis ringens* L.), it can be applied to most commercial undersized sea fish. This sustainable technology can provide the yield of 130% of fish mince. The recommended mass fraction of fish in the finished product should not exceed 55%, as a higher ratio could spoil the sensory profile of the product.

CONTRIBUTION

I.L. Rakityanskaya supervised the project. A.A. Gorbatovskiy performed the experimental research. A.A. Gorbatovskiy and M.V. Kaledina processed the data and prepared the manuscript.

CONFLICT OF INTEREST

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

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κ-casein polymorphism effect on technological properties of dried milk

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Abstract:
Introduction. Numerous molecular genetic studies have revealed a correlation between the polymorphism of milk protein genes and the technological properties of milk raw materials. DNA analysis, in particular, initiated research into the influence of allelic variants of κ-casein (*CSN3*) on thermal stability and cheese suitability of milk. This gives relevance to our study that compares the results of genotypic identification of lactating cows by the κ-casein gene, using raw and processed milk samples.
Study objects and methods. Our study used raw and reconstituted milk samples from first-calf cows of the black motley breed with the *AA* and *BB* genotypes of the κ-casein gene. The samples were analyzed by standardized and generally accepted chemical engineering methods, as well as by capillary electrophoresis and PCR-RFLP analysis.
Results and discussion. We compared the results of tests on thermal stability and cheese suitability of raw and reconstituted milk samples from cows with the *AA* and *BB* genotypes of the κ-casein gene. We tried out an integrated approach to monitoring milk raw materials based on the most relevant technological criteria and correlating the data with the associated *CSN3* gene identification parameters. The PCR-RFLP analysis revealed reproducible results for both raw and dried milk samples in relation to the genotypical identification by the *A*- and *B*- allelic variants of the *CSN3* gene. The tests showed higher thermal stability in the reconstituted milk from the *BB* genotype cow and better cheese suitability in the *AA* genotype sample.
Conclusion. We developed a system for evaluating milk raw materials based on the most important technological parameters in combination with their genotypic characteristics. Our research procedure can unify the accumulation of experimental data and contribute to the formation of bioinformatics algorithms. This approach can be used in mathematical modeling of criteria to evaluate the compliance of the technological properties of milk with the recommended indicators.

Keywords: Casein, genotype, PCR-RFLP, allele, milk, technological properties, whey, thermal stability

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INTRODUCTION

Each breed of lactating animals has a genetic potential that contributes to certain properties of the resulting milk. Breed characteristics determine not only the physicochemical composition of raw milk, but also its technological parameters and yield. As a result, the quality of dairy products also varies significantly [1–3].

In this regard, scientists are interested in determining genetic markers associated with the qualitative characteristics of milk. DNA technologies for developing technological properties of milk *in vivo* aim to model those parameters which are significant in certain areas of the dairy industry. Allelic variants of genes of milk proteins, hormones, and enzymes are the most probable genetic markers of milk quality. Numerous studies have analyzed the influence of cow genotypes on the rennet coagulation and thermal stability of milk. Most research has focused on α_{S1}-casein (*CSN1S1*), β-casein (*CSN2*), β-lactoglobulin (*BLG*), and κ-casein (*CSN3*) encoded by the genes of the same name [4–6].

Studies have proven that the *B* allele of the *CSN1S1* gene is associated with milk yield, while its *C* allele affects the protein milk content. Also, the *A* allele of the *CSN2* gene has a positive effect of on the thermal

stability of milk, while its *B* allele is responsible for rennet coagulation and is a synergist for the similar effect of the *CSN3 B* allele. The *BLG* gene is associated with the biological value of milk, its technological properties, and total protein, namely a high content of casein and fat fractions (*B* allele) and a high content of whey proteins (*A* allele).

The κ-casein (*CSN3*) gene determines both the protein milk content and the technological properties of milk. It is believed that its *A* allele is associated with thermal stability, while its *B* allele is responsible for a high protein content and cheese suitability [7].

Testing biomaterial by determining the κ-casein gene locus is currently one of the most widely used methods. This is due to the frequency of occurrence of its prevailing alleles and the evidence of their association with the technological parameters of milk [8–10].

Many years of research have identified more than ten allelic variants of the *CSN3* gene in cattle. The *A* and *B* alleles are most common and depend on the breed and geographic range. Scientists have also established a significant prevalence of the *A* over the *B* variant [7].

Allelic polymorphism of the κ-casein gene includes three main genotypes: *AA*, *AB*, and *BB*. Their variability affects the composition and properties of cow milk. Numerous studies of the κ-casein genotypes in domestic and foreign breeds have revealed a trend of increasing daily milk yield and decreasing protein mass fraction from the *B* allele to the *A* allele [7].

Despite some conflicting results [11], most researchers agree that the $CSN3^{AA}$ genotype has a positive effect on the thermal stability of milk, while the $CSN3^{BB}$ genotype determines its cheese suitability [12].

The polymorphism of milk proteins was initially studied on lactating cows by means of electrophoresis and isoelectric focusing. Calves and bulls were genotyped by mass testing of milk from their ancestors and offspring through the female line. Such studies took about 5–6 years. Long duration and high costs made it practically impossible to correct the desired alleles in the animal population [13].

New methods of genotyping polymorphic variants of milk proteins at the DNA level opened up wide opportunities for breeding and research. The most widely used method is the polymerase chain reaction (PCR) followed by the analysis of restriction fragment length polymorphism (RFLP) [13].

The PCR-RFLP analysis made it possible to identify the polymorphism of milk proteins using not only the animal's biomaterial, but also raw milk and processed materials. In particular, these methods enabled scientists to interpret PCR-RFLP fragments of the DNA extracted from a number of dairy products. This opened up new prospects for milk processors in predicting the technological properties of raw milk [14, 15].

We aimed to compare the results of the κ-casein gene genotypic identification of lactating cows, using whole raw and freeze-dried milk samples. After interpreting the data, we assessed the physicochemical parameters and technological properties of the reconstituted product in order to determine the need for further processing of dried milk.

To achieve this aim, we set the following objectives:
– to genotype cows by the PCR-RFLP analysis for allelic variants of the κ-casein gene in dried and raw milk samples;
– to try out a comprehensive approach to monitoring the technological properties of reconstituted milk using multidirectional methods;
– to assess the influence of κ-casein genetic polymorphism on the technological properties of milk.

STUDY OBJECTS AND METHODS

Sampling and laboratory analysis. Milk was obtained from first-calf cows of the black-motley breed (marked 357 and 12) at the Mukhametshin farm, the Republic of Tatarstan. Their genotypes for the κ-casein gene, identified by the DNA analysis of raw milk samples, were *AA* and *BB*, respectively. Raw milk was frozen during 3–5 h after milking and delivered to the All-Russian Dairy Research Institute within two days.

After thawing, raw milk from cows with the *AA* and *BB* genotypes was sampled (*AA*[1] and *BB*[1], respectively) for extended physicochemical analysis (Table 1). The rest of the milk of each type was freeze-dried without preliminary heat treatment in order to preserve most of its native qualities.

Freeze-drying. Defrosted raw milk was freeze-dried on 1 dm^3 trays in a TG-50 apparatus (Hochvakuum, Germany) under the following conditions:
– initial temperature: –35 ± 5°C;
– final temperature: +30 ± 5°C;
– condenser temperature: –35 ± 5°C;
– residual pressure: 0.3–1.3 kPa; and
– process time: 42 ± 2 h.

Physicochemical analysis. Raw milk and then reconstituted dried milk were subjected to physicochemical analysis (Table 1) to determine the parameters of milk reconstitution [16, 17].

The moisture content was determined by thermogravimetric methods according to State Standard ISO 6731/IDF 21-2012[I].

The protein content was determined by measuring total nitrogen according to the Kjeldahl method and converting it to protein according to ISO 1871:2009[II]/

[1] State Standard ISO 6731/IDF 21-2012. Milk, cream and evaporated milk. Determination of total solids content (Reference method). Moscow: Standartinform; 2019. 6 p.
[II] ISO 1871:2009. Food and feed products — General guidelines for the determination of nitrogen by the Kjeldahl method. Geneve: International Organization for Standardization; 2009. 12 p.

Table 1 Physicochemical composition of whole milk and reconstituted dried milk samples

Indicator	± uncertainty	Whole milk samples		Reconstituted dried milk samples		
		AA[1]	*BB*[1]	*AA*[2]	*AA*[3]	*BB*[2]
Fat, %	± 0.150	3.90	3.60	3.70	3.30	3.40
Total proteins, %	± 0.060	3.14	2.81	2.96	2.65	2.67
Whey proteins, %	± 0.200	0.80	0.75	0.75	0.68	0.70
Casein proteins, %	± 0.033	2.39	2.08	2.22	2.01	1.95
Moisture, %	± 0.200	87.14	88.80	87.83	89.12	89.24
Non-fat milk solids, %	± 0.400	8.96	7.60	8.47	7.58	7.36
Lactose, %	± 0.350	5.20	4.40	4.90	4.40	4.30
Freezing temperature, °C	± 0.001	–0.526	–0.507	–	–	–
Acidity, °T	± 1.000	17.00	16.90	15.00	13.50	12.00
Ca, mg/kg	± 0.500	116.40	93.12	110.20	98.30	85.00
Active acidity, pH units	± 0.020	6.70	6.69	6.61	6.64	6.69

AA[1] and *BB*[1] are raw milk samples from cows with *AA* and *BB* genotypes

AA[2] and *BB*[2] are freeze-dried milk samples from cows with *AA* and *BB* genotypes

AA[3] is a freeze-dried milk sample with the same protein content as sample *BB*[2]

ISO 8968-1:2014 [IDF 20-1:2014][III] on a Kjeltec-2400 Auto Analyzer (Foss Electric, Denmark).

The content of non-protein nitrogen was determined by precipitating protein nitrogenous substances with trichloroacetic acid and measuring total nitrogen in the filtrate according to ISO 8968-4: 2016 [IDF 20-4: 2016][IV].

Casein and whey proteins were determined by acid precipitation of casein and measurement of total nitrogen in the filtrate according to ISO 17997-1:2004 [IDF 29-1:2004][V]. Based on the content of total and non-protein nitrogen, we calculated the ratio between whey and casein proteins.

The fat content was determined by the Gerber acid method according to State Standard R ISO 2446-2011[VI]/ ISO 19662:2018 [IDF 238:2018][VII]/ISO 11870:2009 [IDF 152:2009][VIII].

The lactose content was determined by the polarimetric method according to State Standard R 54667-2011[IX] and by enzymatic method used as a control according to ISO 26462:2010 [IDF 214:2010][X].

The freezing point for raw milk was determined by the thermistor cryoscope method according to ISO 5764:2009 [IDF 108:2009][XI].

The calcium content was measured by the titrimetric method according to ISO 12081:2010 [IDF 36:2010][XII].

Determination of amino acid composition. The amino acid content was determined by capillary electrophoresis on a Kapel 205M system (Lumex, Russia). This method involves decomposing the samples by acid and alkaline hydrolysis (only for tryptophan), converting amino acids into free forms, obtaining phenylthiocarbonyl derivatives, and their subsequent separation and quantification. The standards of amino acids with at least 98% of the basic substance were used as analytical standards. The electrophoresis system had a quartz capillary with a total length of 60 cm, an effective length of 50 cm, and an inner diameter of 50 μm. The sample solutions were hydrodynamically introduced into the capillary at 30 mbar for 5 s. The separation voltage was 25 kV. UV detection was carried out at a wavelength of 254 nm, with a data collection rate of 2.5 Hz. The electropherograms were processed using the Elforan software. Prior to use, the capillary was washed with a 1 mol/dm^3 NaOH solution, then with ultrapure water, and finally with base electrolyte, for 5 min each.

[III] ISO 8968-1:2014 [IDF 20-1:2014]. Milk and milk products – Determination of nitrogen content – Part 1: Kjeldahl principle and crude protein calculation. Geneva: International Organization for Standardization; 2014. 18 p.

[IV] ISO 8968-4:2016 [IDF 20-4:2016]. Milk and milk products – Determination of nitrogen content – Part 4: Determination of protein and non-protein nitrogen content and true protein content calculation (Reference method). Geneva: International Organization for Standardization; 2016. 11 p.

[V] ISO 17997-1:2004 [IDF 29-1:2004]. Milk – Determination of casein-nitrogen content – Part 1: Indirect method (Reference method). Geneva: International Organization for Standardization; 2004. 8 p.

[VI] State Standard R ISO 2446-2011. Milk. Method for determination of fat content. Moscow: Standartinform; 2012. 12 p.

[VII] ISO 19662:2018 [IDF 238:2018]. Milk – Determination of fat content – Acido-butyrometric (Gerber method). Geneva: International Organization for Standardization; 2018. 15 p.

[VIII] ISO 11870:2009 [IDF 152:2009]. Milk and milk products – Determination of fat content – General guidance on the use of butyrometric methods. Geneva: International Organization for Standardization; 2009. 7 p.

[IX] State Standard R 54667-2011. Milk and milk products. Methods for determination of sugars mass fraction. Moscow: Standartinform; 2012. 27 p.

[X] ISO 26462:2010 [IDF 214:2010]. Milk – Determination of lactose content – Enzymatic method using difference in pH. Geneva: International Organization for Standardization; 2010. 11 p.

[XI] ISO 5764:2009 [IDF 108:2009]. Milk – Determination of freezing point – Thermistor cryoscope method (Reference method). Geneva: International Organization for Standardization; 2009. 17 p.

[XII] ISO 12081:2010 [IDF 36:2010]. Milk – Determination of calcium content – Titrimetric method. Geneva: International Organization for Standardization; 2010. 6 p.

Table 2 Genotyping primers for the *CSN3 A* and *B* alleles generated PCR products and RFLP fragments

Primer nucleotide sequences	PCR-product (bp)	Genotype-specific RFLP-fragments (bp)		
		AA	*BB*	*AB*
JK5: 5′-ATCATTTATGGCCATTCCACCAAAG-3′ JK3: 5′-GCCCATTTCGCCTTCTCTGTAACAGA-3′	350	*Hinf*I		
		134	265	265
		131	85	134
		85		131
				85

PCR-RFLP analysis. The next stage involved the identification of freeze-dried milk by the *A* and *B* alleles of the *CSN3* gene in order to establish correlations with the data on raw milk. The polymorphism of milk proteins in the *CSN3* gene was analyzed by PCR-RFLP.

DNA extraction from dried milk. DNA was extracted in accordance with the DNA-sorb-S-M instructions (Central Research Institute of Epidemiology, Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing). 400 μL of buffer for lysis reagent and 17 μL of lysis reagent were added to 50 mg of dried milk in Eppendorf tubes. The tubes were thermostated at 64°C for 60 min and agitated occasionally in a vortex shaker. The undissolved particles were precipitated by centrifugation at 12 000 rpm for 5 min.

200–350 μL of the supernatant was collected and transferred into tubes with 25 μL of the resuspended universal sorbent. The tubes were tightly closed and left for 10 min, occasionally shaken. After centrifugation for 1 min at 5000 rpm, the supernatant was removed. The test tubes were filled with 300 μL of washing solution No. 1, tightly closed, and resuspended. After another centrifugation for 1 min at 2000 rpm, the supernatant was removed. Then, we added 500 μL of washing solution No. 2, closed the tubes tightly, and stirred until complete resuspension. The mixture was centrifuged for 1 min at 10 000 rpm and the supernatant was removed. After a repeated washing with solution No. 2, the supernatant was removed completely. The tubes were thermostated for 5–10 min at 64°C and 50 μL of elution buffer was added. After complete resuspension, the samples were thermostated again for 5–10 min at 64°C and centrifuged for 1 min at 12 000 rpm. The supernatant with purified DNA was collected for PCR.

CSN3 genotyping by PCR-RFLP. Amplification was performed on a Tertsik programmable amplifier (Russia). A 20 μL reaction mixture (SibEnzyme, Russia) contained 2 μL of dNTP mixture (0.25 mM each), 2 μL of buffer for Taq DNA polymerase (1×), 0.2 μL of Taq DNA polymerase (1 unit), 0.4 μL of JK5 and JK3 primers (0.5 μM each), and 2 μL of the DNA sample. The analysis was conducted in the following modes:

×1:94°C – 4 min; ×35:94°C – 10 sec, 63°C – 10 sec, 72°C – 10 sec;

×1:72°C – 7 min; storage at 4°C [18].

The genotypes for allelic variants *A* and *B* were identified by subsequent endonuclease segregation of amplicons. 10 μL of the PCR sample was treated with 5 units of HinfI restriction endonuclease in "O" SE-buffer (SibEnzyme, Russia) and incubated at 37°C overnight.

The range of genotype-specific RFLP fragments is presented in Table 2.

Detection. The incubated PCR-RFLP samples were mixed with a buffer to be loaded onto an agarose gel (4× Gel Loading Dye, Blue, Evrogen, Russia) in a 3:1 ratio. The stained amplificates were loaded into the wells of a 2% agarose gel prepared by melting 2 g of agarose (Biotechnology Grade, Amresco, USA) in 100 mL of Tris-acetate electrode buffer (500 mL of 1× TAE buffer, 15 μL of 1% ethidium bromide solution). Detection was performed by horizontal electrophoresis using a SE-2 camera (Helikon, Russia) and an Elf-4 power supply (DNA-Technology, Russia). The results were visualized on a Gel Doc XR+ gel documenting transilluminator using the Image Lab software (Bio-Rad, USA). The electrophoresis lasted 45 min and had an output voltage of 180 V and an output current of 150 mA [18].

Milk reconstitution. Dried milk samples from cows with the *AA* and *BB* genotypes of the *CSN3* gene were recombined in such a way that they reached the amount of dry solids in the original raw milk. The samples weighing 12.21 g (*AA* genotype) and 10.79 g (*BB* genotype) were introduced into 87.79 g and 89.21 g of boiled distilled water (pH 6.5 ± 0.1, 40 ± 2°C), respectively (test samples AA^2 and BB^2). Another sample was made from 10.88 g of *AA* genotype dried milk and 89.12 g of water (test sample AA^3), with a protein content unified with sample BB^2. These ratios were chosen to ensure better comparability of the experimental results. After introducing dried milk into water, the samples were kept in a magnetic laboratory stirrer at 500 rpm for 15 min and then cooled to 20 ± 2°C [19, 20].

Determination of cheese suitability. Cheese suitability was determined by a rennet-fermenting test according to State Standard 32901-2014[XIII] with some modification for reconstituted dried milk. Rennet clotting was performed with standardized milk-clotting enzymes derived from the enzymatically produced chymosin CHY-MAX M 2500 IMCU (Chr. Hansen,

[XIII] State Standard 32901-2014. Milk and milk products. Methods of microbiological analysis. Moscow: Standartinform; 2015. 27 p.

Denmark) and Microclerici 2400 IMCU (Sacco Sistem, Italy). To prepare enzyme solutions, we calculated their activity based on the activity of 1 g of rennet enzyme (100 000 IMCU). We used 0.32 g of an enzyme with a 2500 IMCU/g activity and 0.33 g of an enzyme with a 2400 IMCU/g activity. The weighed milk-clotting preparations were dissolved in 100 mL of distilled water at 30 ± 1°C and kept for 30 min in a magnetic stirrer at 300 rpm.

Washed and dried 100 mL high beakers were filled with 90 cm^3 of reconstituted milk (38 ± 1°C) measured with a cylinder. Then, 3 cm^3 of the control enzyme sample was added to each beaker. The samples were thoroughly mixed and placed in a thermostat at 38 ± 1°C until a dense clot formed. We visually assessed the quality of the clot and recorded the duration of rennet clotting. In accordance with State Standard 32901-2014[13], milk was classified as I (good), II (satisfactory), and III (unsatisfactory).

After evaluating the clot, we filtered the separated whey through a lavsan cloth used in cheese-making. The whey was thoroughly mixed and analyzed for a protein content. The percentage of total protein conversion to whey was calculated as:

$$R = (P_s \times 100)/P_m \quad (1)$$

where R is total protein conversion to whey, %; Ps is a mass fraction of total protein in whey, %; P_m is a mass fraction of total protein in reconstituted milk, %.

Based on the data, we performed a comprehensive comparative assessment of the samples' cheese suitability.

Sample preparation for assessing thermal stability. After assessing the quality of rennet clotting, we conducted a number of tests to determine thermal stability. Since samples AA^2, AA^3 and BB^2 differed in active/titratable acidity, we established their buffer capacity to minimize its effect on the protein resistance to heat exposure [21]. Then, based on each of the samples, we modelled a series of test samples with active acidity ranging from pH 6.0 to pH 7.0 and a pH interval of 0.2. Finally, all the test samples in the model series were assessed for thermal stability.

Active acidity (pH) was measured by the potentiometric method using an InoLab pH Level 1 high-precision pH meter equipped with a Sen Tix 61 pH electrode and a temperature sensor for measuring pH in the 0–14 range.

Titratable acidity was determined according to ISO 6092:1980[XIV]/ISO 6091:2010 [IDF 86:2010][XV] and correlated with pH values and subsequent data on thermal stability.

Thermal stability determination by alcohol test. The studies followed State Standard 25228-82[XVI] for reconstituted milk. The method described there is based on the ability of ethanol solutions to denature proteins in milk. The All-Russian Dairy Research Institute modified the method so that it used solutions with ethanol concentrations of 68, 70, 72, 75, 80, 85, 90 and 95% in order to expand the measurement range taking into account different pH values of the model samples. 2 cm^3 of alcohol in a certain concentration was added to Petri dishes with 2 cm^3 of the reconstituted sample and stirred in a circular motion for 2 min. Then, we assessed the consistency of the test mixtures. Coagulation of proteins in the form of visually noticeable flakes meant reduced thermal stability. We compared the data in relation to the concentration of alcohol that did not lead to protein coagulation.

Thermal stability determination by calcium chloride test. The studies involved creating an excess content of calcium ions in the samples, followed by heat exposure [21]. Heat-resistant 10 cm^3 glass vials of HC-3 type (55 mm tall, 22 mm in diameter) were filled with 10 cm^3 of reconstituted milk (20 ± 2°C) and 0.5 cm^3 of a 1% $CaCl_2$ solution. The vials were closed with rubber stoppers and placed in a compact water bath. The lid was tightened with a pressure screw so that the stoppers did not pop out when heated. The bath was quickly filled with boiling water and placed on a heated electric stove to resume boiling immediately. We controlled the time with a stopwatch and monitored the temperature. The samples were kept at 100 ± 1°C for 4 min and then rapidly cooled to 20 ± 2°C by adding cold water. Finally, the contents of the vials were transferred into Petri dishes to assess the stability of the protein fraction.

Thermal stability determination by phosphate test. The study involved adding a KH_2PO_4 solution to reconstituted milk to change the salt balance in terms of the phosphorus content, followed by heat exposure [21]. 1 cm^3 of a KH_2PO_4 solution (68.1 g per 1 L of water) was added to HC-3 vials filled with 10 mL of milk. The vials were sealed with rubber stoppers and placed in a water bath at 100 ± 1°C for 5 min. After cooling, the contents were transferred into Petri dishes to determine the presence of protein flakes.

Thermal stability determination by acid-boiling test. A combination of heat exposure and various amounts of hydrochloric acid was used as a factor of influence on milk protein [21]. Three HC-3 vials were filled with 0.5, 0.8, and 1.2 cm^3 of 0.1N HCl solution and then with 10 cm^3 of reconstituted milk. After mixing, the vials were sealed with rubber stoppers and placed in a water bath at 100 ± 1°C for 3 min. Then, the vials were cooled and checked for coagulation. The level of protein stability was characterized by the amount of the acid added.

[XIV] ISO 6092:1980. Dried milk – Determination of titratable acidity (Routine method). Geneva: International Organization for Standarization; 1980. 2 p.

[XV] ISO 6091:2010 [IDF 86:2010]. Dried milk – Determination of titratable acidity (Reference method). Geneva: International Organization for Standardization; 2010. 4 p.

[XVI] State Standard 25228-82. Milk and cream. Method of determination of termostability on alcohol test. Moscow: Standartinform; 2009. 4 p.

Thermal stability determination by heat test. For this study, we used a UKT-150 thermal stability control apparatus designed by the All-Russian Dairy Research Institute [22]. The method involved comparing the times of protein coagulation in the milk samples placed in a glycerol bath (ultrathermostat) at 130 ± 1°C. 8-mL heat-resistant molybdenum glass tubes were filled with 3 mL of reconstituted milk and tightly closed with rubber stoppers. The tubes were placed in a metal cassette holder and clamped with screws so that the stoppers did not pop out during heating.

A LOIPLT-316a ultrathermostat filled with glycerin was heated to 130 ± 1°C. The cassette holder with the test tubes was placed in the thermostat so that the tubes were immersed in the heated liquid. Immediately after that, the connecting rod was started to move the stand with the test tubes in such a way that milk slowly oscillated back and forth to avoid burning on the tube walls.

The samples were kept in the ultrathermostat at 130 ± 1°C until milk proteins started to coagulate. With the first signs of coagulation in the form of precipitation or protein flakes, the timing was stopped for the respective sample. After registering coagulation in all the samples, we turned off the connecting rod, removed the cassette holder from the UKT-150, and cooled the test tubes. Their contents were transferred into Petri dishes to determine the nature of the clots. The thermal stability of the reconstituted samples was characterized by the time of beginning of coagulation.

Assessment of amino acid balance. The balance of amino acid composition was determined by the generally accepted methods described by Donskova and Zhakslykova [23, 24]. It included the amino acid score, the ratio between essential and nonessential amino acids, the ratio between essential and total amino acids, the utilitarian coefficient, redundancy and comparable redundancy of essential amino acids, as well as the essential amino acid index.

The experiment was carried out in three repetitions. The data were processed using Microsoft Excel 2019.

RESULTS AND DISCUSSION

Qualitative and quantitative composition of raw milk. By PCR-RFLP analysis, raw milk samples were identified by the κ-casein gene as *AA* and *BB*. After freeze-drying, the samples were analyzed by PCR-RFLP again. The analyses revealed reproducible results of identification for both raw and freeze-dried milk samples by the *A*- and *B*- allelic variants of the *CSN3* gene.

The physicochemical evaluation of the milk samples from cows with the *AA* and *BB* genotypes showed significant differences in both qualitative and quantitative terms. The *AA* genotype cow milk had a higher content of fat, protein, lactose, and total dry solids (Table 1), which largely correlated with the results of other studies.

The calcium content in the BB[1] sample was below the average (120 mg/kg), unlike sample *AA*[1]. Despite

Table 3 Amino acid composition of whole milk from cows with different *CSN3* genotypes

Indicator	Actual values, g/100 g protein			Amino-acid score, %	
	FAO/WHO scale	*AA*[1]	*BB*[1]	*AA*[1]	*BB*[1]
Valin	5.00	3.21 ± 0.19	3.19 ± 0.19	64.20*	63.80*
Isoleucine	4.00	3.58 ± 0.21	3.55 ± 0.21	89.50	88.75
Leucine	7.00	4.78 ± 0.29	4.73 ± 0.28	68.29	67.57
Lysine	5.50	5.09 ± 0.31	4.90 ± 0.29	92.55	89.09
Methionine + Cysteine	3.50	2.81 ± 0.17	3.34 ± 0.20	80.29	95.43
Threonine	4.00	2.94 ± 0.18	2.92 ± 0.18	73.50	73.00
Tryptophan	1.00	1.17 ± 0.07	1.09 ± 0.07	117.00	109.00
Phenylalanine + Tyrosine	6.00	5.81 ± 0.35	5.84 ± 0.35	96.83	97.33
Essential amino acids (EAA)	36.00	29.39 ± 1.76	24.32 ± 1.46	–	–
Arginine	–	4.38 ± 0.26	3.88 ± 0.23	–	–
Histidine	–	2.02 ± 0.12	1.89 ± 0.11	–	–
Asparagine	–	5.26 ± 0.32	5.38 ± 0.32	–	–
Serine	–	3.88 ± 0.23	3.66 ± 0.22	–	–
Glutamine	–	12.89 ± 0.77	12.70 ± 0.76	–	–
Glycine	–	1.20 ± 0.07	1.21 ± 0.07	–	–
Alanine	–	2.15 ± 0.13	2.16 ± 0.13	–	–
Proline	–	6.11 ± 0.37	6.32 ± 0.38	–	–
Nonessential amino acids (NEAA)	–	37.92 ± 2.28	37.21 ± 2.23	–	–
Total amino acids (TAA)	–	67.31 ± 4.04	61.53 ± 3.69	–	–
EAA/NEAA relation	0.56–0.67	0.78	0.65	–	–
EAA/TAA relation	0.36	0.43	0.40	–	–

* limiting amino acid

AA[1] and *BB*[1] – raw milk samples from cows with *AA* and *BB* genotypes

Table 4 Balance of amino acid composition of whole milk from cows with different *CSN3* genotypes

Sample	Utilitarian coefficient	Redundancy of essential amino acids, g/100 g protein	Comparable redundancy, g/100 g protein	Essential amino acid index
AA[1]	0.79	6.28	9.78	0.87
BB[1]	0.78	6.59	10.33	0.87

the differences in the protein content between samples *AA*[1] and *BB*[1], the ratio of caseins to whey proteins was consistently equal to 75:25% and 74:26%, respectively. Thus, the polymorphism of the *CSN3* genotypes significantly affected the quantitative composition of all milk components, while the comparable ratios between whey protein and casein in raw milk remained unchanged.

Assessment of amino acid balance in raw milk. To assess the effect of the *CSN3* polymorphism on the qualitative characteristics of the protein phase of milk, we studied the amino acid composition of milk samples obtained from cows with the *AA* and *BB* genotypes (Table 3). In this study, we took into account actual differences in the mass fraction of protein in the samples.

The comparative analysis of essential amino acids in the milk samples from cows with the *AA* and *BB* genotypes (samples *AA*[1] and *BB*[1], respectively) showed similar contents of valine, isoleucine, leucine, threonine, tryptophan, phenylalanine, and tyrosine. Sample *AA*[1] had a higher content of lysine (5.09 g/100 g protein) and a lower content of methionine and cystine (2.81 g/100 g protein), compared to sample *BB*[1]. The amino acid index, i.e. a ratio between essential and nonessential amino acids, was 0.65 for sample *BB*[1] (within the FAO/WHO recommendations of 0.56–0.67 for a balanced diet) and 0.78 for sample *AA*[1], which indicated a slight imbalance in favor of essential amino acids that might slow down their metabolic availability.

The comparison of the amino acid composition of the samples with that of the "ideal" protein featured valine as a limiting amino acid in both cases. Both samples showed insufficient scores (less than 100%) of all essential amino acids, except for tryptophan. The results were used to determine the value of milk protein in the samples with the *AA* and *BB* genotypes (Table 4).

As we can see, the above differences in the amino acid composition did not have a significant effect on the biological value of milk proteins in samples *AA*[1] and *BB*[1].

The effect of the κ-casein genotype on dried milk thermal stability. Thermal stability is one of the most important indicators in the production of dairy products from reconstituted dried milk. The stability of milk during heat exposure is determined by a combination of factors, such as acidity, a concentration of free ions of calcium, magnesium, phosphorus, and citrates, the amount of total protein, the ratio between protein fractions, and the degree of their hydration. Therefore, there is no universal method to determine thermal stability that would take into account all the criteria of system variability [25].

An alcohol test, due to its simplicity, is currently the most common method to determine thermal stability in the dairy industry. However, it does not always account for all the factors affecting the stability of milk protein during heat treatment. Therefore, we used five most common methods to study the stability of casein micelles in milk with κ-casein polymorphism, namely an alcohol test, a calcium chloride test, a phosphate test, an acid-boiling test, and a heat test. The experiment was conducted in a range of 6.0–7.0 pH units with an interval of 0.2 pH units to better understand the mechanism of casein destabilization.

The analysis of thermal stability of the reconstituted milk samples obtained from cows with different *CSN3* genotypes showed significant differences between samples *AA*[2] and *BB*[2] (Table 5). In particular, at pH 6.6, sample *BB*[2] withstood the action of a 85% alcohol solution, while *AA*[2] denatured with the addition of a 68% alcohol solution. The lower thermal stability of *AA*[2] compared to *BB*[2] can be explained by a higher content of protein (by 10%), lactose (by 14%), and colloidal calcium (by 30%) in sample *AA*[2]. Sample *AA*[3], which withstood an 80% alcohol test at pH 6.6, partially confirms this hypothesis.

All milk samples acidified to pH 6.4 showed a decrease in protein thermal stability. Unlike sample *AA*[3], sample *BB*[2] failed the test even at 68% alcohol, which made it unsuitable for processing at this acidity level. We can assume that these results are associated with the *CSN3* genotypic characteristics of the cows. Sample *AA*[3] had a more stable hydration membrane during alcohol exposure at a lower acidity level.

Noteworthily, all the samples withstood the alcohol test at pH 7.0 (95% alcohol) and pH 6.8 (90–95% alcohol). On the one hand, it indicated the absence of

Table 5 Thermal stability of reconstituted dried milk from cows with different *CSN3* genotypes by alcohol test

Sample	pH					
	6.0	6.2	6.4	6.6	6.8	7.0
AA[2]	< 68%	< 68%	< 68%	< 68%	90%	95%
AA[3]	< 68%	< 68%	68%	80%	90%	95%
BB[2]	< 68%	< 68%	< 68%	85%	95%	95%

AA[2] и *BB*[2] – reconstituted dried milk samples from cows with *AA* and *BB* genotypes

AA[3] – a reconstituted dried milk sample with the same protein content as sample *BB*[2]

Table 6 Thermal stability of reconstituted dried milk from cows with different *CSN3* genotypes by calcium chloride test

Sample	pH					
	6.0	6.2	6.4	6.6	6.8	7.0
AA^2	–	–	–	–	–	±
AA^3	–	–	–	–	–	±
BB^2	–	–	–	–	–	±

– negative result; ± conditionally positive result; + positive result

Table 7 Thermal stability of reconstituted dried milk from cows with different *CSN3* genotypes by phosphate test

Sample	pH					
	6.0	6.2	6.4	6.6	6.8	7.0
AA^2	–	–	–	–	+	+
AA^3	–	–	–	+	+	+
BB^2	–	–	–	+	+	+

– negative result; + positive result

Table 8 Thermal stability of reconstituted dried milk from cows with different *CSN3* genotypes by acid-boiling test

Sample	pH					
	6.0	6.2	6.4	6.6	6.8	7.0
AA^2	–	–	–	–	0.5 mL*	0.8 mL*
AA^3	–	–	–	–	0.5 mL*	0.5 mL*
BB^2	–	–	–	0.5 mL*	0.5 mL*	0.5 mL*

– negative result; *amount of 0.1N HCl withstood by milk, mL

Table 9 Thermal stability of reconstituted dried milk from cows with different *CSN3* genotypes by heat test

Sample	pH					
	6.0	6.2	6.4	6.6	6.8	7.0
AA^2	15 min	15 min	35 min	35 min	35 min	60 min
AA^3	5 min	12 min	27 min	33 min	35 min	60 min
BB^2	15 min	25 min	33 min	43 min	90 min	> 180 min

destabilizing factors for casein micelles in this pH range. On the other hand, there was a genetic dependence: the milk from the *BB* genotype cows revealed higher thermal stability than the *AA* genotype milk (pH 6.8–7.0).

All the samples failed a test with calcium chloride used as a destabilizing agent followed by heat exposure at pH 6.0–6.8 (Table 6). At pH 7.0, the samples contained sporadic protein agglomerates, which made them conditionally thermostable. Increased amounts of calcium ions in milk, especially at higher acidity, reduced the negative charge of casein micelles and the aggregation of casein particles. An increase in the size of aggregated particles decreased their thermal stability, ultimately leading to complete destabilization during heat exposure. Thus, we found that the *CSN3* genotypes had an insignificant effect on the thermal stability of milk whose salt balance was shifted by calcium ions.

The milk thermal stability test, which involves exposure to a solution of monosubstituted potassium phosphate in combination with heat treatment, showed the stability of samples AA^2, AA^2, and BB^2 in a pH range of 6.8–7.0 (Table 7). With a decrease in active acidity to pH 6.6, samples AA^3 and BB^2 remained stable, unlike AA^2, and only AA^3 had a denatured protein, which generally agreed with the data from the alcohol test. At pH 6.4, none of the samples was heat-resistant.

The acid-boiling test revealed insignificant differences between the milk samples with different *CSN3* genotypes, which can be clearly seen at pH 6.6 (Table 8). At this acidity level, sample BB^2 withstood the addition of 0.5 mL of 0.1N HCl, remaining thermostable. The stability of all the samples at pH 6.8 can be due to a partial neutralization of the acid by the alkali solution introduced during the preparation of milk samples. According to this method, milk is considered heat-resistant if it withstands an acid-boiling test with the addition of 0.8–1.0 mL of 0.1N HCl. Thus, only sample AA^2 at pH 7.0 can be considered heat-resistant.

The heat test, which takes into account all the factors affecting protein stability in the assessment of milk thermal stability, showed a partial correlation with the results of the other tests. The reconstituted dried milk from cows with the *BB* genotype (sample BB^2) showed better protein stability at pH 6.0–7.0 during high-heat treatment, compared to the other samples (Table 9). Sample BB^2 withstood high-heat treatment at pH 7.0 for more than 180 min (after 180 min, the experiment was terminated due to the absence of such regimes in world practice), while the samples from the *AA* genotype cows were stable for only 60 min.

Decreasing active acidity to pH 6.8 reduced the thermal stability by half for all the samples, while a decrease to pH 6.6 had a significant effect only on sample BB^2, reducing its thermal stability to 43 min. It appeared that an increase in pH from 6.6 to 7.0 decreased the positively charged groups on the surface of the casein micelle, which ultimately increased the total negative charge of the casein molecule and, therefore, its thermal stability. The genotypic differences of the κ-casein fractions in samples AA^3 and BB^2 most likely affected the rate of the reaction.

Lowering active acidity to pH 6.6 and more had the opposite effect, i.e. decreased the total negative charge of the casein molecule while maintaining the reaction rate for samples AA^3 and BB^2. These genotypically different samples with a similar physicochemical composition revealed a dependence between the time of heat treatment before the onset of protein destabilization and milk acidity. A decrease in milk acidity from pH 6.6 by 0.2 units lead to a decrease in the time of high-heat treatment by an average of 10 ± 3 min.

The effect of the κ-casein genotype on dried milk cheese suitability. The ability of milk, including

Table 10 Assessment of cheese suitability of reconstituted dried milk from cows with different *CSN3* genotypes by rennet-fermenting method

Parametr	MICROCLERICI (chymosin 2400 IMCU/g)		CHY-MAX (chymosin 2500 IMCU/g)	
	AA^3	BB^2	AA^3	BB^2
Rennet clotting time, min	100	360	60	150
Clot quality group	II	III	I	II
Protein content in whey, %	0.73	1.3	0.73	0.955
Total protein conversion to whey, %	28.6	48.0	28.6	35.7

reconstituted milk, to form a dense clot under the action of milk-clotting enzymes is the most important factor in the production of cheese and curd. Literature describes quite contradictory studies into the influence of the *CSN3* genotype of cows on the cheese suitability of raw milk. Some researchers found the presence of this effect, while others reported its absence. This can be due to concomitant factors that have a significantly greater effect on the cheese suitability of milk than the *CSN3* polymorphism.

For example, Fox *et al.* reported that the level of glycosylation directly affected the susceptibility of κ-casein to hydrolysis by chymosin: it decreased with an increase in carbohydrates [26]. Other authors found that the quality of the clot and the time of its formation were affected by milk acidity, protein and fat contents, as well as the quality of enzyme preparations (mainly chymosin) obtained from different manufacturers [27, 28].

We took into account the results of previous works and tested samples BB^2 and AA^3, which was rehydrated to a similar amount of protein as BB^2 had (2.67% and 2.65%, respectively). This method of rehydrating dried milk from a cow with the $CSN3^{AA}$ genotype minimized the differences in the main components between samples AA^3 and BB^2. In addition, in order to exclude the influence of enzyme preparation quality, we used chymosin produced by two large European companies. The test results are presented in Table 10.

When using 2400 IMCU/g chymosin (MICROCLERICI, Sacco Sistem), a dense clot formed in samples AA^3 and BB^2 after 100 min and 360 min, respectively. The tests using 2500 IMCU/g chymosin (CHY-MAX, Chr. Hansen) showed a higher rate of clotting in both AA^3 and BB^2 (60 min and 150 min, respectively). We found that the rate of clotting was higher in the milk from the *CSN3 A* allele cows, compared to the *B* allele cows. In addition, we observed a similar correlation when assessing the clot quality.

When using CHY-MAX, the quality of the AA^3 clot belonged to group I, while that of BB^2, to group II. When coagulating milk with MICROCLERICI, the quality of the AA^3 clot belonged to group II, while that of BB^2, to group III. Assessing the degree of protein loss associated with its conversion into whey, we found that sample AA^3 had a protein mass fraction of 0.73%, which corresponded to 28.6% of the total protein content in milk. In this sample, the degree of protein conversion into whey did not depend on the enzyme used. However, sample BB^2 showed a protein loss of 48% (MICROCLERICI) and 35.7% (CHY-MAX), which corresponded to a conversion to whey of 1.3% and 0.96% of total protein, respectively.

CONCLUSION

Our study revealed the effect of the *CSN3* gene polymorphism on the technological properties of dried milk. We found that the reconstituted milk sample with the *BB CSN3* genotype had higher thermal stability, while the sample with the *AA* genotype had better cheese suitability. In this regard, we cannot rule out an additional effect of milk drying and reconstitution. In addition, in order to minimize the influence of other factors, we used various methods to determine thermal stability and cheese suitability. Although we found no strict correlation between them, their combination allowed us to conduct a thorough analysis of milk stability during heat treatment and rennet coagulation. This approach to a comprehensive monitoring of technological properties opens up opportunities for accumulating a significant array of experimental data. Systemic research in this direction will produce a sufficient amount of material to create bioinformatics algorithms for mathematical modeling. In the future, this might give rise to a new system of criteria to evaluate the compliance of the technological properties of milk with the recommended indicators.

CONTRIBUTION

The authors were equally involved in working on the manuscript.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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Halal food supply chains: A literature review of sustainable measures and future research directions

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Abstract:
Introduction. Although sustainability represents a high-profile topic in supply chain management, it remains an unexplored research area for Halal food supply chains (HFSCs). Hence, to bridge this knowledge gap, we conducted a systematic literature review to identify the measures necessary for the development of sustainable HFSCs and potential research gaps at the nexus of sustainability and Halal food literature.
Study objects and methods. We carefully analyzed forty (40) papers selected from leading, highly-ranked journals to answer the following research question: "What are the measures necessary for the development of sustainable Halal food supply chains?"
Results and discussion. The findings revealed that the improvement of Halal processes through the implementation of quality management systems, the effectiveness of Halal labeling, and the use of technology could enhance the economic performance of HFSCs. Furthermore, HFSC's sustainability efforts are strengthened by enhancing trust and transparency benefitting human resource skills development, promoting animal welfare issues, and increasing regulatory compliance. The implementation of environmental protection measures is a primary driving factor for environmental sustainability activities. Environmental sustainability could be fostered by a shift to the application of greening practices and the support of environmentalism in the Halal food industry.
Conclusion. The findings of this study provide critical managerial implications for Halal food practitioners as they can have a summary of the previous studies and thus use it as a benchmark for introducing sustainable measures in their Halal food firms.

Keywords: Halal, food industry, food supply chain, supply chain management, Islamic, sustainability

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INTRODUCTION

Halal is an Arabic word that denotes anything which is lawful, legitimate, sharia-compliant, or permitted. In the context of food, Halal reflects a set of Islamic dietary laws that are important to satisfy the demand of Muslim consumers for food quality and compliance to Islamic principles [1]. Similarly, to be fit for consumption, the entire Halal food supply chain (HFSC) should ensure that the final product is genuinely Halal at the point of the consumer purchase [2]. This implies that all materials used in the production of food should be Halal, and all machinery, utensils, tools and packaging should not be contaminated by non-Halal elements (e.g., porcine, alcohol, dirty materials).

The integrity of the Halal food chain is crucial for the successful operations of Halal food businesses as it entails both compliance and assurance of the proper preparation, production, processing, distribution, and delivery of safe products to the final consumer. Apart from being a religious obligation, Halal food attracts non-Muslim consumers who increasingly perceive Halal-certified food as a symbol of hygiene, quality, safety, and additional health benefits [3].

Today, the Halal food industry is a large revenue market and according to PEW Research, the global

Muslim population will increase by 73% to an estimated 2.8 billion by 2050 [4]. For example, the Halal food market has amounted to USD 1.292 trillion, almost triple the size of Japan's food and beverage market [5]. The increase in the demand for Halal food is due to numerous factors including population growth, economic prosperity, globalization of the Halal food chain, as well as social and religious obligations to consume only Halal [6]. Moreover, a growing number of non-Muslims consume Halal products, due to the perception that Halal foods are safe [7].

As Muslim and non-Muslim consumers are becoming aware and knowledgeable about their food consumption, sustainability remains a growing concern in the food industry [8]. According to Abdullah *et al.*, the fast-evolving Halal food industry will face growing pressure responding to consumers' concerns [9]. Therefore, a systemic approach to address sustainability issues in the HFSC is imperative to optimize the value chain and support food preparation, processing, storage, and logistics. As such, challenges related to sustainable HFSC practices, nutrition, food safety, food authenticity and corporate social responsibility should be a high priority on the agenda of Halal food suppliers and retailers. Therefore, the purpose of this study was to investigate the necessary measures that can be taken to develop sustainable HFSCs.

This research responds to Abdullah *et al.* and Rahman *et al.*, who note that there is still a lack of research that documents the sustainable practices in the HFSC [9, 10]. Their calls have provided a justification for conducting this study. As such, we conceptualize Halal sustainability as a significant shift from today's Halal food ecosystem, where the primary goal is compliance with the Islamic dietary law, to the development of holistically sustainable HFSCs. We recognize that the transition toward sustainable HFSCs gives rise to necessary changes in the governance of how Halal foods are prepared, produced, handled, and delivered to consumers. Hence, we conducted a systematic literature review and a bibliometric analysis to identify the sustainable practices that are implementable within HFSCs.

The literature on Halal food and sustainability remains fragmented, poorly conceptualized, and a patchwork of non-coherent insights. To bridge this knowledge gap, we attempted to provide clear answers to the following research question: "*What are the measures necessary for the development of sustainable Halal food supply chains (HFSCs)?*"

The originality of this paper stems from the identification of practices that can be embraced by HFSCs to achieve sustainability. To the authors' best knowledge, this review represents the initial attempt to cover this increasingly emerging topic. Thus, providing a deeper understanding of sustainability within the context of the Halal food industry. Halal food scholars and practitioners can gain relevant insights from this relatively unexplored area. Our academic contributions are significant and threefold. Firstly, the ultimate goal of this work was to condense current knowledge from a wide variety of sustainable practices in the HFSC transparently and thoroughly. Secondly, despite some partial attempts to uncover sustainability themes in HFSCs, they are limited to one dimension (e.g., environmental sustainability) while the other dimensions (i.e., economic and social sustainability) are ignored or barely discussed. Unlike previous studies, this study provides a comprehensive analysis of all practices transcending the three dimensions of sustainability, namely, the economic, social, and environmental dimensions. Lastly, the review investigation is useful to evaluate knowledge generation, circulation, and discourses among the Halal food scientific community. We posit that a balanced scholarly focus on sustainability from different perspectives can be insightful and valuable for identifying actions and practices that drive sustainability in HFSCs. Accordingly, this contribution is what this study primarily intends to achieve and deliver.

STUDY OBJECTS AND METHODS

The study aimed to review the existing academic literature regarding HFSCs and sustainability and to explore overlaps. A systematic literature search summarized existing knowledge on the sustainable measures applicable to the Halal food industry. The literature review procedure utilized guidelines from Malik *et al.* [11]. As such, the authors first identified the relevant search keywords for the query process. The search keywords were determined based on the central objective of the study and consisted of two string groups. The first combination of keywords included: "*sustainab * OR environ * OR eco * OR green * OR social OR societal OR ethic * OR CSR OR eco- OR efficiency OR "triple bottom line" OR TBL.*" The second combination contained the following terms: "*Halal food" OR "Halal drink" * OR "Halal meat.*"

The authors accessed Scopus and Web of Science (in December 2019) to query article titles, abstracts, and keywords. Scopus provided comprehensive coverage of peer-reviewed academic literature and is considered the largest abstract and citation database of peer-reviewed literature covering a wide range of disciplines [12]. While Scopus provides the opportunity to access high-quality, peer-reviewed data for systematic reviews, it is not a complete source [13]. For this reason, we used Web of Science to identify any additional studies not currently indexed in Scopus. The initial search was limited to peer-reviewed journal articles published in English. Consistent with Ramos-Rodríguez and Ruíz-Navarro, this procedure helps to ensure that the literature originates from academic sources, increasing the credibility in their findings [14].

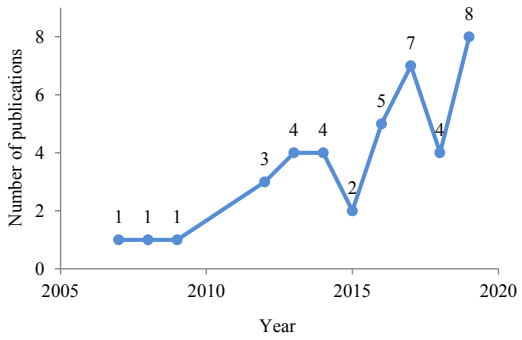

Figure 1 Time distribution of the 40 papers

Scopus returned one hundred and twenty-four (124) articles, and Web of Science returned eighty-four (84) articles. The majority of the Web of Science articles (n = 84) were included in the Scopus search results and removed. We applied filter criteria to retain only articles with titles, abstracts, and keywords that were relevant to the scope of the present study. This filtering procedure reduced the number of publications to sixty (60), with fifty-six (56) from Scopus and four (4) from Web of Science. After reading and evaluation of full content, forty (40) journal articles were considered relevant for the research and retrieved for the final analysis. Each of the papers was carefully examined to ensure that insights considered the overlap of sustainability practices with HFSCs. To facilitate the analysis of the selected articles, we created a database to categorize and group the topics.

RESULTS AND DISCUSSION

Distribution of papers by year. We plotted the number of articles in different periods to reveal the evolutionary trend of research interest. Figure 1 shows the yearly distribution of journal articles retained for the review. Despite the historical roots of sustainability, literature related to sustainable practices in HFSCs did not emerge until 2007. The scholarly interest in sustainability within HFSCs commenced in 2007 and grew steadily until 2015. Generally, there is an upward pattern in the number of journal articles published from 2007 on. However, from 2015 onward, the focus on sustainability within the context of the Halal food industry increased, resulting in eight (8) articles in 2019. We observed that twenty-six (26) out of forty (40) journal articles published between 2015–2019 reflected the nascent and growing interest of the Halal food research community into the concepts of sustainability and HFSCs.

Journal source distribution. As illustrated in Fig. 2, the selected publications were spread across twenty-nine (29) journals. Notably, the British Food Journal published six (6) articles, Meat Science published three (3), Journal of Islamic Marketing published three (3), and the International Journal of Supply Chain Management and Agriculture and Human Values published two (2) articles each. The category "others" consisted of twenty-four (24) journals with one (1) publication each.

Distribution of papers according to the research approach. Figure 3 describes the classification of the forty (40) selected studies according to the research approach applied. We applied two main research methods for categorization; theoretical and empirical. Empirical articles focused on assessing consumers' attitudes and perceptions regarding the consumption of Halal food, evaluating the impact of adopting lean practices and quality management systems. The collection of primary data encapsulated a wide variety of methodological approaches such as surveys, case studies, content analyses, and interviews. The theoretical articles were conceptual, review-type research discussing Halal food from diverse perspectives such as marketing, production, supply chain management, and religion. The reviewed papers provided a synthesis on Halal slaughter, stunning techniques, and other issues mainly related to animal welfare. One paper used mixed methods, developing a framework and validating it with empirical data through a survey. To increase the transparency of the review process,

Table 1 summarizes all pertinent articles selected for this study and their classification according to the research methods applied.

Discussion of measures for sustainable HFSCs and future research directions. In this section, we discuss the possible measures to be taken to drive the development of sustainable HFSCs. The

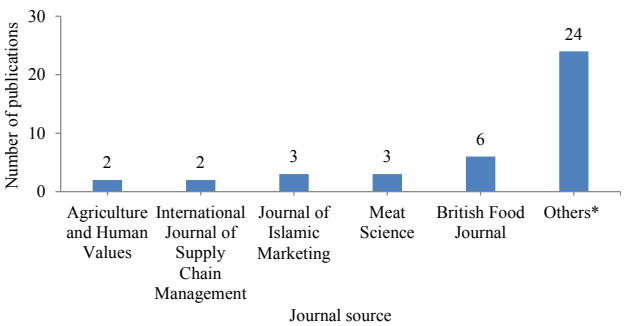

Figure 2 Journal source distribution

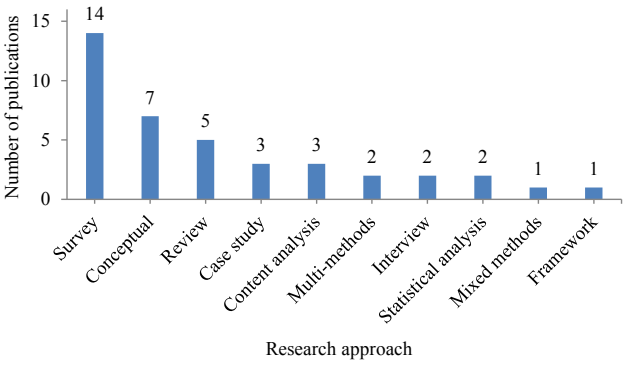

Figure 3 Distribution based on research methodologies

Table 1 Classification of publications according to research methodologies

Research approach	References
Survey	Ab Rashid and Bojei; Ashraf; Fuseini *et al.*; Idris; Jayakrishnan *et al.*; Kuzaiman *et al.*; Manzouri *et al.*; Manzouri *et al.*; Muhamed *et al.*; Rezai *et al.*; Verbeke *et al.*; Weng and Khin [15–27]
Conceptual	Bonne and Verbeke; Kabiraj *et al.*; Mukherjee; Nasaruddin *et al.*; Soon *et al.*; Tieman and Hassan; White and Samuel [8, 28–33]
Review	Chandia and Soon; Fuseini *et al.*; Nakyinsige *et al.*; Sabow *et al.*; Sohaib and Jamil [34–38]
Case study	Bergeaud-Blackler; de Souza *et al.*; Lever and Miele [39–41]
Content analysis	Kamarulzaman *et al.*; Mostafa; [42–44]
Multi-methods	Ali and Suleiman; Khan *et al.* [45, 46]
Interview	Ahmad *et al.*; Kurth and Glasbergenand [47, 48]
Statistical analysis	Ismail; Mohd Noor *et al.* [49, 50]
Mixed methods	Abdullah *et al.* [9]
Framework	Adham *et al.* [51]

sustainability measures drew on the triple-bottom-line (TBL) framework identified from the literature and supplemented with unaddressed research gaps and recommendations for future research. The TBL framework dates back to Elkington who argues that organizations have to emphasize the importance of economic, social, and environmental performance [52]. Similarly, Carter and Rogers suggest that firms who pursue the three foundational dimensions of the TBL framework would be able to achieve better economic performance. In the context of HFSCs, the company's vision of sustainability implies that these dimensions are equally important [53].

Sustainable economic measures in HFSCs. HFSC quality management systems. Research by Kuzaiman *et al.* highlighted that Halal food producers must consistently improve their products by applying a high-quality, holistic process management system [20]. This goal can be achieved by the careful monitoring of raw materials and upgrading labor skills [50]. In Ahmad *et al.*, the authors argue that the implementation of quality management systems (QMS) could help Halal food manufacturers to reinforce trust, lower operating costs, and increase the overall profitability of the HFSC [47]. The authors further note that the design of a QMS is invaluable for continuous improvements in the Halal food company.

QMS aids in the monitoring of activities and tasks which are necessary to maintain a desired level of excellence in quality-related policy and planning, quality assurance, quality control, and quality improvement. This implies that Halal food businesses should take a QMS approach to develop more effective HFSCs, reducing the risk of product recalls and reducing the overall risk to Halal food chain integrity. Integrity in this context implies maintaining food quality during transit, especially in cold chains, ensuring food safety is maintained, eliminating the risk of cross-contamination, and protecting the authenticity of the product against the risk of counterfeit or economically motivated adulteration by bad actors along the extended Halal food chain.

Hence, while fostering quality improvements within the organization is crucially important, a Halal QMS should extend beyond the boundaries of the firm to maintain HFSC integrity of the food while in transit [47]. The lack of QMS in HFSC activities may lead to several problems and consequently, to additional internal and external costs [54]. In Manzouri *et al.*, the authors argued that the implementation of QMS may be problematic for small-size food companies due to the lack of resources [22]. Therefore, to ensure the quality of their Halal food products, these businesses need to develop specialized and customized solutions that can increase their ability to deliver safe and authentic Halal food products that meet consumers' demands and comply with applicable regulatory requirements.

Halal labeling. Halal product labeling represents a critical communication medium by which consumers can obtain knowledge about Halal food products [55, 56]. Hence, an appropriate labeling scheme can increase consumer confidence and willingness to purchase Halal products [33]. Further, the usage of an industry-standard barcode (i.e., such as the industry-standard GS1 barcodes) combined with a lot or batch number (or a QR code) on the label facilitates consumer inquiry using a mobile phone scan or a website [57]. Within the supply chain itself, product traceability using GS1 standards on packages and pallets of goods enables the tracking of Halal food to a specific supply chain actor as well as across the entire supply chain processes [23].

Future research should investigate the requirements to build an efficient and effective traceability system that can preserve the integrity of Halal food and assure adequate verification and control processes [26]. To enhance the marketability of Halal food products, stakeholders in the HFSC may be interested in restructuring their strategies to broaden their market reach, design effective branding and attractive packaging for their offerings [23]. Importantly, communicating the nutritional value of Halal food is a regulatory requirement and may enhance trustworthiness, which is the key to establishing trustful relationships in HFSCs [16].

Whenever consumers are skeptical about the authenticity of Halal food products, market research on new and creative promotion techniques (i.e., signalling) should be conducted to improve marketing effectiveness and reduce consumers' anxiety [18]. Marketers should, therefore, design Halal labels that reduce information asymmetry and increase consumers' confidence in the quality and authenticity of Halal food. As food fraud includes the false representation of a Halal certification logo on product packaging, research should focus on consumer-facing tools to assist Halal food consumers to authenticate the integrity of the Halal certification logo on food products [58–60]. Additional research could investigate and clarify the role of new policies and institutions to help Halal food producers to embrace sustainable practices to market their food to wide market segments [25]. On this latter point, the application of the United Nations Sustainable Development Goals (SDGs) for HFSCs is crucially important [61].

Technology. The use of modern information technology systems and tools is crucial to enhance the efficiency, productivity and integrity of Halal food chains [62]. For example, the integration of the Internet of Things (IoT) devices in HFSCs could revamp several Halal logistics activities and enhance the integrity of cold chain processes such as the assurance of food quality through IoT-based temperature monitoring [63]. In discussing the implication of IoT in HFSCs, Ab Rashid and Bojei argued that RFID (Radio Frequency Identification) brought benefits to HFSCs, including the improvement of Halal meat traceability, and optimizing resource utilization [15]. Drawing on this study, researchers may conduct cost-benefit analyses of investing in new technologies to enhance the operations of HFSCs. The application of IoT in the HFSC has the potential to improve the operational efficiencies of HFSCs, strengthen the coordination of food processes, provide insightful information for real-time decision-making, and enhance marketing efficiency and effectiveness. Added to those benefits, Ahmad *et al.* argue that Halal food businesses could use the Internet and social media platforms to increase their technology resilience capacity (e.g., utilizing scalable cloud-based platforms), establish their online presence, increase consumer engagement, and achieve higher product visibility online [47].

A recent study by Kamarulzaman *et al.* found that social media could play a significant role in supporting businesses to communicate their efforts at addressing Halal food market imperfections to consumers [42]. The authors further reported that social media websites could be useful for overcoming several challenges, such as the lack of knowledge of Halal food sources, food quality, and authenticity. Moreover, the efficient management of HFSC activities can also be facilitated through the adoption of business intelligence tools and big data. In this regard, Ahmad *et al.* submitted that big data could enhance the capability of HFSCs to manage several problems associated with supply chain documentation, logistics packaging, as well as Halal and other critical business tasks [47]. In another study, Jayakrishnan *et al.* argued that the use of big data and business intelligence techniques could help HFCS optimize their organizational performance through increasing the effectiveness of decision-making processes and knowledge creation [19]. Therefore, scholars may be interested in examining the factors and barriers to adopting these technologies HFSCs.

Sustainable social measures in HFSCs. Trust and transparency. In order to retain competitiveness, Halal food companies should enhance transparency and trust in HFSC processes and activities. A lack of trust can have adverse effects on HFSCs performance and consumers will not buy Halal food products or engage with Halal food suppliers [16]. Halal food producers have to enhance the level of trust in their supply chains and products because trust is a key determinant in brand image (brand trust) and better operational performance [28]. Trust could be established by enhancing information transparency and by disclosure more timely, understandable, and reliable information relating to HFSC processes [15].

Halal food companies have to recognize the value of trust and transparency in securing the authenticity of Halal foods. The provision of food production information and a trustworthy Halal quality signal (such as a Halal certification logo) can be beneficial for consumers and a key driver to seek additional reassurance [8, 33]. From a consumer perspective, a participatory approach involving consumers in the cooperation and circulation of reliable information regarding Halal food status could ensure higher Halal food integrity [15].

Soon *et al.* argued that the key to maintaining Halal integrity in international trade is transparency, and consequently HFSC actors, policymakers, regulators, Halal certification authorities, and research institutions should devote concerted effort to create a more holistically integrated Halal food ecosystem [31]. Furthermore, there is a necessity to develop trustworthy Halal-certified products as they may foster an increased sense of consumer trust and confidence in food products [15]. Therefore, research on how to increase trust and what levels of transparency to consider in necessary to assure that Halal food products are properly prepared, handled, processed, and delivered to final consumers.

Human resource development. The development of human resources constitutes a strategic capability to sustain the competitive advantage of HFSCs. The empowerment of workers operating in the Halal food industry is valuable for increasing the efficiency of HFSC operations [50]. The employees should possess full knowledge of Halal food guidelines and practice the concept of hygiene allowed by Islamic dietary law [15].

This means that it is critical to abide by Halal requirements throughout the HFSC, from slaughtering, production, and distribution to the delivery of Halal food products to consumers.

The support of the Halal workforce can be determined by the utilization of new technologies, the continuous upgrade of labor skills, and the establishment of training programs sustained by the related parties such as the regulatory bodies, Halal food organizations, and Halal business incubators [49]. Idris argues that to reap the benefits of new technologies in HFSCs, organizations should focus on implementing the necessary infrastructure and develop a highly skilled and proficient workforce capable of responding to consumers' needs [18].

To adapt to lean manufacturing principles and the introduction of new technologies, the Halal workforce should be well informed about the goal of HFSCs and involved in lean supply chain implementation, decision-making, and value creation processes [21, 49]. This approach could reinforce Halal assurance practices and significantly increase organizational performance [64]. The lack of employee motivation can have adverse effects on HFSCs. Due to this issue, Manzouri *et al.* attributed the lack of adoption of QMS in Halal food companies to the weaknesses of human resources skills and knowledge [21]. As a result, scholars could investigate how Halal food companies can motivate their employees and improve their capabilities by adopting a food safety culture extending to the dynamics facing HFSCs such as globalization, adoption of new technologies, and the increasing need to develop professional skills.

Animal welfare. HFSC actors should include ethical principles, particularly in animal testing activities and quality inspections [30]. The livestock industry should strive to account for all ethical principles related to food animals from farms to the slaughterhouse. At the farm level, HFSC actors should allocate a unique identification number to every animal. Farmers should capture and record all necessary information relating to the health conditions and veterinary interventions with their animals, including the supply chain details of feed and nutrients used to raise the livestock.

New food industry directives should convert meat-based ingredients to plant-based ingredients and additives, replace porcine by bovine sources, and support plant-based animal foods [32]. In doing so, Halal food companies could address the growing concerns of segments of consumers who care about the provenance (i.e., origin) of the food that animals are fed and the history of the farm upon which livestock were raised or grazed [33]. Moreover, care should be taken to ensure proper slaughter processes. Specialized staff should be placed in charge of overseeing animal welfare during the slaughter process [36]. Sabow *et al.* argue that stunning using electricity should be carried out at high voltage because of its ability to keep the animal alive in compliance with the fundamental requirements of Halal slaughtering [37].

For example, Fuseini *et al.* indicate that the majority of scholars (> 95%) agreed that if pre-slaughter stunning does not result in the death of the animal, the meat would be Halal [17]. HFSC actors should maintain an appropriate range of voltage, ensure the integrity of the equipment, and the availability of skilled operators in order to comply with the Halal rules for animal slaughter. Therefore, a unified global Halal standard is imperative to incorporate animal welfare-friendly slaughter practices and measures in HFSCs [17]. This would help to provide more transparency on the slaughter process and enable HFSC stakeholders to make more informed decisions regarding stunning practices.

Regulatory support. Several regulatory bodies frame Halal certification standards across Muslim majority countries with the United Arab Emirates perceived as a leading authority [42, 65]. In order to ensure a cohesive and coherent global approach to Halal regulations, intervention efforts are needed to ensure enforcement and strict compliance of HFSC actors. The success of the Halal industry is highly dependent on governments to regulate HFSCs through Halal regulations, food safety standards, enforcement, and incentives that could accelerate the growth and development of HFSCs [15].

More robust government support should be in place through the encouragement of business incubators who provide training to entrepreneurs in the Halal food industry, advice and external consulting, and assistance in meeting the requirements of the Halal food market. For example, KIMAR is a business incubator in Malaysia that works to improve the awareness of potential entrepreneurs and their access to finance, training, and advice through many platforms [51]. The role of this incubator also involves the building of firms' capabilities to establish networks and partnerships of knowledge sharing and collaboration in the Halal sector.

The importance of regulatory support is highlighted in the study of Kurth and Glasbergen, who emphasized the need for improvement in areas related to stakeholder engagement, transparency, accessibility, impartiality, and efficiency [48]. The value of a supportive regulatory environment can prevent malicious actors from compromising the Halal integrity of the food chain [29]. Herein, recall that the Halal principle entails that all stakeholders in HFSCs must act based on trade fairness, ethical business practices, honesty, and good conduct. By the same token, the commitment of all HFSC stakeholders and the heavy involvement from the concerned authorities should be invested to improve the quality of Halal food products as the lack of regulations, standards, and enforcement could result in the failure of HFSCs, the spread of fraudulent practices, and unethical conducts [30, 42].

Thus, monitoring and enforcement of Halal standards and regulations have to be consistently evaluated so that consumers' confidence and trust in Halal food can be enhanced [24]. As supported by Soon *et al*., countries that commit to implementing standardized Halal practices can increase efficiency and boost Halal food trade [31]. Hence, future research should be directed toward discussing the opportunities and challenges of Halal food standardization in the HFSC.

Sustainable environmental measures in HFSCs. Implementation of greening practices. The food industry is recognized as one of the contributors to detrimental environmental impacts, incurring a large amount of food waste and creating a high carbon footprint [9]. Therefore, the sustainable functioning of HFSCs hinges on their contribution to environmental protection and the proper consideration of the potential environmental risks threatening the food ecosystem. One way to reinforce environmental sustainability in HFSCs is to implement greening practices.

According to Weng and Khin, the application of greening practices in HFSCs could prevent health hazards to humans and animals [27]. The authors argue that greening practices should protect endangered flora and species. Further, a significant reduction of waste is required in food processing and packaging. Of crucial importance is to protect animal welfare and ensure food quality through the efficient use of resources. Moreover, engagement in greening activities is beneficial for HFSC actors as the World Business Council for Sustainable Development argued that companies incorporating greening practice were able to achieve higher financial performance, lower production costs, improve product quality, increase market share, increase environmental performance, and build long-term relationships with exchange partners [9].

If Halal guidelines are aligned with supply greening practices, HFSCs can lower production costs, reduce negative environmental impacts, and improve the overall performance of the Halal food company. Aside from providing sufficient assurance of Halal food integrity, Halal transportation should be optimized in a way that reduces "food miles" by sourcing more products from local suppliers [63]. The logic behind this approach is attributed to the fact that products sourced from afar it can be argued that bulk transportation is cheaper in products sourced from afar result in more emissions and vulnerabilities.

Nevertheless, this scenario is invalidated if distant HFSC suppliers provide green products to customers or embrace greening practices, including the use of production facilities that are less energy-demanding and with low carbon emissions. For example, de Souza *et al.* illustrate that Saudi Arabia pursues an import strategy that aims to source green feed for the production of Halal food [40]. Therefore, this opens new opportunities for scholars to explore the trade-offs between sourcing locally or from geographically disperse suppliers.

Environmental protection. As segments of Halal consumers are becoming more oriented toward the purchase of eco-friendly products, HFSCs should adapt their operations. Rezai *et al.* argued that sustainable development in agriculture and the food industry is a determinant for a productive Halal ecosystem [25]. To maintain environmentally sustainable HFSCs, an improvement in agriculture must occur through soil conservation, an increase of arable land, efficient water use, and reduction of chemicals and fertilizer consumption [9].

Besides, the mounting public awareness of the necessity to protect the environment should motivate HFSCs to introduce new business practices that can respond to stakeholders' pressures and demands for reduced ecological damages and better environmental measures. In this regard, Rezai *et al.* found that the majority of consumers considered Halal food production a better reflection and driver for the support of sustainable farming activities [25]. To create more value for consumers, HFSC stakeholders should be eco-sensitive in their operations, decisions, and policies with regards to sourcing, manufacturing, logistics, and transportation.

Similarly, Halal regulatory bodies need to provide incentives for companies to adopt the best practices and to place the environmental responsibility at the heart of business strategy and mission. Research in this area should focus on the role of Halal organizations in institutionalizing the measures of environmental protection throughout the entire HFSC. Consistent with Abdullah *et al*., an integrative and inclusive approach for environmental sustainability should be applied in the Halal food industry to reduce food waste, encourage sustainable food processes, reduce the environmental impacts, and cater to the needs of all stakeholders in the Halal food industry [9].

CONCLUSION

The purpose of this study was to explore the sustainable measures that could be employed in the Halal food industry. Through the conduction of a systematic literature review, forty (40) studies were thoroughly analyzed using content analysis and a bibliometric method. The results presented in this study are useful in offering valuable insights and a holistic view of the transition toward the development of HFSCs.

The review focused on identifying the trends in sustainability research specific to HFSCs. The trends revealed that the Halal food sector is an emerging research area with an increasing number of publications over in recent years. Moreover, the use of empirical research methods was dominant. From the analyzed literature, it has been demonstrated that the economic and social dimensions of sustainability have attracted

more attention than the environmental dimension. HFSCs necessitate radical changes to be sustainable.

To this end, this study represents a unique opportunity to contribute to the Halal food research in several aspects. Firstly, it draws on the TBL framework to comprehensively synthesize the necessary measures for the development of sustainable HFSCs. Secondly, the focus on improving Halal food processes through the implementation of holistic quality management systems, the establishment of adequate operating infrastructure, and the development of an interoperable supply chain can enhance the competitiveness of Halal food firms. The use of effective labeling practices could support Halal food consumer purchase decisions. Halal food firms can capitalize on labeling to signal the sustainability of their products, communicate the nutritional value of Halal food, and inform consumers of the truthfulness of food attributes.

The adoption of new technologies, such as the Internet of Things, social media, big data analytics, and business intelligence tools, can enhance the operational performance of HFSCs. These technologies have the potential to be the gateway toward the fulfillment of economic sustainability by enhancing the integration of Halal food processes, optimizing production operations, facilitating traceability, and ameliorating the marketability of Halal products. In order to maximize the benefits of social sustainability, HFSC stakeholders have to reinforce trust and transparency, place a priority on the development of quality human resources, respond to animal welfare concerns, and acquire regulatory support. From the environmental perspective, the implementation of greening and environmental protection measures is imperative to encourage the delivery of environmentally sustainable Halal food.

The findings of this study provide critical managerial implications for Halal food practitioners as they can have a summary of the previous studies and use it as a benchmark for introducing sustainable measures in their Halal food firms. Similarly, Halal food researchers may be inspired to carry out further studies as they have a clear idea of the existing gaps in the extant literature as a starting point of their future research projects.

Lastly, we would like to acknowledge the limitations of our review. Although we queried two major academic databases, we cannot claim that we covered all published works falling in the scope of our study. The literature collection process was guided by a set of keywords, which provide us with a certain level of confidence that we have synthesized an extensive knowledge base on the overlaps between sustainability and HFSCs and the existing gaps in the literature.

CONTRIBUTION

All the authors contributed equally to the study and bear equal responsibility for information published in this article.

CONFLICT OF INTEREST

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

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Optimization of canthaxanthin extraction from fermented biomass of *Paracoccus carotinifacuens* VTP20181 bacteria strain isolated in Vietnam

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Abstract:

Introduction. The bacterium strain *Paracoccus carotinifaciens* VTP20181 isolated in Vietnam produces canthaxanthin, a carotenoid widely used in the food and pharmaceutical industries. The aim of this work was to determine optimal parameters for canthaxanthin extraction from fermented biomass of *P. carotinifaciens* VTP20181.

Study objects and methods. First, a series of single factor investigations were carried out in regard to maximal carotenoid content in the biomass extract obtained by using ultrasonic waves. Four parameters of the extraction process, such as extraction temperature, solvent/material ratio, extraction time, and ultrasonic output power, were studied. The obtained results were then optimized by using Response Surface Methodology (RSM) and Box-Behnken experimental design.

Results and discussion. The optimal technological parameters of the extraction process included extraction temperature of 35°C, solvent/material ratio of 9.5:1 (v/w), extraction time of 90 min, and ultrasonic output power of 145 W. Under optimal conditions, canthaxanthin and total carotenoid contents were determined as 14.95 ± 0.12 and 18.21 ± 0.11 mg/g respectively, which were compatible with theoretical calculations – 15.074 and 18.263 mg/g, respectively.

Conclusion. Current results confirmed that the strain of halophilic *P. carotinifaciens* VTP20181 is a potential source for canthaxanthin biosynthesis.

Keywords: *Paracoccus carotinifaciens* VTP20181, canthaxanthin, total carotenoid, optimization, response surface methodology

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INTRODUCTION

Natural pigments derived from β-carotene exhibit many outstanding features, such as strong antioxidative properties and enhanced color persistence, and have been studied for feasibility in industrial and food applications [1, 2]. Experimental data and clinical trials proved beta carotene and its derivatives, such as canthaxanthin, astaxanthin, zeaxanthin and lutein, to be able to confer beneficial effects on human health. They are able to remove free radicals, strengthen cell walls, as well as have anti-tumoral, immunity-enhancing and cell protecting capabilities that greatly contribute to prevention and treatment of cardiovascular diseases and obesity [3–6].

Currently, carotenoids are one of the natural pigment groups widely used in the food, pharmaceutical, cosmetic, and livestock industries [7–9]. The total value of products derived from carotene worldwide in 2015

reached $1.21 billion and is increasing gradually, 3–5% each year. By 2025, the commercial value is estimated to reach USD $2 billion [10, 11].

Canthaxanthin (4,4'-diketo-β-carotene) is a carotenoid with stable polyene backbone frame structure, which provides a better antioxidant effect [12–14]. This compound was discovered for the first time in an edible fungus called *Paracoccus carotinifaciens* and is now obtained from some plant species, crayfish, salmon, mushrooms, seaweed, and bacteria [15–18].

Although chemical synthesis of carotenoids has been long established, recent restrictions imposed on the use of synthetic chemicals in the food and pharmaceutical industries have called for more efficient production of pigments from natural sources. Engineered biosynthesis of canthaxanthin from microbial sources is an alternative to chemical sinthesis due to its advantages including health safety and independence from natural conditions [19]. Particularly, *D. natronolimnaea* HS-1 bacterial strain was identified as a suitable microorganism for industrial production of canthaxanthin, with canthaxanthin yield of 5.31 mg/L under optimal culture conditions in batch fermenter system [20, 21]. In Vietnam, some strains with high canthaxanthin production capacity, such as *Staphyloccocus* CNTP 4191, *Staphyloccocus* CNTP 4192, *Haloferax alexandrinus* NBRC 16590, and *P. carotinifaciens* VTP20181, were also isolated.

Extraction of carotenoids and beta carotene derivatives from bacterial biomass can be carried out by using various techniques. They are microwave assisted-extraction, ultrasound-assisted extraction, pulsed electric field assisted extraction, and supercritical fluid extraction [22–25]. However, efficiency of such processes vary greatly depending on technological parameters, surfactants, and used solvents [26–30]. Therefore, further optimization of technological parameters with respect to output is necessary in process intensification at larger scale [31, 32].

This study was aimed to optimize canthaxanthin extraction process from the biomass of the bacterium *P. carotinifaciens* VTP20181 isolated in Vietnam.

STUDY OBJECTS AND METHODS

The object of the study was dry biomass of *Paracoccus carotinifaciens* VTP20181 bacteria obtained after fermentation and spray drying. The biomass was packed in vacuum bags and refrigerated at (–4)°C to be prepared for the extraction process.

Extraction. Exactly 100 g of dry biomass was weighed by using a PA214 analytical scale (Ohaus-US, China) and transferred to a 2000 mL three-neck flask. Different volumes of solvents (7:1, 9:1, and 11:1, v/w) were added into the flask and shook to allow solvent penetration into the biomass. The solvent was 96% food-grade ethanol supplemented with 0.5% glyceryl monostearate for more effective extration. The temperature was maintained at pre-determined levels by using the reflux condenser apparatus and a water bath.

A UP200Ht ultrasonic homogenizer with sonotrode S26d14 ultrasound head (Germany, 26 kHz, 0–200 W) was usedfor extraction. The ultrasonic power and time were also adjusted for investigation at certain research levels. The extract was separated from the solid phase through a Buchner funnel and concentrated to obtain the total extract that contained canthaxanthin and totsl caratenoids.

Determination of total carotenoid content. To determine total caratenoids, we used the method of de Carvalho *et al.* with slight modifications [33]. First, 5 g of the biomass extraxt was extracted with 25 mL of acetone and filtered under vacuum. The permeate was re-extracted three times until the color of the obtained extract was transparent. Purified water was added to the obtained extract to prevent emulsion formation, then transferred to a 500 mL separatory funnel containing 40 mL of petroleum ether. Carotenoids will move to the petroleum ether phase (light phase), and the soluble acetone phase (dense phase) was removed. The separation was performed four times till no solvent was observed. Afterwards, water was removed from the petroleum ether phase by using anhydrous sodium sulfate in a 50 mL flask. To determine tottal caratenoid content (mg/g), the absorbance of the sample was read at 450 nm.

$$Carotenoids\ content = \frac{A \times V \times 10}{A_{1\ cm}^{1\%} \times P} \quad (1)$$

where A is the absorbance; V is the total extract volume, mL; P is the sample weight, g; $A_{1\ cm}^{1\%} \times P$ is the β-carotene extinction coefficient of petroleum ether ($A_{1\ cm}^{1\%} \times P = 2592$).

Determination of canthaxanthin content [34]. The reference standard, canthaxanthin (99% HPLC), was purchased from Sigma-Aldrich (Merck). Sigma-Aldrich also supplied all solvents (CHROMASOLV® grade methanol and chloroform, ethanol p.a., n-hexane p.a., and i-propanol p.a). Deionized water ($\sigma \leq 0.4$ µS/cm) was used in the experiment.

Analytical HPLC was performed with a C-18 column Hypersil Gold (5µm; 150×4.6 mm) on a Thermo system and detector PDA. The methanol-water mobile phase (97:3, v/v) was filtered through a 0.45 µm membrane and degassed. The analysis was carried out at a flow rate of 1.0 mL/min at room temperature. Chromatograms were

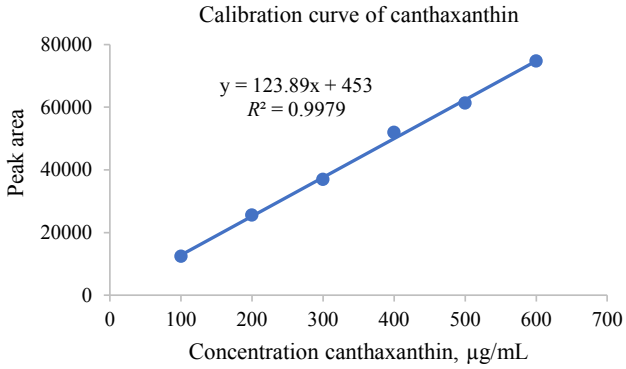


Figure 1 Calibration curve of canthaxanthin

Table 1 Independent variables and their corresponding levels

Independent Variables	Codes	Variable range (Δ)	Levels		
			–1	0	1
Z_1: Extraction temperature, °C	A	5	30	35	40
Z_2: Solvent/material, v/w	B	2	7	9	11
Z_3: Extraction time, min	C	30	60	90	120
Z_4: Ultrasonic power, W	D	20	120	140	160

Table 2 Experimental design and response values

Run	A	B	C	D	Y_1, mg/g	Y_2 mg/g
1	–1	–1	0	0	11.08 ± 0.17	12.86 ± 0.13
2	+1	–1	0	0	11.39 ± 0.15	14.33 ± 0.17
3	–1	+1	0	0	12.73 ± 0.18	15.79 ± 0.19
4	+1	+1	0	0	12.60 ± 0.21	14.86 ± 0.15
5	0	0	–1	–1	12.35 ± 0.11	15.02 ± 0.11
6	0	0	+1	–1	9.68 ± 0.08	11.61 ± 0.18
7	0	0	–1	+1	12.17 ± 0.15	14.87 ± 0.19
8	0	0	+1	+1	12.45 ± 0.13	15.27 ± 0.16
9	–1	0	0	–1	12.06 ± 0.12	14.40 ± 0.12
10	+1	0	0	–1	12.01 ± 0.11	14.43 ± 0.13
11	–1	0	0	+1	13.21 ± 0.14	15.60 ± 0.18
12	+1	0	0	+1	13.26 ± 0.17	16.20 ± 0.12
13	0	–1	–1	0	11.72 ± 0.16	14.69 ± 0.11
14	0	+1	–1	0	11.58 ± 0.12	15.30 ± 0.17
15	0	–1	+1	0	8.87 ± 0.09	9.67 ± 0.08
16	0	+1	+1	0	11.81 ± 0.11	14.01 ± 0.11
17	–1	0	–1	0	11.22 ± 0.18	13.58 ± 0.18
18	+1	0	–1	0	12.74 ± 0.19	15.41 ± 0.19
19	–1	0	+1	0	12.55 ± 0.14	15.18 ± 0.15
20	+1	0	+1	0	11.31 ± 0.15	13.69 ± 0.16
21	0	–1	0	–1	11.93 ± 0.12	14.44 ± 0.13
22	0	+1	0	–1	10.51 ± 0.13	12.72 ± 0.13
23	0	–1	0	+1	10.17 ± 0.17	12.31 ± 0.15
24	0	+1	0	+1	13.26 ± 0.16	16.05 ± 0.18
25	0	0	0	0	14.39 ± 0.10	17.42 ± 0.14
26	0	0	0	0	15.21 ± 0.19	18.41 ± 0.12
27	0	0	0	0	15.20 ± 0.16	18.40 ± 0.13

recorded at 475 nm and used to determine the content of canthaxanthin in the sample, which was expressed as mg/g extract.

To prepare calibration standards, 2.5 mg of canthaxanthin standard was dissolved in 25 mL of chloroform to form the stock solution, which was stored at 12°C. The stock solution was then diluted with methanol to obtain standard solutions with known concentrations ranging from 100 to 700 µg/mL. The standard solutions were then measured for peak area to build the calibration curve.

Experiment design and optimization. The canthaxanthin extraction process was optimized using the Response Surface Methodology (RSM), with canthaxanthin and total carotenoid contents as dependent variables. Experimental factors of the process included extraction temperature, solvent/material ratio, extraction time, and ultrasonic power. First, the factors were individually investigated to define a set of empirical values and the central range. Further, the obtained values were used in Box-Behnken model using Design-Expert 7.0 software to produce 27 sets of process parameters (Tables 1 and 2). Those sets were then actually attempted to collect data on canthaxanthin content and total carotenoid content, which were fitted in two second-order polynomial equations [35].

Table 3 Estimated coefficients of the response models with canthaxanthin content and total carotenoid content as dependent variables

Source	Y_1, mg/g		Y_2, mg/g	
	F-value	*P*-value	*F*-value	*P*-value
Model	22.07	< 0.0001*	17.84	< 0.0001*
A	0.096	0.7620^{ns}	0.034	0.8560^{ns}
B	25.74	0.0003*	22.96	0.0004*
C	12.51	0.0041*	12.47	0.0041*
D	17.12	0.0014*	15.37	0.0020*
AB	0.28	0.6050^{ns}	0.63	0.4450^{ns}
AC	10.86	0.0064*	8.6	0.0126*
AD	0.013	0.9113^{NS}	0.09	0.7694^{ns}
BC	13.59	0.0031*	8.09	0.0148*
BD	29.21	0.0002*	23.12	0.0004*
CD	12.49	0.0041*	10.15	0.0078*
A^2	29.17	0.0002*	22.22	0.0005*
B^2	127.2	< 0.0001*	98.30	< 0.0001*
C^2	115.01	< 0.0001*	92.81	< 0.0001*
D^2	56.73	< 0.0001*	46.41	< 0.0001*
R^2	0.9626		0.9542	
Adj-R^2	0.9190		0.9007	
Adeq-Precision	18.589		16.489	

*$P < 0.05$; ns = not significant

The analysis of variance (ANOVA) was carried out to determine the compatibility of the model. The second-order equation representing the general function form of one desired outcome with respect to independent variables looks as follows:

$$\hat{Y} = b_0 + \sum_{j=1}^{k} b_j X_j + \sum_{u,j=1}^{k} b_{uj} X_u X_j + \sum_{j=1}^{k} b_{jj} X_j^2 \quad (2)$$

where, $\hat{Y}$ is the respone variable, X is independent variables, and k is number of tested variables. b_0 is the intercept coefficient; b_j is the linear coefficient; b_{jj} is the square coefficient and b_{uj} is the interaction coefficient.

RESULTS AND DISCUSSION

Single factor investigation. Based on some preliminary investigations of canthaxanthin extraction from microbial biomass, we selected the initial parameters for this process: extraction temperature of 35°C, solvent/material ratio of 9:1 (v/w), extraction time of 90 min, and ultrasonic power of 160 W. In our investigations, we varied only one of the selected

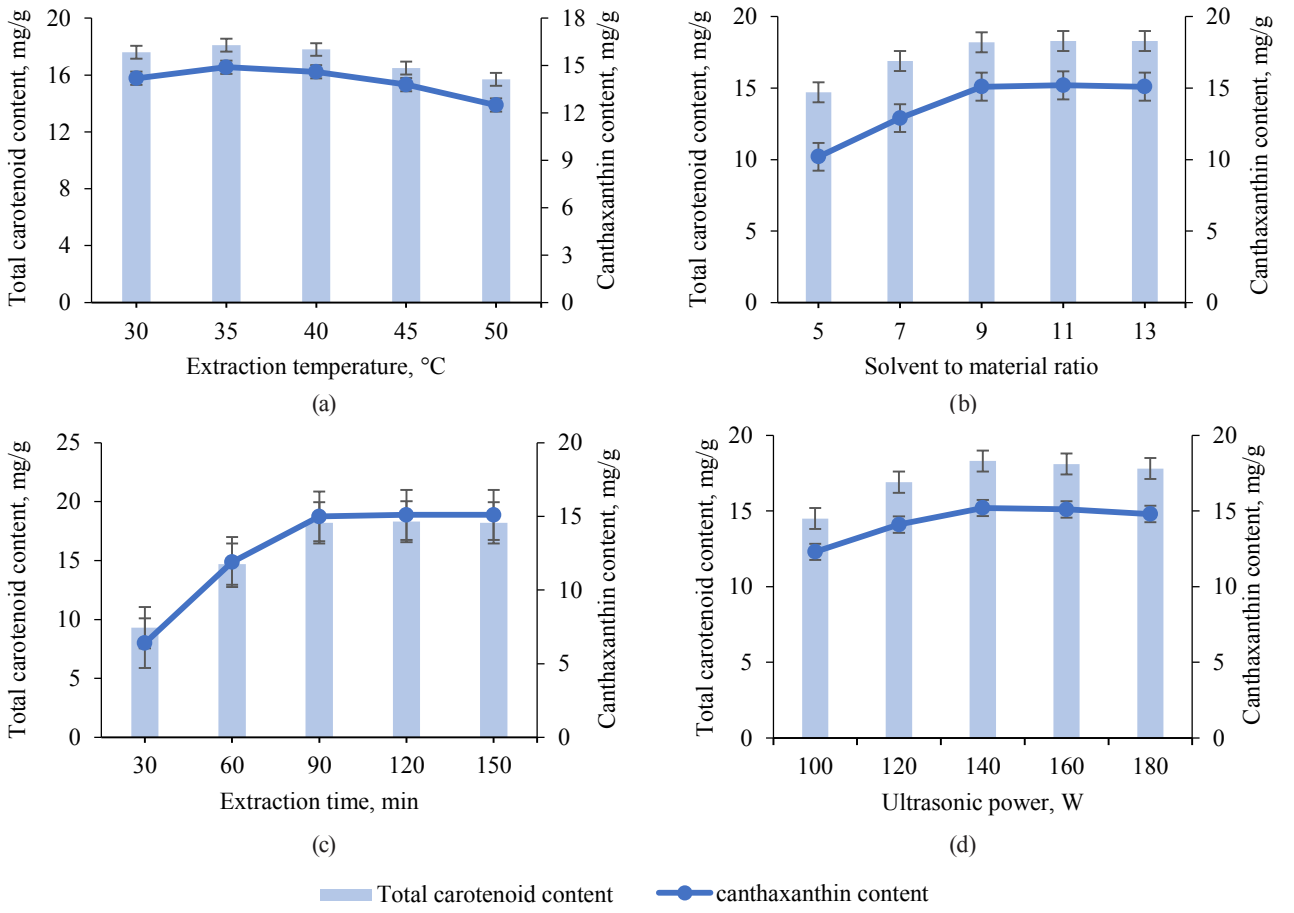


Figure 2 Effect of extraction temperature (a), solvent/material ratio (b), extraction time (c), and ultrasonic power (d) on the total carotenoid content and canthaxanthin content from dried extract

conditions, other three conditions were kept at their initial levels.

Firstly, we investigated the effects of temperature on the extraction process (Fig. 2a). Temperature ranged from 30 to 50°C with a 5°C interval. At 30°C, the obtained total carotenoid content and canthaxanthin content reached 17.6 and 14.2 mg/g, respectively. As the temperature was increased from 30 to 35°C, the extraction efficiency increased rapidly and reached its maximum at 35°C, with a total carotenoid content of 18.1 mg/g and canthaxanthin content of 14.9 mg/g. When the temperature continued being increased to 40 and to 50°C, the total carotenoid and canthaxanthin contents tended to decrease gradually and reached the lowest values of 15.7 and 12.5 mg/g, respectively, at 50°C. The results were completely consistent with Aflaki as well as Das and Bera who studied effects of temperature on carotenoid extraction [36, 37].

The increasing of the temperature from 30 to 35°C improved efficiency extraction due to the increased carotenoid diffusion capacity and decreased solvent viscosity, which allows for better penetration of the solvent into the material. However, elevating the temperature past 40°C can degrade or oxidize carotenoid compounds. Therefore, to ensure the highest efficiency of canthaxanthin and total carotenoid extraction, we selected 35°C as the optimum temperature for successive experiments. The low level (–1) and the high level (+1) were 30 and 40 °C, respectively (Table 1).

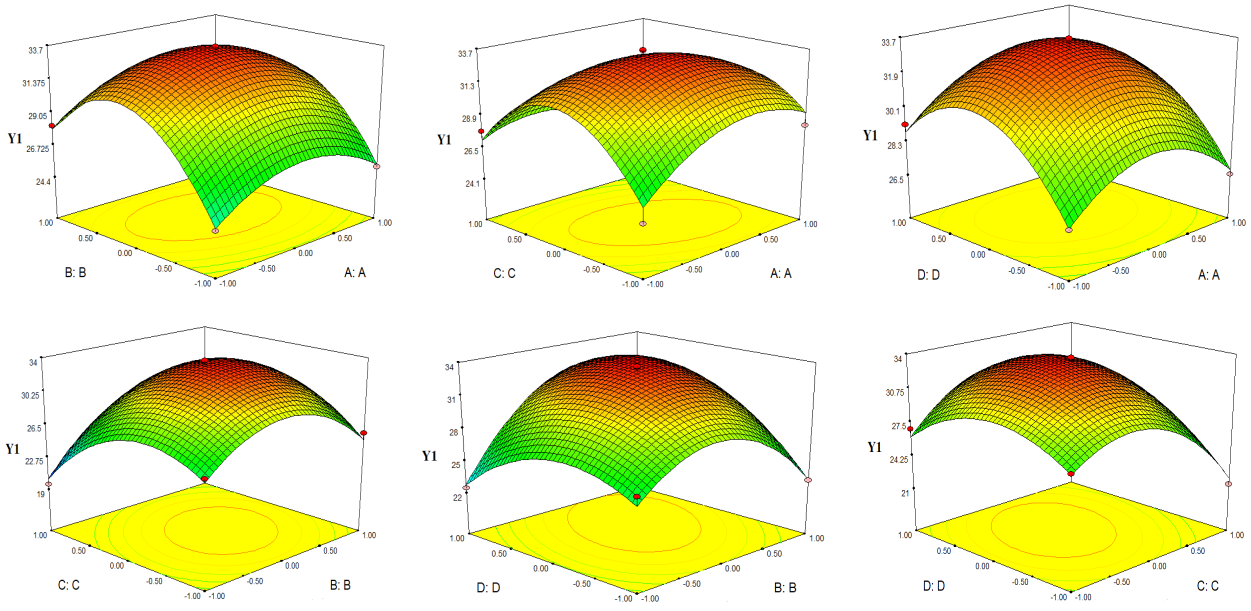
In the next experiment, a solvent/material ratio was varied from 5:1 to 13:1 (v/w). Figure 2b shows that the increase of the solvent/material ratio from 5:1 to 9:1 increased significantly canthaxanthin and total carotenoid contents from 10.2 to 15.1 mg/g and from 14.7 to 18.2 mg/g, respectively. When the ratio was increased to 11:1 and 13:1, canthaxanthin and total carotenoid contents were almost unchanged. Our results were entirely consistent with those of other researchers [37, 38].

Solute concentrations of the material and solvent significantly differed, according to the equilibrium principle, the outward movement of solutes from intracellular spaces into the solvent occurred at high speed. When equilibrium was attained, the diffusion process would gradually slow down despite the increase in solvent volume. Therefore, the solvent/material ratio of 9:1 was selected as the optimum ratio. The low level (–1) and the high level (+1) were 7:1 and 11:1, respectively (Table 1).

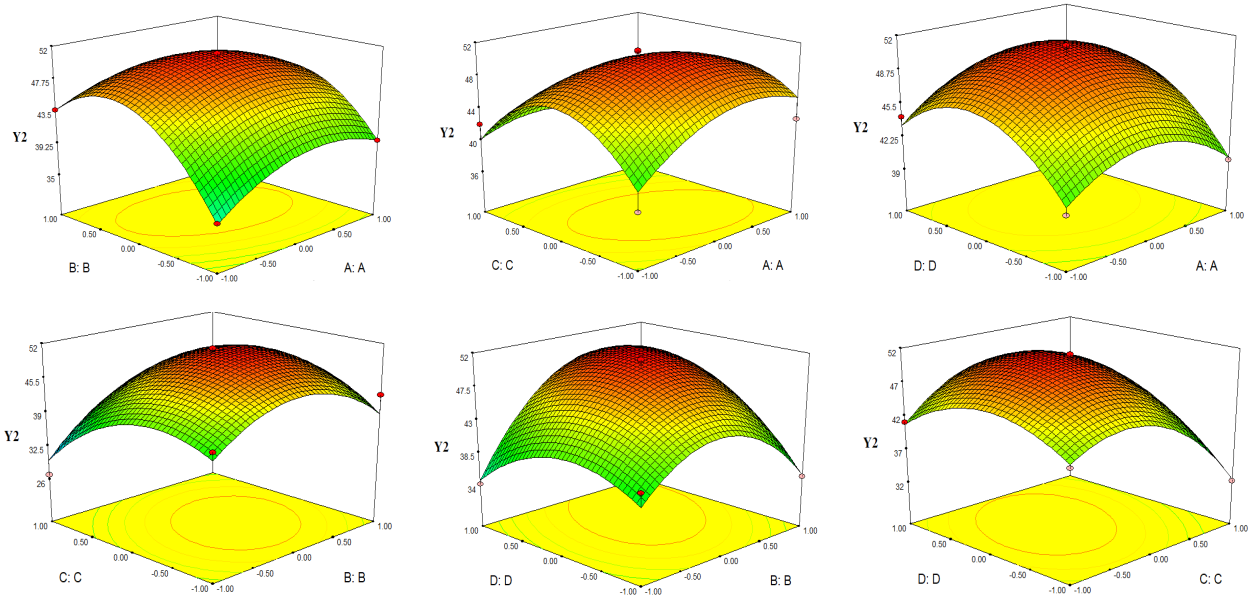
Figure 2c demontrates the effect of time (30 to 150 min) on the extraction process. With increasing extraction time from 30 to 90 min, canthaxanthin

Table 4 Empirical second-order polynomial model of canthaxanthin and total carotenoid contents

Response	Model Equations	R^2	P – value
Y_1 (total canthaxanthin content)	$Y_1 = 14.94 + 0.61B - 0{,}43C + 0.5D - 0.69AC + 0.77BC + + 1.13BD + 0.74CD - 0.98A^2 - 2.04B^2 - 1.94C^2 - 1.36D^2$ (1)	0.9626	< 0.0001
Y_2 (total carotenoid content)	$Y_2 = 18.07 + 0.79B - 0.58C + 0.64D - 0.83AC + 0.81BC + + 1.36BD + 0.9CD - 1.16A^2 - 2.44B^2 - 2.37C^2 - 1.67D^2$ (2)	0.9542	< 0.0001



(a)



(b)

Figure 3 Response surface of canthaxanthin content (a) and total carotenoid content (b)

content increased from 6.4 to 15.0 mg/g, and total carotenoid content increased from 9.3 to 18.2 mg/g. When the extraction time was increased from 90 to 150 min, canthaxanthin and total carotenoid contents did not change significantly. Similar results were found by Roohinejad *et al.* and Strati and Oreopoulou [39, 40].

Initially, an increase in extraction time allowed more solutes to diffuse into the solvent. However, at a certain threshold, equilibrium will be established, and even a significant increase in extraction time does not produce any noticeable change in extraction yield. Therefore, to ensure the extraction efficiency and the energy and time

Table 5 The values of the independent and real variables

Independent variables				Real variables			
A	B	C	D	Extraction temperature, °C	Solvent/material ratio	Extraction time, min	Ultrasonic power, W
0.01	0.23	–0.02	0.27	35	9.5	90	145

Table 6 Predicted response values and experimental response values obtained under optimum conditions

Dependent variables	Optimum value	
	Experimental	Predicted
Y_1, mg/g	14.95 ± 0.12	15.074
Y_2, mg/g	18.21 ± 0.11	18.263

optimization of the process, we selected an extraction time of 90 min as the baseline for the next experiment. The low level (–1) and the high level (+1) were 60 and 120 min, respectively (Table 1).

Lastly, we investigated the effect of ultrasonic output power, which was in a range of 100 to 180 W, on canthaxanthin and total carotenoid contents (Fig. 2d). The results show that increasing the ultrasonic power from 100 to 140 W increased sharply canthaxanthin and total carotenoids from 12.3 to 15.2 mg/g and 14.5 to 18.3 mg/g, respectively. Further increasing the ultrasonic power to 160 W caused a slight reduction in the canthaxanthin and total carotenoid contents to 15.1 and 18.1 mg/g, respectively. When output power reached 180 W, both canthaxanthin and total carotenoids decreased rapidly to 14.8 and 17.7 mg/g.

This is because ultrasonic waves act as agents breaking down the cell membrane of materials, which makes the diffusion process easier. However, when the ultrasonic power exceeds the threshold, the excessive number of air bubbles slows down the extraction process. The contact surface between the raw material and the solvent diminishes, resulting in reduced performance. Similar results were revealed by Yan *et al.* [41]. Therefore, ultrasonic power of 140 W was selected for the futher experiments. The low level (–1) and the high level (+1) were 120 and 160 W, respectively (Table 1).

Estimation of RSM model and statistical analysis. Two models corresponding to the two target functions, namely canthaxanthin content (Y_1) and total carotenoid content (Y_2), were estimated using data from the experiments guided by the Box-Behnken design. Four independent variables were extraction temperature, extraction time, solvent/material ratio, and ultrasonic output power. After the estimated function had been obtained and optimal conditions had been calculated, real experiments were performed to verify those optimal conditions and to determine model validity.

Based on separate single factor investigations, 27 experiments were conducted according to the experimental design matrix. To justify Y_1 and Y_2 response functions, *F*-, *P*-, and R^2-values were used to evaluate the linear regression analysis results (Table 3). Based on the regression results, canthaxanthin content (Y_1) and total carotenoid content (Y_2) could be described as independent variables of second-degree functions, as shown in Table 4.

The significance of the regression was also confirmed by *F*-values for Y_1 and Y_2, which achieved the value of 22.07 and 17.84, respectively and the very low *P*-value ($P < 0.0001$) (Table 3). The coefficients of determination (R^2) of the two equations were 0.9626 and 0.9542, suggesting that 96.26 and 95.42% of variability in canthaxanthin and total carotenoid content were explainable by experimental results, respectively. Besides, the value of Adj-R^2 (0.9190 and 0.9007, respectively), also showed the high accuracy of the experimental data in the limited range.

Regression equations (1) and (2) showed that all four technological factors (A, B, C, and D) affected the target function's value. Of which, extraction temperature (A) exerted the minimal direct impact on the value of the target function and influenced the responses mostly through its indirect interaction with extraction time (AC) and square interaction (A^2). The remaining three variables had a significant influence on the target functions through linear effects (B, C, D). Among them, single variables B and D had a positive effect on the target functions, while variable C had an inverse effect on the target functions corresponding to their coefficients in the regression equation (1) and (2). The influence of the technological variables on the target function could be ranked descendingly by the impact magnitude as follows: B > D > C > A.

Response surface analysis. Three-dimensional response surface spaces were plotted by using Design Expert software. The *X* and *Y* axes of the response surfaces in three dimensions represented the two varying factors, while other factors where held at their central values. The Z-axes represented one of the two target functions: canthaxanthin content, and total carotenoid content.

The red areas on the response surfaces represented pairwise combinations that gave desirable outcomes of Y_1 or Y_2 and depended on the magnitude and the sign of the component variable that formed the response. For example, C exhibited negative signs in both (1) and (2). Therefore, the area that corresponded to optimal outcomes of Y_1 and Y_2 in the surfaces constituted by C was smaller than those of remaining pairs without C (AD, AB and BD).

Optimization and model verification. The optimal values of independent variables were determined by

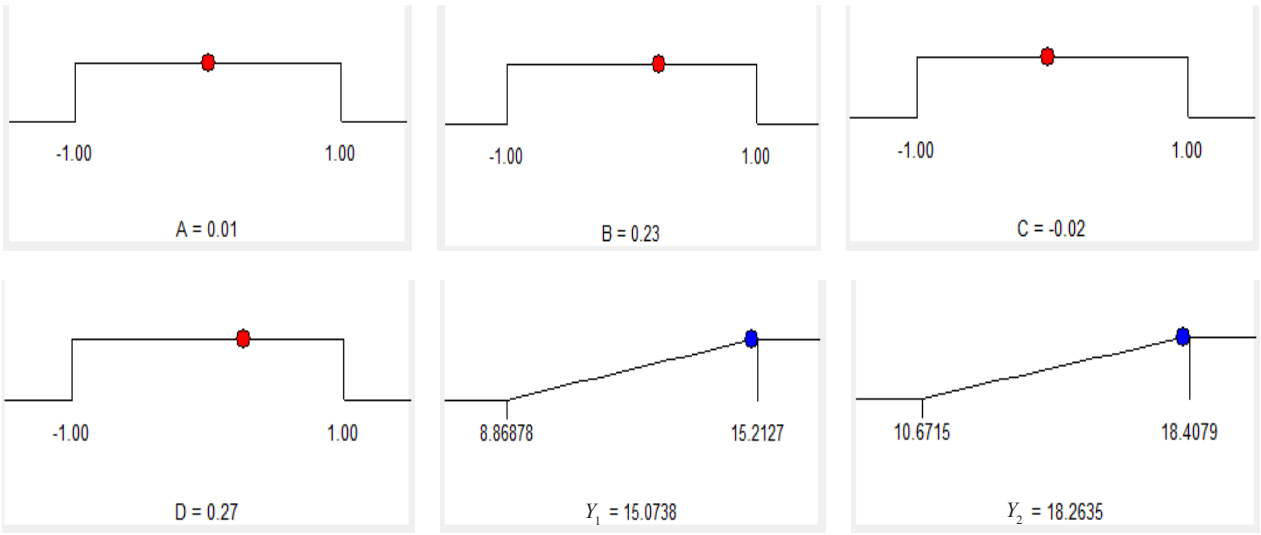


Figure 4 Optimum conditions by solution of ramps

solving the quadratic regression equation with respect to maximal canthaxanthin and total carotenoid content. The importance of canthaxanthin content (Y_1) and total carotenoid content (Y_2) was selected as at level 4 and level 3, respectively. Predicted values showed that canthaxanthin content and total carotenoid content attained their maxima with the following conditions: extraction temperature of 35°C, extraction time of 90 min, ultrasonic output power of 145 W, and solvent/material ratio of 9.5:1 (v/w). (Table 5, Fig. 4). Under optimized conditions, predicted and actual values of canthaxanthin content and total carotenoid content (Table 6) were approximately equal to each other, we could once again assert that our established model had a high compatibility.

CONCLUSION

In this study, the surface response methodology combined with the experimental design of Box-Behnken model was applied to optimize the extraction process of canthaxanthin from fermented biomass of *Paracoccus carotinifaciens* VTP20181 bacteria isolated in Vietnam. We used four independent variables: extraction time, extraction temperature, ultrasonic output power, and solvent/material ratio (v/w). Dependent variables were canthaxanthin content and total carotenoid content.

The obtained optimized conditions for the extraction process were: extraction time of 90 min, extraction temperature of 35°C, ultrasonic output power of 145 W, and solvent/material ratio of 9.5:1. Under these conditions, canthaxanthin and total carotenoid contents were 14.95 ± 0.12 and 18.21 ± 0.11 mg/g, respectively. Theoretical calculation models were completely compatible with the experiment.

CONTRIBUTION

Investigation – L.X. Duy, T.Q. Toan, D.V. Anh, N.P. Hung, T.T.T. Huong, N.M. Dat, D.T.T. Le, D.T.N. Pham, N.P.T. Nhan, and D.V. Manh. Supervision – P.Q. Long. Original draft writing – L.X. Duy and P.N.T. Dung. Review writing and editing – D.T.N. Pham.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Geroprotective potential of *in vitro* bioactive compounds isolated from yarrow (*Achilleae millefolii* L.) cell cultures

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Abstract:
Introduction. There is an urgent need for geroprotectors that prevent premature aging, especially antioxidants of plant origin. Due to the shortage of medicinal plant materials, scientists look for alternative sources of bioactive compounds of phenolic nature, for example, cell cultures and organs of higher plants. This paper describes a study of the geroprotective potential of *in vitro* bioactive compounds isolated from yarrow (*Achilleae millefolii* L.) cell cultures.
Study objects and methods. Callus, suspension and hairy root cultures of *A. millefolii* were obtained by *in vitro* cultivation on modified nutrient media. High performance liquid chromatography (HPLC) was used to analyze the composition of the cell cultures and ethanol extracts. The extracts' antimicrobial activity was studied by the disk diffusion method and their antioxidant activity was measured based on titration of a potassium permanganate solution.
Results and discussion. The biomass of all yarrow cell cultures contained essential oils, flavonoids, glycosides, phenolic acids, carotenoids, as well as vitamins C and E. The suspension culture had a higher content of essential oils, flavonoids and glycosides than the callus and hairy root cultures. The extracts of the *A. millefolii* suspension culture also contained geroprotectors – phenylpropanoids, flavonoids, and simple phenols, with a prevalence of caffeic acid, cynaroside, 4,5-dicofeylquinic acid, apigenin, and luteolin. In addition, HPLC revealed the presence of cumic aldehyde, umbelliferone, 3-caffeylquinic acid, and caffeic acid – the bioactive compounds previously not reported in yarrow. *In vitro* experiments with the extracts proved their antimicrobial and antioxidant activity.
Conclusion. The complex of bioactive compounds isolated from the biomass of yarrow suspension culture provides this plant with potential geroprotective properties. Thus, yarrow can be used to create nutraceuticals that prevent premature aging.

Keywords: Medicinal plant, yarrow, *Achilleae millefolii* L., bioactive compound, geroprotector, plant cell culture, extraction, antimicrobial properties, antioxidant properties

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INTRODUCTION

Aging is one of the global problems of humanity. In 2018, Russians aged 60+ accounted for 25.4% of the country's population. By 2050, their proportion is estimated to reach 31.2%. Aging is an inevitable process and one of the factors that can trigger the development of certain chronic diseases (cancer, type 2 diabetes mellitus, atherosclerosis, hypertension, myocardial infarction, etc.). Despite plentiful research on the biology of aging, its mechanisms are still far from being fully clear [1–3].

Premature aging is age-related changes that occur earlier than in healthy people of the same age group. According to A. Zhuravlev and Yu. Golubeva, "with premature aging, an individual's biological age is ahead of their calendar age" [4]. The causes of premature aging go beyond the negative impact of environmental

factors and lifestyle (bad habits, unhealthy diet, irregular daily routine), including genetic predisposition to such changes (Hutchinson-Guildford syndrome, Werner's syndrome) [5].

Gerontology is a science that studies aging and related processes. Some of its branches include geriatrics, gerocomia, and gerontopsychology that study prevention and treatment of old age diseases, care for older people, as well as their psychological and behavioral patterns. Gerontologists argue that some old age diseases can be prevented or delayed by certain chemicals, thus increasing life expectancy. As a result, pharmacologists are trying to create drugs capable of protecting the body from aging. These drugs include geroprotectors [6–8].

Geroprotectors are substances with a proven ability to increase the life span of animals (antioxidants, cross-linking inhibitors, enterosorbents, immunomodulators, antidiabetic agents, and adaptogens). Antioxidants play an important role in fighting against premature aging and thus preventing the negative effects of oxidative stress [9, 10].

Highly valuable are plant-based antioxidants (flavonoids, stilbenes, phenolic acids). They have a wide range of preventive and therapeutic effects and, unlike their synthetic analogues, do not have a toxic effect on the body or cause addiction [11–13].

In this regard, recent years have seen a sharply increased interest in the production of natural bioactive compounds with gerontological properties. The commercialization of such technologies could enable Russia to discontinue importing functional food ingredients, support domestic production and export bioactive compounds abroad. However, there is a shortage of medicinal raw materials due to a rapidly increasing demand for them and the unfavorable environmental situation. A new solution might be to use cell cultures (suspension, callus) and organs (hairy roots) of higher plants as an alternative source of renewable environmentally friendly materials.

Common yarrow (*Achilleae millefolii* L.) is rich in antioxidant substances and therefore has high geroprotective potential. It is a widespread plant traditionally used to heal wounds and treat inflammatory and respiratory infections since it contains bioactive compounds with anti-inflammatory, anti-ulcer, and anti-tumor effects [14]. These bioactive substances include amino acids, fatty acids, other acids (salicylic, succinic, ascorbic, folic, caffeic, chlorogenic) and flavonoids (luteolin, apigenin, quercetin) [15, 16].

In this work, we aimed to study the geroprotective potential of *in vitro* bioactive substances isolated from cell cultures (callus, suspension) and hairy roots of common yarrow (*A. millefolii* L.)

STUDY OBJECTS AND METHODS

The study objects were callus and suspension cell cultures and hairy roots obtained from the yarrow (*Achilleae millefolii* L.) seeds germinated in the Botanical Garden of Immanuel Kant Baltic Federal University (Kaliningrad).

To sterilize the seeds, we washed them with a detergent and kept first in 70% ethanol for 1 min and then in a 5% sodium hypochlorite solution for 50 min. After sterilization, the seeds were planted on agar media in 60 and 90 mm Petri dishes to grow sterile seedlings.

The nutrient medium for growing callus cultures (per 1 liter of distilled water) contained 50.00 mL MS (Murashige and Skoog) macro-salts (20×), 1.00 mL MS micro-salts, 5.00 mL Fe-EDTA, 0.10 mg thiamine; 0.50 mg pyridoxine; 0.50 mg nicotinic acid; 30.00 g sucrose; 1.00 mg kinetin; 2.00 mg β-indoleacetic acid; and 20.00 g agar [17]. The first seedlings appeared after 6–8 weeks of cultivation. To induce callus cultures, we used eight-week-old sterile seedlings with 2–4 true leaves. For this, the leaves and stems of the seedlings were cut into pieces and planted on an agar medium in 60 and 90 mm Petri dishes. Primary callus were formed on days 7–14 of cultivation. They were separated from the remains of plant explants and transferred to fresh nutrient media. The further cultivation cycle was 4–5 weeks.

To obtain cell suspensions, 300–400 mg of yarrow callus cultures was placed in 250 mL flasks with 25 mL of liquid nutrient medium and cultivated on a circular shaker at 95–100 rpm. After 18–20 days, we subcultured the cell supernatant, gradually increasing the dilution rate from 1/2 to 1/8 (inoculum to fresh nutrient medium) and shortening the subculturing cycle. Suspension cultures were grown in 250 mL flasks (30–40 mL of suspension per flask) on a shaker at 100 rpm. The nutrient medium (per 1 liter of distilled water) contained 50.00 mL MS macrosalts (20×), 1.00 mL MS microsalts, 5.00 mL Fe-EDTA, 0.10 mg thiamine, 0.50 mg pyridoxine, 0.50 mg nicotinic acid, 30.00 g sucrose, 1.00 mg kinetin, and 2.00 mg β-indoleacetic acid.

Hairy roots were obtained from the leaves of 14–28 day old yarrow seedlings. The leaves were transformed with *Agrobacterium rhizogenes* strain 15834 Swiss (Moscow, Russia) [18]. The strain was grown on the YEB agar medium (5 g/L peptone, 1 g/L yeast extract, 5 g/L sucrose, 0.5 g/L $MgCl_2$) for 24 h in the dark at 23°C on a shaker with circular rotation (5–10 cm amplitude, 90 rpm). The explants were pierced with a sterile needle and placed on Gamborg B5 medium containing a suspension (OD_{600} = 0.4) of *A. rhizogenes* [19].

After 12–48 h of incubation in the bacterial suspension, the explants were washed with sterile water, wiped with sterile filter paper until dry, and placed on solid B5 medium containing 500 mg/L of cefotaxime (Claforan, UK). The antibiotic was added to remove the agrobacterium residues. After

30 days of cultivation, we assessed the frequency of transformation (the number of transformed explants to their total number). The transformed explants were selected on the basis of their phenotypic traits (lateral branching, absence of geotropism, ability to grow in a hormone-free environment). The obtained roots were passaged two times on solid hormone-free B5 medium containing a reduced amount of cefotaxime (250 mg/L). They were cultivated in the dark at 23°C on a shaker at 100 rpm during 5 weeks. Subsequent passage to fresh medium was carried out as soon as the agrobacterium contamination appeared. After 14 days, individual explants featured rhizogenesis.

The growth of callus biomass was calculated as:

$$P_i = \frac{X_i - X_0}{X_0} \quad (1)$$

where X_i is the weight of culture on the i-th day of cultivation (standard: i = 7, 14, 21, 28 and 35 days); X_0 is the initial weight of culture (transplant weight).

Suspension cultures were analyzed for all growth parameters (dry mass, cell viability). To determine the content of wet and dry biomass in a liter of medium (M_{max_dw}, g/L), a fixed amount of suspension (not less than 15 mL, in triplicate) was filtered through a paper filter using a Buchner funnel under vacuum. The biomass was dried to constant weight in a stream of air at 30°C. The viability (v) of cell cultures was determined by counting live (unstained) and dead (stained) cultures under a microscope using phenosafranine (0.1% solution) or 0.025% Evans blue.

The growth index (I_{dw}) and the specific growth rate in the exponential phase (μ_{dw}, day^{-1}) were calculated as:

$$I_{dw} = X_{max}/X_0 \quad (2)$$

where X_{max} is the maximum content of dry biomass in a liter of medium; X_0 is the initial content of dry biomass in a liter of medium.

$$\mu_{dw} = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \quad (3)$$

where X_2 is the content of dry biomass in a liter of medium at point t_2; X_1 is the content of dry biomass in a liter of medium at point t_1.

The growth index was used to characterize the growth of root cultures *in vitro*.

The composition of yarrow callus, suspension and hairy root culture biomass, as well as the extracts, was analyzed by high performance liquid chromatography (HPLC) according to [20]. For this, we used the following eluent compositions: 1) tetrahydrofuran: acetic acid:5% H_3PO_4:water (19:20:2:59); 2) tetrahydrofuran:dioxane:MeOH:acetic acid:5% H_3PO_4:water (14.5:12.5:5:2:2:66). The substances were separated on a Shimadzu LC-20 Prominence chromatograph (Japan) with a Shimadzu SPD20M diode-matrix detector, using the columns Kromasil C18, 5 µm, 250×4.6 mm and HyperClone 5 µm, BDS 130Å, C18 250×4.6 mm.

Bioactive substances were extracted with ethyl alcohol (Kemerovo Pharmaceutical Factory, Russia). Dried plant biomass was ground in an LZM-1M mill (Olis, Russia) and sieved through a 1-mm hole sieve. 3 g of dried plant biomass was extracted in 260 mL of ethyl alcohol (30, 50, and 70 % ethanol) under static conditions in a PE-4310 water bath (EKROSKHIM, Russia) with a reflux condenser (30, 50, and 70°C). The extraction was performed twice during 2, 4, and 6 h.

To assess the geroprotective potential of the bioactive compounds isolated from the yarrow callus, suspension, and hairy root biomass, we analyzed their antimicrobial and antioxidant properties.

The antimicrobial activity of the extracts was analyzed according to Methodological Guidelines 4.2.1890-2004 by the disk diffusion method. The microorganisms used as test strains included *Escherichia coli*, *Candida albicans*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Enterococcus faecium*, *Klebsiella pneumonia*, *Helicobacter pylori*, *Streptococcus viridans*, *Streptococcus bovis*, *Porphyromonas gingivalis*, *Acinetobacter baumannii*, *Borrelia burgdorferi*, *Propionibacterium acnes*, *Aggregatibacter actinomycetemcomitans*, and *Streptococcus intermedius*.

A suspension of microorganisms under study was prepared from a broth culture according to the turbidity standard. Antimicrobial activity was determined using a standard meat-and-peptone agar (MPA). After inoculation, extract-impregnated discs were placed onto the medium, with an antibiotic disc used as a control. Then, the Petri dishes were put in a thermostat upside down and incubated at 35–37°C for 18–24 h (depending on the microorganism). To record the results, we placed the dishes upside down on a dark matte surface so that the light fell at an angle of 45°. The diameter of the growth inhibition zones was measured with an accuracy of 1 mm using a vernier caliper.

To determine the extracts' antioxidant activity, we filled a 50 mL titration glass with 8 mL of freshly boiled and cooled distilled water, 1 mL of a 20% sulfuric acid solution, 1 mL of 0.05 N potassium permanganate solution. After stirring, the solution was titrated with

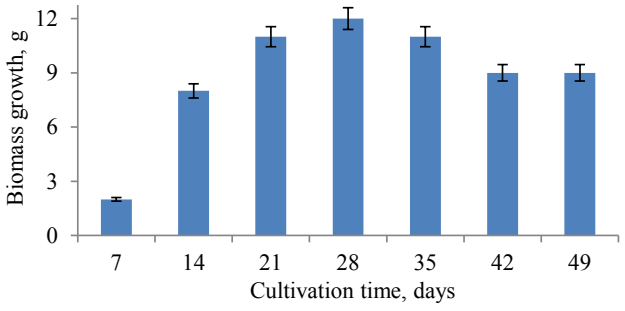


Figure 1 *In vitro* yarrow (*Achilleae millefolii* L.) hairy root culture growth index vs. cultivation time

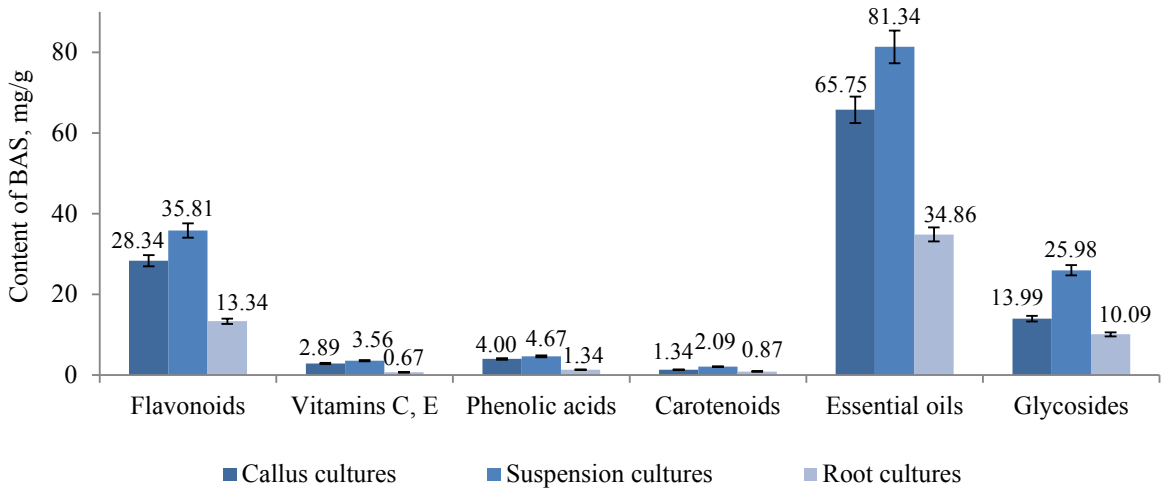


Figure 2 Bioactive substances in callus, suspension and hairy root cultures of yarrow (*Achilleae millefolii* L.)

an extract under study from a 1 mL microburette with a division value of 0.01 mL until the pink color disappeared.

Statistical data was analyzed using Microsoft Office Excel 2007 and the paired Student's test. The differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

The dry biomass growth index for the callus culture of yarrow (*Achilleae millefolii* L.) was 8.7–10.6 with a subcultivation cycle of 28–32 days.

The suspension culture showed high growth characteristics, namely:

– the content of dry and wet biomass in a liter of medium M_{max_dw} = 14.53–16.12 g/l;

– the viability of cell cultures v = 82–91%;

– the specific growth rate in the exponential phase μ_{dw} = 0.16–0.19 day^{-1}; and

– the exponential growth index I_{dw} = 9.56–10.23.

We found two types of cells in the yarrow suspension culture: mainly meristemetic and a number of parenchyma-like cells. The latter tended to increase by the end of the stationary phase of subcultivation. Although the aggregates were predominantly round in shape, there was also a number of elongated aggregates in the form of chains consisting of 5–20 small cells. Noteworthily, the primary suspensions of yarrow showed extremely unstable growth and significant

Table 1 Parameters for extracting bioactive compounds from yarrow (*Achilleae millefolii* L.) suspension culture biomass

No. extract	Extraction parameters		
	Tempe-rature, °C	Ethanol, %	Extraction time, h
1	30	70	2
2	30	70	4
3	50	50	2
4	50	30	4
5	50	50	4
6	50	70	4
7	50	70	6
8	70	30	2
9	70	50	6

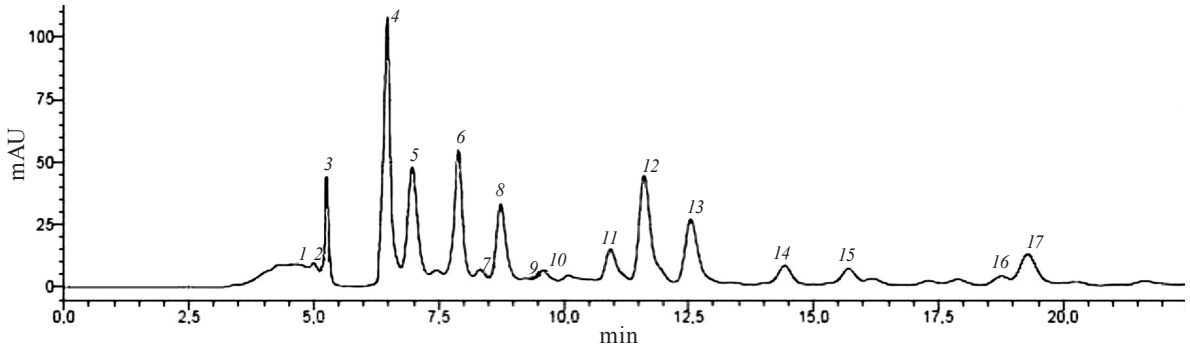


Figure 3 Chromatogram of aqueous-alcoholic extract (70% ethanol, 30°C, 2 h) of yarrow (*Achilleae millefolii* L.) suspension culture: *1* – benzyl alcohol; *2* – cumin aldehyde; *3* – 3-caffeylquinic acid; *4* – caffeic acid; *5* – 4,5-dicofeylquinic acid; *6* – apigenin; *7* – isoramnetin; *8* – casticin; *9* – 1-caffeoyl-3-feruloylquinic acid; *10* – 3,4-dicofeylquinic acid; *11* – vitsenin-2; *12* – cynaroside; *13* – luteolin; *14* – rutin; *15* – cosmosiin; *16* – esculetin; *17* – umbelliferone

Table 2 Qualitative and quantitative analyses of bioactive compounds in aqueous-alcoholic extract (70% ethanol, 30°C, 2 h) of yarrow (*Achilleae millefolii* L.) suspension culture by HPLC

No. peak	Retention time, min	Component	Content, mg/mL
1	4.39	Benzyl alcohol	0.21 ± 0.05
2	5.03	Cumin aldehyde	0.38 ± 0.05
3	5.25	3-caffeylquinic acid	3.05 ± 0.50
4	6.47	Caffeic acid	22.21 ± 0.88
5	6.97	4,5-dicofeylquinic acid	12.70 ± 0.32
6	7.89	Apigenin	11.15 ± 0.60
7	8.32	Isoramnetin	0.52 ± 0.05
8	8.74	Casticin	7.65 ± 0.50
9	9.22	1- caffeoyl-3-feruloylquinic acid	0.30 ± 0.02
10	9.58	3,4-dicofeylquinic acid	1.03 ± 0.20
11	10.93	Vitsenin-2	4.18 ± 0.27
12	11.61	Cymaroside	14.55 ± 0.32
13	12.54	Luteolin	9.27 ± 0.50
14	14.42	Rutin	2.96 ± 0.30
15	15.70	Cosmosin	1.80 ± 0.11
16	18.76	Esculetin	1.50 ± 0.20
17	19.29	Umbelliferone	5.30 ± 0.50

differences in the cell shape and size. A decrease in the inoculum amount led to an increase in the duration of biomass growth.

According to Fig. 1, the growth curve of the *in vitro* yarrow root culture is S-shaped. After a lag phase of 7–14 days, the culture entered the exponential phase on the 21st day and slowed down in growth on the 28th day, entering the stationary phase. The growth index was 26.

Figure 2 shows the qualitative and quantitative composition of bioactive substances in the callus, suspension and hairy root cultures of yarrow.

Table 4 Antioxidant properties of extracts obtained from yarrow (*Achilleae millefolii* L.) suspension culture biomass

Sample No.	Antioxidant activity, mg/g	Sample No.	Antioxidant activity, mg/g
1	0.2020 ± 0.0070	6	0.2220 ± 0.0065
2	0.2100 ± 0.0071	7	0.2270 ± 0.0078
3	0.2250 ± 0.0067	8	0.2080 ± 0.0074
4	0.2050 ± 0.0075	9	0.1900 ± 0.0075
5	0.2150 ± 0.0069		

As we can see in Fig. 2, the biomass of callus, suspension and hairy root cultures of *A. millefolii* L. had a high content of essential oils, flavonoids, and glycosides and lower concentrations of phenolic acids, carotenoids, and vitamins C and E. Compared to callus and hairy root cultures, the suspension culture was richer in essential oils (by 23.7 and 133.3%), flavonoids (by 26.4 and 168.4%), and glycosides (by 85.7 and 157.5%).

Based on the results, we decided to use a suspension culture of yarrow to extract a complex of bioactive compounds with potential geroprotective properties.

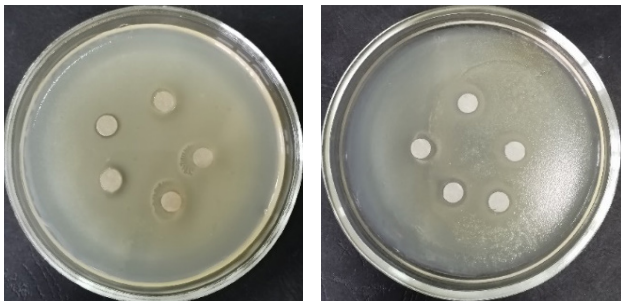
We examined nine extracts obtained from the suspension culture biomass with different extraction parameters (Table 1).

Extract No. 1 (70% ethanol, 30°C, 2 h) was qualitatively and quantitatively analyzed for bioactive compounds by HPLC (Fig. 3, Table 2).

As we can see, the aqueous-alcoholic extract of *A. millefolii* suspension culture contained phenylpropanoids, flavonoids, and simple phenols – potential geroprotectors. It also had a high content of caffeic acid, cynaroside, 4,5-dicofeylquinic acid, apigenin, and luteolin. In addition, we found cumin aldehyde, umbelliferone, 3-caffeylquinic acid, and caffeic acid – the compounds that have never been reported in yarrow before.

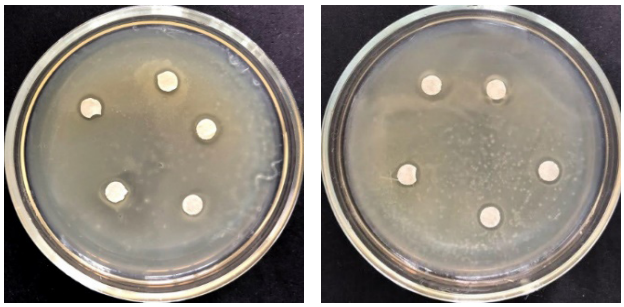
Table 3 Antimicrobial properties of extracts obtained from yarrow (*Achilleae millefolii* L.) suspension culture biomass

Sample No.	1	2	3	4	5	6	7	8	9
Test strain	Growth inhibition zones, mm								
Escherichia coli	9.4 ± 0.5	9.6 ± 0.5	9.2 ± 0.5	9.6 ± 0.5	9.8 ± 0.5	9.3 ± 0.5	9.4 ± 0.5	9.8 ± 0.5	9.5 ± 0.5
Candida albicans	9.2 ± 0.5	9.5 ± 0.5	9.1 ± 0.5	9.5 ± 0.5	9.6 ± 0.5	9.2 ± 0.5	9.4 ± 0.5	9.5 ± 0.5	9.6 ± 0.5
Bacillus cereus	8.8 ± 0.4	9.1 ± 0.5	8.6 ± 0.4	9.1 ± 0.5	9.3 ± 0.5	8.6 ± 0.4	8.9 ± 0.5	9.1 ± 0.5	9.0 ± 0.5
Pseudomonas aeruginosa	6.5 ± 0.3	6.7 ± 0.3	6.2 ± 0.3	6.8 ± 0.3	6.8 ± 0.3	6.3 ± 0.3	6.5 ± 0.3	6.9 ± 0.3	6.7 ± 0.3
Enterococcus faecium	7.9 ± 0.4	8.2 ± 0.4	7.8 ± 0.4	8.2 ± 0.4	8.2 ± 0.4	7.7 ± 0.4	7.9 ± 0.4	8.2 ± 0.4	8.1 ± 0.4
Klebsiella pneumonia	9.2 ± 0.5	9.4 ± 0.5	9.0 ± 0.5	9.5 ± 0.5	9.4 ± 0.5	9.0 ± 0.5	9.3 ± 0.5	9.4 ± 0.5	9.3 ± 0.5
Helicobacter pylori	8.8 ± 0.4	9.2 ± 0.5	8.9 ± 0.4	9.2 ± 0.5	9.5 ± 0.5	8.9 ± 0.5	8.9 ± 0.5	9.3 ± 0.5	9.2 ± 0.5
Streptococcus viridans	7.9 ± 0.4	8.1 ± 0.4	7.6 ± 0.4	8.2 ± 0.4	8.2 ± 0.4	7.7 ± 0.4	7.9 ± 0.4	8.2 ± 0.4	8.0 ± 0.4
Streptococcus bovis	7.6 ± 0.4	7.9 ± 0.4	7.5 ± 0.4	7.9 ± 0.4	8.0 ± 0.4	7.6 ± 0.4	7.8 ± 0.4	8.0 ± 0.4	7.9 ± 0.4
Porphyromonas gingivalis	9.5 ± 0.5	9.8 ± 0.5	9.4 ± 0.5	9.9 ± 0.5	10.0 ± 0.5	9.5 ± 0.5	9.7 ± 0.5	10.1 ± 0.5	9.8 ± 0.5
Acinetobacter baumannii	9.6 ± 0.5	9.9 ± 0.5	9.4 ± 0.5	9.9 ± 0.5	10.0 ± 0.5	9.4 ± 0.5	9.7 ± 0.5	9.9 ± 0.5	9.8 ± 0.5
Borrelia burgdorferi	7.9 ± 0.4	8.2 ± 0.4	7.8 ± 0.4	8.3 ± 0.4	8.4 ± 0.4	7.9 ± 0.4	7.9 ± 0.4	8.3 ± 0.4	8.1 ± 0.4
Propionibacterium acnes	9.8 ± 0.5	10.0 ± 0.5	9.5 ± 0.5	9.9 ± 0.5	10.2 ± 0.5	9.7 ± 0.5	9.8 ± 0.5	10.0 ± 0.5	9.9 ± 0.5
Aggregatibacter actinomycetemcomitans	8.3 ± 0.4	8.5 ± 0.4	8.0 ± 0.4	8.5 ± 0.4	8.6 ± 0.4	8.1 ± 0.4	8.3 ± 0.4	8.6 ± 0.4	8.4 ± 0.4
Streptococcus intermedius	7.6 ± 0.4	7.9 ± 0.4	7.5 ± 0.4	7.9 ± 0.4	8.1 ± 0.4	7.6 ± 0.4	7.8 ± 0.4	8.1 ± 0.4	7.8 ± 0.4



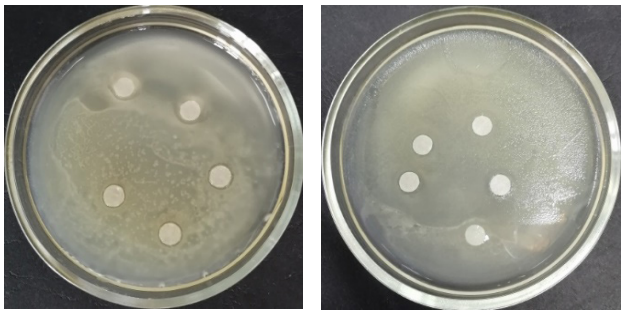
(a) (b)

Figure 4 Antimicrobial activity of yarrow suspension culture extracts against *Escherichia coli*: a) extract No. 5 (9.8 ± 0.5 mm); b) extract No. 8 (9.8 ± 0.5 mm)



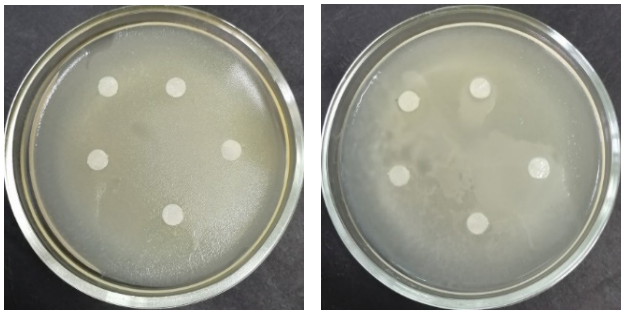
(a) (b)

Figure 5 Antimicrobial activity of yarrow suspension culture extracts against *Candida albicans*: a) extract No. 5 (9.6 ± 0.5 mm); b) extract No. 8 (9.5 ± 0.5 mm)



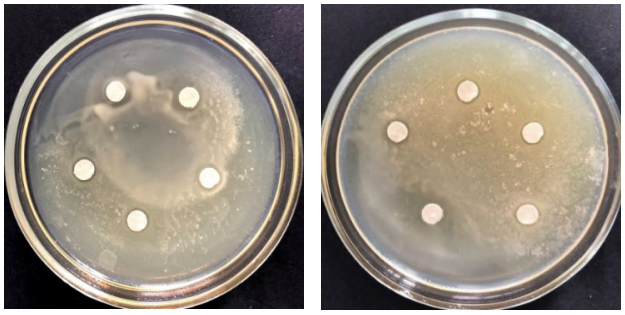
(a) (b)

Figure 6 Antimicrobial activity of yarrow suspension culture extracts against *Bacillus cereus*: a) extract No. 5 (9.3 ± 0.5 mm); b) extract No. 8 (9.1 ± 0.5 mm)



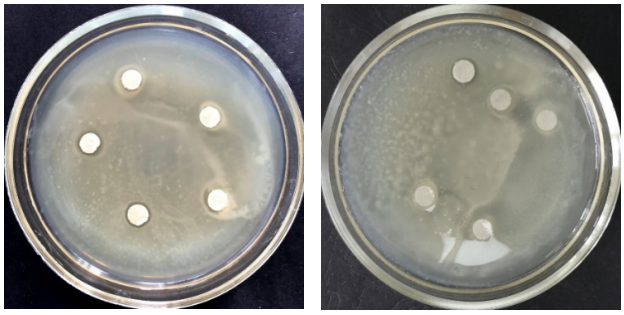
(a) (b)

Figure 7 Antimicrobial activity of yarrow suspension culture extracts against *Pseudomonas aeruginosa*: a) extract No. 5 (6.8 ± 0.3 mm); b) extract No. 8 (6.9 ± 0.3 mm)



(a) (b)

Figure 8 Antimicrobial activity of yarrow suspension culture extracts against *Enterococcus faecium*: a) extract No. 5 (8.2 ± 0.4 mm); b) extract No. 8 (8.2 ± 0.4 mm)



(a) (b)

Figure 9 Antimicrobial activity of yarrow suspension culture extracts against *Klebsiella pneumonia*: a) extract No. 5 (9.4 ± 0.5 mm); b) extract No. 8 (9.4 ± 0.5 mm)

In order to assess the geroprotective potential of the extracts, we studied their antimicrobial (Table 3) and antioxidant properties (Table 4) in *in vitro* experiments.

According to Table 3, all the samples performed antimicrobial activity against the test strains used. The maximum antimicrobial activity was observed in samples No. 5 (50% ethanol, 50°C, 4 h) and No. 8 (70% ethanol, 30°C, 2 h) (Fig. 4–18).

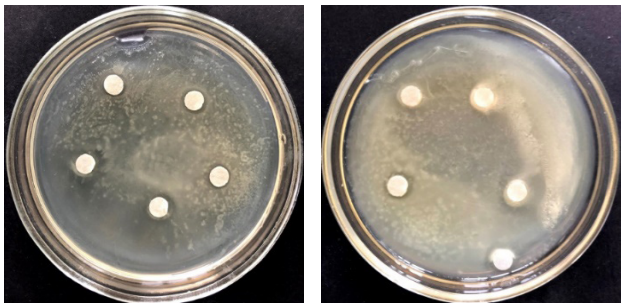
The antioxidant activity of the yarrow suspension culture extracts is shown in Table 4.

According to Table 4, the antioxidant activity of the aqueous-alcoholic extracts obtained from the yarrow suspension culture biomass varied from 0.1900 to 0.2270 mg/g.

Due to their antimicrobial and antioxidant activity and a high content of phenylpropanoids, flavonoids, and simple phenols, the extracts can be considered as having geroprotective properties and can be used to create nutraceuticals that prevent premature aging.

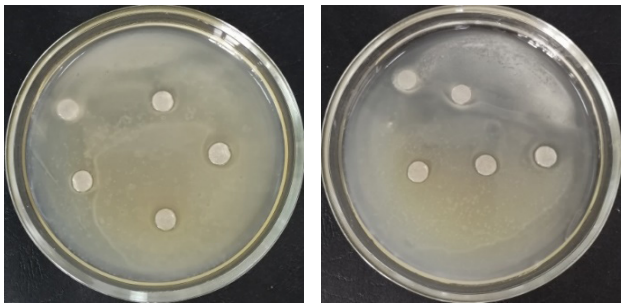
CONCLUSION

We cultivated *in vitro* cell cultures of yarrow (*Achilleae millefolii* L.), namely callus cultures, with a dry biomass growth index of 8.7–10.6; suspension



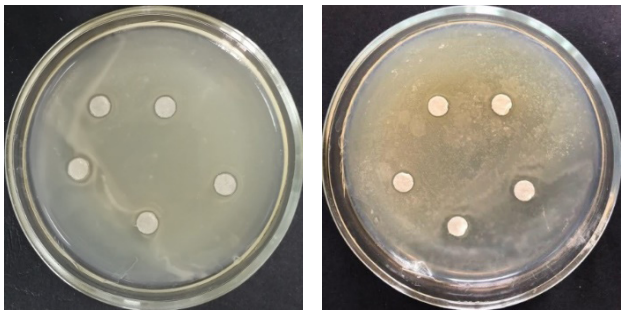
(a) (b)

Figure 10 Antimicrobial activity of yarrow suspension culture extracts against *Helicobacter pylori*: a) extract No. 5 (9.5 ± 0.5 mm); b) extract No. 8 (9.3 ± 0.5 mm)



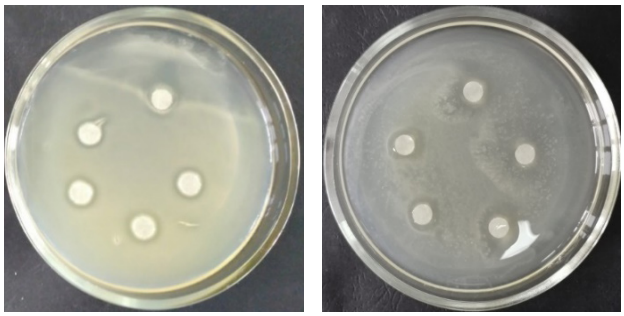
(a) (b)

Figure 11 Antimicrobial activity of yarrow suspension culture extracts against *Streptococcus viridans*: a) extract No. 5 (8.2 ± 0.4 mm); b) extract No. 8 (8.2 ± 0.4 mm)



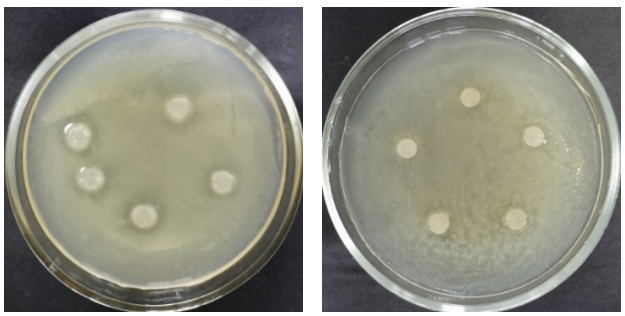
(a) (b)

Figure 12 Antimicrobial activity of yarrow suspension culture extracts against *Streptococcus bovis*: a) extract No. 5 (8.0 ± 0.4 mm); b) extract No. 8 (8.0 ± 0.4 mm)



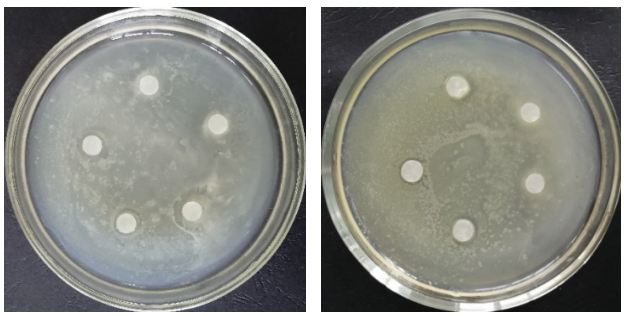
(a) (b)

Figure 13 Antimicrobial activity of yarrow suspension culture extracts against *Porphyromonas gingivalis*: a) extract No. 5 (10.0 ± 0.5 mm); b) extract No. 8 (10.1 ± 0.5 mm)



(a) (b)

Figure 14 Antimicrobial activity of yarrow suspension culture extracts against *Acinetobacter baumannii*: a) extract No. 5 (10.0 ± 0.5 mm); b) extract No. 8 (9.9 ± 0.5 mm)



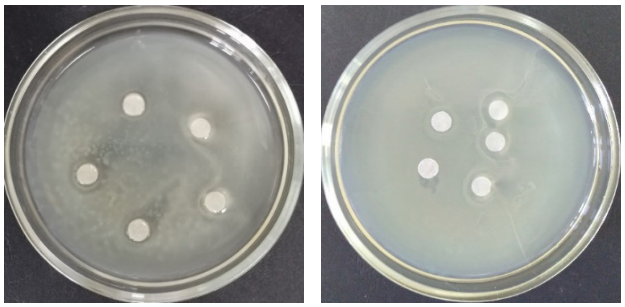
(a) (b)

Figure 15 Antimicrobial activity of yarrow suspension culture extracts against *Borrelia burgdorferi*: a) extract No. 5 (8.4 ± 0.4 mm); b) extract No. 8 (8.3 ± 0.4 mm)

cultures, with 82–91% viability and a growth index of 9.56–10.23; and hairy root cultures (hairy roots), with a growth index of 26.0.

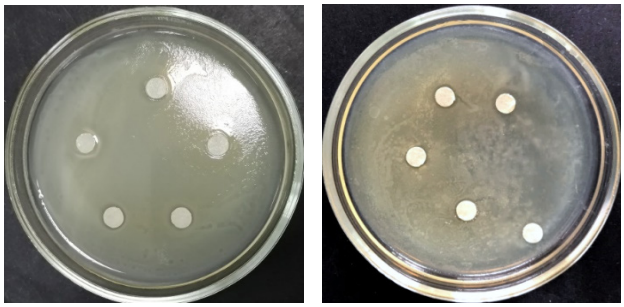
All the cell cultures contained essential oils, flavonoids, glycosides, phenolic acids, carotenoids, as well as vitamins C and E. The suspension cultures had a high content of dominant compounds – essential oils, flavonoids, and glycosides.

We obtained nine samples of ethanol extracts from the biomass of yarrow suspension culture using different extraction parameters: temperature (30, 50, and 70°C), volume of ethanol (30, 50, and 70%), and extraction time (2, 4, and 6 h). HPLC showed the presence of compounds with potential geroprotective properties: phenylpropanoids, flavonoids, and simple phenols. Quantitatively, the dominant compounds were caffeic acid, cynaroside, 4,5-dicofeylquinic acid, apigenin, and luteolin. In addition, HPLC revealed a number of compounds that have not been previously reported in yarrow, such as cumic aldehyde, umbelliferone, 3-caffeylquinic acid, and caffeic acid.



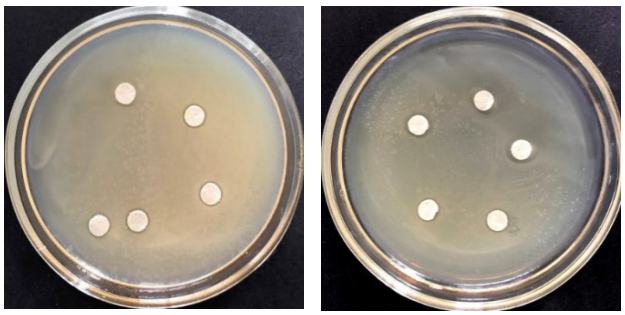
(a) (b)

Figure 16 Antimicrobial activity of yarrow suspension culture extracts against *Propionibacterium acnes*: a) extract No. 5 (10.2 ± 0.5 mm); b) extract No. 8 (10.0 ± 0.5 mm)



(a) (b)

Figure 17 Antimicrobial activity of yarrow suspension culture extracts against *Aggregatibacter actinomycetemcomitans*: a) extract No. 5 (8.6 ± 0.4 mm); b) extract No. 8 (8.6 ± 0.4 mm)



(a) (b)

Figure 18 Antimicrobial activity of yarrow suspension culture extracts against *Streptococcus intermedius*: a) extract No. 5 (8.1 ± 0.4 mm); b) extract No. 8 (8.1 ± 0.4 mm)

Our *in vitro* experiments with the extracts established the presence of antioxidant properties and antimicrobial activity against pathogenic and opportunistic strains, including *Escherichia coli*, *Candida albicans*, *Helicobacter pylori*, and *Pseudomonas aeruginosa*. Due to their geroprotective potential, yarrow cell cultures can be used to create nutraceuticals that prevent premature aging.

Further research in this area could focus on developing techniques to isolate and purify individual bioactive compounds with geroprotective potential from the extracts of yarrow suspension culture biomass. Particular attention should be drawn to those substances which were found in yarrow cell cultures for the first time, namely cumin aldehyde, umbelliferone, 3-caffeylquinic acid, and caffeic acid.

CONTRIBUTION

The authors are equally responsible for the findings and the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Social responsibility as the dominant driver of the evolution of reporting from financial to non-financial: theory and methodology

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Abstract:
Introduction. For over half a century, corporate social responsibility has been in the center of scientific discourse. Its basic concept has become part of strategic management, changing the content of financial reporting and leading to new forms of corporate reporting.
Study objects and methods. The article substantiated the importance of studying corporate social responsibility (CSR) concepts and national models. The study covered the CSR basic concept, targets and paradigms. The evolution of CSR was considered in terms of its impact on the formation of non-financial reporting.
Results and discussion. The authors identified two stages of non-financial reporting development and two directions for the convergence of financial and non-financial reporting. They proposed an assessment matrix to measure facts, actions, and resources in the past, present, and future. This matrix can help companies to generate information for integrated reporting by showing the impact of each type of capital (financial, production, human, intellectual, social, and environmental) on their value creation. Within a promising direction for developing non-financial reporting in conjunction with financial reporting, the authors set requirements to reflect the impact of climate risks on the company's activities in accordance with the recommendations of the Task Force on Climate-Related Financial Disclosures. The authors discussed both standardized and their own approaches to CSR indicators. Finally, they addressed the problem of reliability of non-financial reporting, discussed various forms of its verification (taking evidence from food industry enterprises), and set specific principles to control non-financial reporting indicators.
Conclusion. The authors identified further promising areas of research in the theory and practice of CSR. Their findings can be used in scientific debates on CSR and in the practice of corporate reporting.

Keywords: Measurement, indicators, corporate social responsibility, reporting, food industry, sustainable development

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INTRODUCTION

Corporate reporting is developing during the ongoing transformation of the economic paradigm under the influence of the concept of social responsibility. This concept underlies the shift of attention from financial to non-financial reporting. As a result, new indicators are being constructed to assess the performance of economic entities. Today's increased interest in non-financial reporting is similar to that in financial reporting in the 20th century. At the same time, there is a reconsideration of the concept of financial accounting that defines its information boundaries.

This review aims to fill some gaps in scientific discourse as to how the concept of social responsibility affects the evolution of corporate reporting. It also raises awareness of methodological and theoretical approaches to corporate reporting and identifies recent trends in this area. Despite general interest in corporate responsibility and non-financial reporting, there is clearly a shortage of studies into the methodology of information support for economic decisions and assessment of socially oriented activities of food industry organizations.

Literature analysis focuses on two main areas – corporate social responsibility and corporate reporting. The latter is gradually moving towards an idea that companies need to disclose their results in three dimensions: economic, environmental, and social. Economic indicators reflect the company's financial position and performance. The environmental dimension takes into account its impact on the planet. Finally, the social aspect covers such issues as social justice and improving the quality of life.

The article presents a critical analysis of scientific publications on the topic and systematizes approaches to defining social responsibility and its role in the development of corporate governance and non-financial reporting, as evidenced by food industry enterprises. It gives a retrospective view of financial and non-financial reporting and offers directions for their further development in the form of an assessment matrix that takes into account changes of indicators over time.

STUDY OBJECTS AND METHODS

Social responsibility: definition and evolution. Scientists define social responsibility as taking different forms (individual, collective, group), being of different types (moral, legal, civil, corporate, etc.), and relating to different subjects (individual, organization, state, world community) [1, 2].

Individual social responsibility concerns the actions of particular individuals and is studied by philosophy, ethics, and psychology [1]. Most researchers in this area analyze human behavior associated with providing assistance, i.e. actions motivated by empathy, compassion, and selflessness [1]. Group social responsibility tends to correlate with professional responsibility as a whole or with particular professions (doctor, auditor, lawyer, journalist, etc.) [3]. Collective social responsibility is usually defined as the activities of organizations [4, 5]. The focus of discussion in this area is on corporate responsibility of commercial organizations.

Social responsibility of the state is considered either in the narrow sense, as a relationship between authorities and society, or multidimensionally. The latter approach covers legislative support of a socially acceptable level of well-being for the main social groups; availability of the declared social benefits in health care, education, employment, etc.; creating favorable conditions for small business and providing support to the economically active population, etc. [6, 7]. Discourse in this area has led to an assumption that there are national models of social responsibility focused on the relationship between the state and business [8].

The global context of social responsibility refers to solving problems of global importance. It is usually associated with such international organizations as the International Organization for Standardization (ISO), the International Finance Corporation (IFC), the United Nations (UN), as well as with the European Union (EU) and international standards that regulate organizational social responsibility and non-financial reporting [3, 9–11].

Almost all forms of social responsibility reported in scientific literature are related to organizations and their activities. They refer to corporate social responsibility (CSR) – the center of interaction between all economic agents that is closely connected with other forms of social responsibility. CSR is a focus of attention for economists discussing how responsible companies are in relation to their employees, consumers, and suppliers, as well as society and the planet as a whole [10, 12].

The theoretical concept of CSR goes back a long way. Blagov connects it with the theory of strategic corporate governance and identifies two stages in its development. The first stage (mid-1950s–mid-1990s) is associated with the formation of concepts, while the second stage (mid-1990s up to date) was when concepts developed on their own basis [4]. According to Blagov, the period until the 1990s saw a search for a paradigm to formulate and solve problems of interaction between business and society, based on the logic "principles – processes – results" [4]. This stage produced three basic concepts: 1) corporate social responsibility (CSR-1), which defines the moral principles of business and determines the reasons for its actions; 2) corporate social responsiveness (CSR-2) or the ability of a business to perceive social impact on management, which determines how the company operates; 3) corporate social performance (CSP), which combines CSR and CSP [4]. Thus, the first stage resulted in a transfer of CSR issues into a practice-oriented plane.

The second stage of CSR evolution gave rise to three alternative concepts: 1) the concept of stakeholders (CS), which specifies CSP as certain activities aimed at meeting the expectations of specific stakeholders; 2) the concept of corporate citizenship (CCC), which allows corporations to formulate their own program of becoming a "good corporate citizen"; and 3) the concept of corporate sustainability (CCS), which defines CSR principles as a unity of three types of responsibility (economic, social, and environmental) [4]. The latter concept is commonly referred to as the concept of sustainable development. This concept introduced a "triple bottom line" of sustainable development into

the theory and practice of business, covering economic, social, and environmental dimensions. According to Blagov, it was this concept that formed a basis for the international non-financial reporting standards of the GRI (Global Reporting Initiative) and the ISO 26000 Guidance on Social Responsibility [4].

Kanaeva looks at the theory of CSR through the key results of its development, namely: 1) conceptualization, i.e. defining the CSR problems and forms; 2) operationalization and instrumentalization, which led to a rethinking of the reasons (motives) for increasing CSR and incorporating CSP into strategic corporate governance; 3) professionalization, i.e. developing approaches to training specialists in implementing CSP; and 4) institutionalization, i.e. transplantation, formation and consolidation of relevant norms and restrictions, and incorporating CSR into the institutional environment of the new economic model [13].

According to Kanaeva, the key result of CSR conceptualization was a paradigmatic shift in the attitudes to this phenomenon. Previously understood as complementary, CSP was now recognized as a vital part of the company's development strategy, a prerequisite for its long-term competitive advantages and corporate sustainability [11]. This understanding gave rise to a new economic model and now we are witnessing the formation of a special CSR institution built into this model.

As early as 2010, Fedorov and Polyakov described CSR as a socio-economic institution involved in the regulation of the economy [5]. Kanaeva refined this approach by specifying CSR functions that contribute to the key principles of sustainable development [3]. Zavyalova offered another approach to CSR evolution [14]. The author defined CSR as a generalizing umbrella theory that covers various concepts described by Blagov (CCC, CCS, CSP, CSR-2, etc.). According to her approach, CSR developed through a succession of three theories: corporate selfishness, corporate altruism, and convergent collaboration. The theory of corporate selfishness defines the goal of a business as maximizing the profits of capital owners, which excludes the idea of social responsibility [14]. The theory of corporate altruism implies a voluntary participation of companies in CSR programs. Finally, the theory of convergent collaboration is a compromise that institutionalizes CSR. In this theory, CSR remains voluntary but is subject to assessment [14].

Thus, the above researchers unanimously agree that by the beginning of the 21st century, the evolution of CSR had resulted in a transformation of ideas about the role of business in society. Blagov and Kanaeva substantiated the new socio-economic paradigm in which businesses have to incorporate CSR into their corporate strategies in order to survive.

The practical implementation of CSR can be exemplified by two national models, American and European.

The American CSR model began to take shape at the turn of the 20th century, but it was not until the mid-20th century that it gained significant acceptance in practice, as reported by Danshina [8]. This model implies minimal government interference in business policies. The government can influence them indirectly by introducing tax benefits and various other concessions at the legislative level [6, 8].

The main CSP areas in the United States are corporate philanthropy, targeted programs, and corporate pension funds. Goal-oriented marketing strategies are common, which involve allocating part of company earnings for socially significant projects. Businesses and non-governmental organizations (NGOs) often form "social alliances" to solve socially significant problems together. American companies actively use their CSP results to attract public attention, so they make their activities transparent through non-financial reporting, among other means [8].

The European CSR model developed under the influence of trade unions and legislative norms [8, 10, 15]. There, CSP is regulated by legislation at the international, national and local levels [8]. European companies pay high taxes that the government uses to provide social welfare services to its citizens. However, many companies not only comply with the law and pay high taxes, but also implement their own environmental and social programs [9]. Most of them, like American companies, regularly prepare non-financial reports.

Thus, the CSR evolution has resulted in a regular voluntary practice of CSP and financial reporting by companies in different countries, despite significant differences in its implementation [16–18].

RESULTS AND DISCUSSION

Non-financial reporting. Most researchers agree that European companies were the first to publish social reports in the 1990s [19, 20]. However, Pyatov *et al.* found that the first evidence of non-financial reporting dates back to the first half of the 19th century [21]. The practice of non-financial reporting became the subject of discussions about information disclosure, accountability format, and independent reviewing [16].

Companies began to prepare reports on social issues, corporate social responsibility and sustainable development, as well as standardized reports [16, 22]. The evolution of non-financial reporting was a progressive transition from social and environmental reports to reports on sustainable development, later to take an integrated form, as stated by Vakhrushina and Tolcheeva [23].

According to Malinovskaya, integrated reporting was brought about by the evolution of economic theories and the emergence of institutional investors in the late

1980s. In the 1990s, institutional investors adopted the principles of responsible investment, creating a demand for information on environmental and social activities of investment objects and their ability to create value over time in order to reduce investment risks [24]. The financial and non-financial reports existing at the time could hardly satisfy that demand. A response to that was an idea of integrated reporting that emerged in the early 2000s, later to become standardized [24].

The conceptual framework for integrated reporting is based on the principle that the company's main goal is to create value in the interests of all stakeholders by increasing the key types of its capital: financial, environmental and social, as reported by Melnik and Kogdenko [25]. Thus, the new type of reporting emerged from two basic concepts of social responsibility – the concept of stakeholders and the concept of corporate sustainability.

Today, the development vector of non-financial reporting is determined by the requirement for the company to disclose its climate risks. These risks have been developed by the Financial Stability Board's Task Force on Climate-Related Financial Disclosures (TCFD) since 2015. According to Efimova and Rozhnova, the guidelines issued by the TCFD in 2017 became the next, more qualitative, step in the development of non-financial reporting [26].

The most debatable issue of non-financial reporting has been how to measure CSR. Debates are held at the national, international and corporate levels in order to achieve data comparability.

The assessment of sustainable development is discussed at the national level using the indicator and integral approaches. The indicator approach became a basis for the Sustainable Development Goals designed by the UN for all countries to achieve by 2030 [3, 11, 27]. There are 17 goals that have 169 targets, each responsible for a number of indicators [11].

Integral indicators are a set of weighted indicators of sustainable development, which some researchers consider as analogues of GDP (gross domestic product) [28]. Two main integrated indicators today are the UN's Human Development Index and the World Bank's Adjusted Net Savings. The former reflects social aspects of sustainability and aggregates sub-indices of longevity, education and material well-being. The latter characterizes environmental and economic sustainability and takes into account economic losses from depletion of natural resources and damage from environmental pollution [11]. Noteworthily, food industry enterprises of all levels make a special contribution to the human development index.

The indicator and integral approaches to social responsibility assessment are applicable both at the sectoral or regional level and at the organizational level [7, 28, 29]. According to Barilenko *et al.*, the generally accepted integral indices of organizational sustainable development include the Dow Jones Sustainability Index, Global 100 Index, and Blomberg ESC Index [30]. Modern scientists continue to search for alternative options. The Russian economist Sheremet (1929–2020) was among the first to propose a comprehensive approach to assessing organizational sustainable development [31]. Several recent approaches can be found in [28, 32–34]. Kuznetsova and Kuznetsov designed a system of sustainable development indicators for industrial enterprises and developed an integral indicator for a comprehensive assessment of economic, environmental and social factors [28]. Komendenko and Svetashova proposed to evaluate the company's integral efficiency adjusted for environmental and social risk factors [32].

The indicator approach also attracts a lot of scientific attention [35–38]. For example, Vertakova and Chulakova assess the impact of CSP on the growth of business value and propose indicators for the company's interaction with regional and municipal authorities, population and personnel, as well as for its contribution to the socio-economic development [36]. Noteworthily, it was Blagov who proposed to evaluate the company's relations with stakeholders to assess its sustainable development as early as 2004 [35]. Based on the concept of stakeholders, he developed a set of reputation indices that characterize the company's relations with the government, consumers, business partners, personnel, and shareholders. The indices were tried out using the data of food industry enterprises located in the North-West of Russia [35].

In addition to the indicator and integral approaches, Barilenko *et al.* identified another four approaches to assessing the company's sustainable development [30]. They are: 1) a system of indicators for standard reports set in accordance with the standards (e.g., GRI); 2) a matrix of key performance indicators (KPI) that reflects the achievement of sustainable development goals; 3) a system of indicators to assess the efficiency of a business model (resources, business processes and results); and 4) a project portfolio approach focused on the development of the company's strategy of sustainable development [30]. These four approaches have a different foundation compared to the indicator and integral approaches described above. They are based on the calculation of analytical indicators and integrators and on the development of reporting indicators for non-financial reporting.

Efimova and Moiseeva developed their own proposals based on the KPI matrix [39, 40]. In particular, Efimova created a method for monitoring and assessing the process of creating value using six types of capital (financial, production, human, intellectual, social, and environmental). This method also takes into account a company's business model reflected in its integrated reporting [39]. It can be used to analyze how the company forms and uses the six types of capital –

its key resources – in its business processes. For this, the indicators for each type of capital are classified into three groups: resources, business processes, and results [39].

Of special interest is the use of corporate social responsibility indicators in practice. For example, Danshina analyzed non-financial reporting of nine business organizations, leaders of the Dow Jones Sustainability Index in 2016. The researcher classified the reporting indicators disclosed by the US and European companies into five areas: environment, social work, CSR in relation to employees, handling suppliers, and corporate indicators [8]. The comparative analysis showed that all US companies and only one European (Nestle SA, a leader in the food industry) provided reports for all the sections [8]. The rest of the European companies did not disclose their corporate indicators.

Barilenko *et al.* analyzed GRI sustainability reports and found that the companies preferred to disclose those indicators which directly related to their business model and value, or profitability indicators for their investors [30]. The researchers also empathized that the companies' reports complied with relevant guidelines and standards. The same conclusion was made by Bobrova and Malaykina, who analyzed non-financial reports of Russian energy companies [41].

Thus, while scientists are looking for new integral indicators, systematizing CSR and reporting indicators, organizations are already using an established set of sustainable development indices to build their ratings and follow the recommended standards for integrated non-financial reporting. However, it is generally believed that companies often provide heterogeneous and incomparable data that require clarification.

Financial reporting. Many researchers believe that non-financial reporting is a natural result of the development of financial reporting [21]. Although financial reporting had established long before the first attempts at non-financial reporting, the rules of its formation were determined by the same developmental principles that were subsequently used to design national CSR models.

Financial reporting developed as a consequence of accounting practices that were determined by dominant factors differing from country to country. Sokolov (1938–2010) identified significant differences in the development of accounting in countries such as Italy, France, Germany, England, and America [42]. In different periods, these countries have had a significant impact on the evolution of accounting around the world. They clarified the purposes of accounting, making a transition from controlling those involved in the business process to controlling the company's management. Also, they changed the choice of sciences to determine accounting rules, making a transition from law to psychology. In the 20th century, they redefined the content of reporting forms, replacing a simple balance of rights and obligations with an equality between resources and the amount of obligations and capital.

Modern financial statements have derived from the evolution of accounting. It was as early as 1673 that the French merchant Jacques Savary (1622–1690) designed the Commercial Code to regulate accounting practice in Europe [43]. The modern stage is generally associated with 2001, when most European countries recognized the need to bring national accounting conventions in compliance with the International Financial Reporting Standards (IFRS) based on the Anglo-American system of accounting [44]. In the context of economic globalization, the IFRS contributed to the comparability of companies' reports prepared in accordance with the national accounting conventions.

Modern accounting standards are based on the national accounting regulation models, two of which are especially important today – European and Anglo-American. The first model developed under strict state regulation, while the second one implied minimal government interference in business [45]. The main differences between these models are most visible when comparing two key aspects: the criteria for recognition and assessment of financial statement elements (assets, liabilities, capital, income and expenses). In the European model, the criteria are based on legal aspects, while in the Anglo-American model, on their economic content. The rules of assessment are different in all countries, depending on the object of assessment. They are established at the moment of their recognition and then change over the natural course of time: past-present-future [46].

Today, financial reporting is developing under the influence of CSR. The rethinking of business targets has expanded the boundaries of financial reporting. Experts are clarifying the rules for recognizing elements of financial reporting and adding new objects of assessment [47]. This is based on inquiries about the companies' CSP.

Correlation between financial and non-financial reporting. Current works on financial and non-financial reporting are focused on their relationship and interchangeability. Most authors justify the need for their combination and complementarity [23, 26]. At the same time, much attention is drawn to their information links, differences and similarities.

Melnik and Kogdenko associate their differences with the disclosure of differing goals in the company's reports. Traditional financial reports contain economic data to reflect the company's financial results. Non-financial reports inform about financial performance and business externalities, in particular environmental and social impacts. They show what financial and non-financial factors have affected the company's ability to create value and reflect the company's impact on the economic, natural and social environments [25].

The authors think that integrated reporting, like other types of non-financial reporting, is impossible to

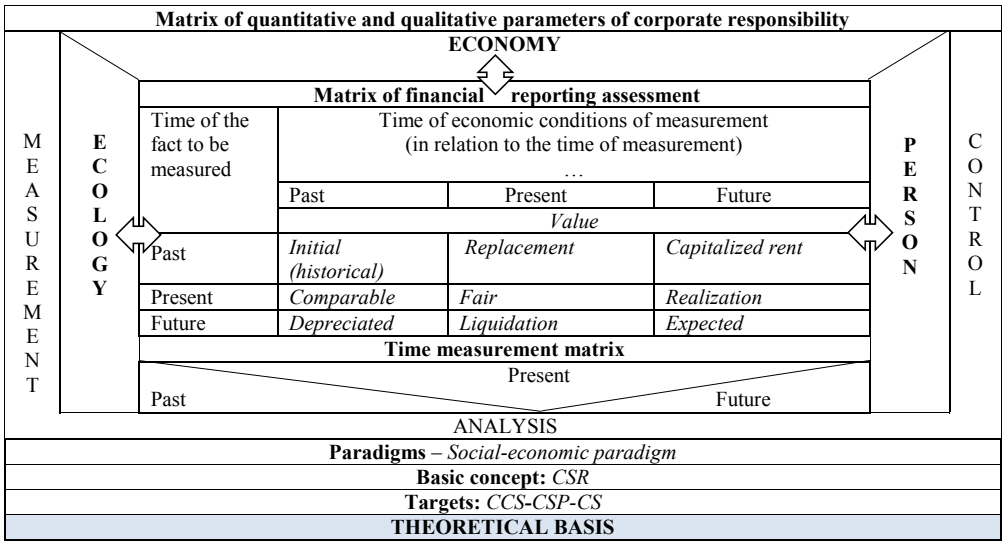

Figure 1 Matrix of quantitative and qualitative parameters of corporate responsibility (by the authors)

standardize since it covers a wide range of qualitative characteristics: the company's business model and competitive advantages, segment information, interaction with stakeholders, as well as basic non-financial indicators of industry and business [25]. They insist on expanding the boundaries of integrated reporting, compared to financial reporting [25].

According to Getman, however, the boundaries of financial reporting match those of non-financial reporting [48]. The author insists on the need to standardize integrated reporting so that it better reflects a "motley" range of non-financial indicators [48]. To substantiate his position, Getman shows a relationship between integrated and financial reporting. Not only does he define the boundaries of reports, but he also suggests using aggregation and disaggregation of information in integrated reporting, taking into account the segment approach used in financial reporting. The author finds it important that integrated reporting covers controlled companies within the perimeter of consolidation for financial statements and that time frames should be set for short, medium and long terms, taking into account the operating and investment cycles used in financial accounting [48].

The evolution of non-financial reporting gives rise to new accounting directions, for example, business accounting, which is described in detail by Plotnikov and Plotnikova [49, 50]. The authors consider business accounting as a product of standards and guidelines for integrated reporting. Its goal is to provide stakeholders with information on the business model's sustainable development and stages of value creation. Business accounting focuses on the organization's business model structured by type of capital, life cycle, and value creation and transformation over time. Its main task is to synchronize the created value of business models and to collect information for integrated reporting to reflect changes in each type of capital [50].

Although Plotnikov and Plotnikova set a fundamental task to assess changes in the business model (capital) over time and emphasized the need to assess the input-output system based on past, present and future events, they did not offer any method. A solution was proposed by Sokolov who developed a matrix of measuring financial reporting indicators that change over time (Fig. 1). This matrix reflects the impact of temporal factors on accounting.

According to Sokolov, assessments differ in two parameters – the moment of measurement to which the assessment criterion belongs and the moment for which the result is intended. Correlations between the two parameters form nine time estimates: past, present and future estimates of the past, present and future [46].

The matrix aims to assess the present against the past and the future, thus contributing to the assessment of value creation over time. Using the matrix in non-financial reporting will solve the main problem of business accounting – synchronization of the business model's created value and measurement of the company's impact on the three-fold result of sustainable development. Thus, the matrix can be used in both financial and non-financial reporting.

Considering the relationship between financial and non-financial reporting, we should draw special attention to the modern trend laid down by the TCFD. Unlike all previous guidelines and standards for non-financial reporting, the TCFD recommendations emphasize the need for disclosing information about climate risks in financial reporting. This marked the beginning of a new stage in the development of reporting based on mutual convergence of reporting information.

According to Efimova and Rozhnova, "the developers of reporting standards recognized the impairment of financial information prepared in isolation from non-financial information" [51]. The researchers stressed the need to harmonize financial and

non-financial reporting based on their common goals and principles. They believe that this will "improve the relevance, reliability (quality) and consistency of information for its users about the development of an enterprise in the context of climatic risks" [51]. As a result, this approach should increase the users' trust in reporting by demonstrating the company's understanding of financial and non-financial reporting as a strategic management tool and an important way of communicating with its users [48].

The theoretical basis for harmonizing financial and non-financial reporting, proposed by Efimova and Rozhnova, consists of the following principles: relevance, timeliness, multi-stage detailing, overall interconnectedness, strategic focus and orientation towards the future, scenario analysis, balanced judgement of materiality, responsibility, reasonableness, balance of power and responsibility, continuous improvement of the quality of information display and methods of data convertibility, and reasonable skepticism [51].

Thus, we distinguished two stages in the evolution of non-financial reporting. The first was a stage of substantiating a new reporting area – disclosure of information about CSR in non-financial reporting based on qualitative and quantitative indicators. The second was a stage of mutual enrichment and convergence of financial and non-financial reports based on critical analysis of their differences.

We identified two directions for convergence of financial and non-financial reporting. The first is to use a unified assessment matrix that reflects the influence of temporal factors on the assessment of past, present and future events. The second is to use the TCFD recommendations to assess the impact of climate risks on business and reflect it in the changes of financial indicators, performance results, and cash flows. This approach is based on the fact that non-financial information about climate change risks is only valuable when reported together with financial indicators. Of special relevance here are the recommendations of Efimova and Rozhnova on the harmonization of financial and non-financial reporting based on common principles and methods.

Control of indicators. The evolution of non-financial reporting has been accompanied by discussions about the need and methodology for external, independent verification of the quality and reliability of reporting information [16, 52, 53]. The need for verification of non-financial information led to the emergence of social audit as a measure of CSR. Kizilov and Bogataya believe that social audit originated in the United States in the 1940s as a result of compiling company social ratings [54].

However, social audit and its methods are still defined and interpreted differently [55–57]. For example, Kizilov and Bogataya define it as a confirmation of social reporting (narrow sense) and as an analysis of the company's social programs for effectiveness and compliance with relevant standards (broad sense) [54]. Saprykina and Krylova prefer the term "CSR audit" and define it as an independent verification of corporate reporting on CSR. The content of this process relates to the degree of detail of the information to be verified, while its result is an expression of opinion on the reliability of reporting [58].

Whether internal or external, social audit aims to check if the company's CSR practice reflected in non-financial reporting complies with its goals. The analysis is based on the following criteria: the company's understanding of CSR, its place in the company's system of values, the nature and forms of the company's interaction with stakeholders, key directions of social programs, changes in social activity indicators, as well as problems and directions for further development of CSR [59].

In addition to the auditor's opinion, the external independent verification of non-financial reporting also includes public assurance, comments from third parties, and verification of compliance with the standard [60, 61]. In order to use many forms of independent verification of non-financial statements provided by the food industry enterprises under study, we need a simultaneous development of norms, standards and control practices. The same approach is also used to harmonize financial and non-financial reporting, as we noted above.

There are general and specific principles for verification of non-financial reporting. General principles include adequacy, objectivity, complexity, reliability, comparability, etc., while accessibility is regarded as a specific principle. The principle of accessibility is interpreted as a basis for assessing CSR data that are available in open sources and can be used by any interested party [62]. Alternatively, some principles are directly borrowed from auditing, such as professional ethics, professional skepticism, professional judgment, professional responsibility in collecting sufficient and appropriate evidence to reduce the audit risk, as well as assurance of the materiality and reliability of the company's reporting [58].

CONCLUSION

The directions for further development of the CSR theory and practice include:

– theoretical research and critical analysis of applying the CSR paradigm, basic concept and targets using a systems approach, including the historical method and the technology of foresight;

– temporal measurements of CSR information to develop reporting indicators, analysis and control procedures for measuring environmental, economic and social activities using financial and non-financial reporting methods (presented in Fig. 1 as a matrix of

quantitative and qualitative parameters of corporate responsibility).

Our critical analysis of scientific literature on CSR revealed the following:

1. The understanding of social responsibility may vary depending on the level (individual, corporate, national, global), but CSR is at the center of theoretical debate and practice, since it is at the corporate level that most CSP participants interact with each other.

2. The development of CSR replaced the economic paradigm with the socio-economic paradigm based on a new theoretical basis (concept and targets) determined by three key concepts: CCS, CSP, and CS. Changes in the strategy and quality of company management in the CSR context led to the development of non-financial reporting as a form of informing stakeholders about the company's social, economic, and environmental activities.

3. Non-financial reporting evolved in the following stages: 1) company-initiated preparation of non-financial reporting on certain aspects; 2) development of CSR reporting standards; 3) prioritizing integrated non-financial reporting (including a report on sustainable development).

4. The theoretical perspectives on non-financial reporting show the following:

4.1. Non-financial reporting is currently governed by the standards for preparing comprehensive reports, such as reports on sustainable development and integrated reports. However, their content has not yet been unified. Therefore, there is a need for further research into making reports on sustainable development for food industry enterprises.

4.2. Companies do not have a single approach to measuring sustainable development since there is no basis for unification. The recent trend is to formulate new requirements for disclosure of information on the impact of climate risks on the company's activities, which is a next stage in the development of non-financial and financial reporting.

4.3. Scientists hold fundamentally opposite opinions on the relationship between financial and non-financial reporting. One perspective is that non-financial reporting is only a supplement to financial reporting. Another view is that the content of non-financial reporting is completely different from that of financial reporting. Finally, some researchers believe that the future of company reporting lies in the harmonization and mutual development of financial and non-financial reporting.

4.4. Scientists are still searching for forms and methods of verifying non-financial reporting to ensure its reliability.

One of the key benefits of the CSR concept is a rapid development of non-financial reporting that has a significant impact on the content of financial reporting. Another important result is the idea of combining financial and non-financial reporting on the basis of CSR. However, the content of non-financial reporting is still in its infancy and requires clarification in terms of the information perimeter, data measurement, analysis, and audit.

To conclude, we believe there is a need for unifying the existing rules for preparing CSR reports by adopting an integrated reporting model as the most comprehensive form of non-financial reporting among the models proposed so far. This type of reporting is based on the disclosure of the company's business model and capital flows. Its methodology assumes a plurality of capitals: financial, production, human, intellectual, social, and environmental. Monitoring the effectiveness of these capitals' creation, maintenance and use contributes to creating value and, ultimately, allows the company to implement CSR.

For this monitoring to work, we need to unify the content of integrated reporting as a CSP report that discloses information on the quantitative and qualitative parameters of CSR based on the modern approach to financial reporting represented by the temporal measurement matrix.

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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Influence of blackberry juice addition on mead fermentation and quality

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Abstract:

Introduction. Mead, one of the oldest alcoholic beverages that man consumed is obtained by fermentation of honey solution, and contains from 8 to 18% vol. ethanol. Honey can be considered as an excellent source of carbohydrates for the fermentation process, but low concentrations of other substances in the honey can slow down the process. Blackberry (*Rubus fruticosus* L.) contains dietary fibers, vitamin C (ascorbic acid), vitamin A, vitamin E, potassium and calcium, along with the phenolic metabolites that are a source of possible health benefits.

Study objects and methods. In this study was investigated the influence of blackberry juice addition on mead fermentation process, chemical composition and antioxidative activity. Dynamics of the fermentation process were controlled based on weighing the flasks in time on a scale every 24 h throughout the alcoholic fermentation. At the end of fermentations, oenological parameters of mead: dry matter content, pH, volatile acidity and ethanol content and reducing sugars. For the determination of antioxidative capacity the content of total phenolics, total flavonoids and total flavonols were measured and two tests were performed: DPPH and ABTS.

Results and discussion. Addition of blackberry juice had a positive effect on fermentation dynamics (almost 25% higher rate of fermentation than in control samples), and improved all physicochemical characteristics and composition of resultant meads. Also, meads with the addition of blackberry juice had a significantly higher concentration of total phenolics, total flavonoids and total flavonols and significantly stronger antioxidative properties compared to the control meads without juice addition. The highest total phenolics, total flavonoids and total flavonols content was determined in the mead with the maximum addition of blackberry juice (B20W): it reached 490.88, 50.34 and 62.57 μgQE·mL^{-1}, respectively, and was 6-fold higher for total phenolics and total flavonoids content, and 10-fold higher for total flavonols content than in the mead without juice addition (CW). The strongest antioxidative activity was determined in the B10W mead; it accounted for 6.98 μgTE·mL^{-1} (DPPH assay) and 0.65 μgTE·mL^{-1} (ABTS assay), what was 1.5-fold and 3-fold higher, respectively, than in the mead without juice addition (CW).

Conclusion. The conducted study demonstrated that the use of blackberry juice influenced the course of fermentation of meads as well as their physicochemical and antioxidative properties (positive effect on fermentation dynamics – almost 25% higher rate of fermentation than in control samples, and improvement of all physicochemical characteristics and composition of resultant meads).

Keywords: Fruit, blackberry, beverages, fermentation rate, mead, antioxidant activity, kinetic model

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INTRODUCTION

Mead, one of the oldest alcoholic beverages that man consumed is obtained by fermentation (similar to the process of making white wine) of honey solution, and contains from 8 to 18% vol. ethanol [1]. Honey can be considered as an excellent source of carbohydrates for the fermentation process, but low concentrations of other substances in the honey (e.g. nitrogen) can slow down the process. Therefore, there is a need for adding nitrogen and various additives such as fruit pulps or juices, citric acid, coconut milk blend etc., but their addition should not hide the smell and taste of honey [2–5]. These additives are used to improve fermentation rates, alcohol yields and sensory characteristics of meads [1].

To enhance the character and complexity of meads, a variety of fruits, vegetables, herbs, or spices (ginger,

cardamom, cloves, thyme, rosemary, bay leaves, sage, parsley, fennel, cinnamon, nutmeg, lemon or orange peels, among others) may be added. Fruits and their pulps have been highly recommended because of their richness in carbohydrates, fibers, minerals, vitamin C, carotenoids, phenolic and sulfuric substances. Also, their antioxidant action can help to maintain a balance between production and elimination of reactive oxygen species and other related compounds, thereby attenuating free radical-induced damage to cells [4]. Blackberry (*Rubus fruticosus* L.) fruit is traded globally due to its delicious taste, pleasant flavor and excellent nutritional profile. These fruits are consumed fresh or processed to make food products such as jam, wine, tea, ice cream, desserts, seedless jellies and bakery products. Blackberries contain dietary fibers, vitamin C (ascorbic acid), vitamin A, vitamin E, potassium and calcium, along with the phenolic metabolites that are a source of possible health benefits [6]. The content of biologically active compounds in meads and their antioxidative activity depend on many factors: type of honey, heat processing of wort, parameters of fermentation process, the type of used herbs, spices, fruits, etc [7–9]. Several structured and unstructured mathematical models have been developed in order to describe the fermentation reaction. A number of studies have been done on kinetic modeling of ethanol as regards its fermentation time. Fermentation rate i.e. kinetic of ethanol production can be described by modified Gompertz equation defined as in Eq. (1) [10, 11]:

$$P = P_{\mathrm{m}} \cdot \exp\left\{-\exp\left[\frac{r_{\mathrm{p,m}} \cdot \exp(1)}{P_{\mathrm{m}}}\right](t_{\mathrm{l}} - t) + 1\right\} \quad (1)$$

where P_m is the maximum ethanol content, g; $r_{p,m}$ the maximum rate of ethanol production, g·day^{-1}; t is duration of fermentation, days; and t_l is the lag time, days. In the available literature there is insufficient data on the production of Blackberry mead, so the objective of this study was to determine the effect of Blackberry juice addition to honey wort on fermentation performances, chemical composition, content of phenolic compounds and on the antioxidative properties of the produced meads.

STUDY OBJECTS AND METHODS

Chemicals and equipment. All chemicals used in this study were of analytical grade. The equipments were as follows: scales (H54AR, Mettler-Toledo, Columbus, USA and PFB 1200-2, KERN & SOHN, Balingen, Germany), hand blender (MSM7150, Bosch, Stuttgart, Germany), ultrasonic bath (U300, Ultrawave Limited, Cardiff, UK), magnetic stirrer (ARE, Velp Scientifica, Usmate, Italy), vortex (ZX3, Velp Scientifica, Usmate, Italy), rotary evaporator (Devarot, Elektromedicina, Ljubljana, Slovenia), spectrophotometers (6315 Jenway, Cole-Palmer, Staffordshire, UK and Spectronic 1201, Milton Roy, Ivyland, USA), pH meter (HI-2211, Hanna Instruments, Smithfield, USA), waterbath (Wisecircu, J.P. Selecta, Abrera, Barcelona, Spain), refractometer (Leica Abbe Mark II, Reichert Technologies, Depew, USA), conductivity meter (HA-2315, Hanna Instruments, Smithfield, USA), Bunsen burner, muffle furnace (Vims elektrik, Novi Sad, Serbia).

Samples. Blossom honey and blackberry fruit (*Rubus fruticosus* L.) from Thornfree cultivar needed for this study were aqiured on September 2016 in Mrkonjić Grad, municipality Mrkonjić Grad, Bosnia and Herzegovina. They were transported to the laboratory (being protected from the sunlight) and in the laboratory they were store in the dark at 2–4°C during 48 h.

Physicochemical analyses of honey. In accordance with the requirements established in Bosnia and Herzegovina legislation, the characteristics and satisfactory quality of the honey were assured through an analysis of the following parameters: moisture content, diastase activity, HMF content, acidity, reducing sugars, saccharose, electrical conductivity and ash content as described by Official Methods of Analysis [12, 13]. The pH was measured with a pH meter of honey dissolved in bidistilled water.

Honey must preparation. Blossom honey was stirred with water in the ratio 1:5 (honey/water). The resultant wort was pasteurized at 65°C for 10 min with regular stirring and skimming off the scum then cooled and poured into fermentation flasks. Blackberry fruit was pressed through a laboratory press to obtain juice that was further used in the study. Resultant juice was also pasteurized at 65°C for 10 min, cooled and poured into fermentation flasks in amounts required for this study. Afterwards, pH values of the wort and juice were corrected to 3.7–4 and four samples were prepared: control wort (CW) and three worts with added blackberry juice in the amount of 5% (B5W), 10% (B10W) and 20% (B20W) of fermentation wort volume. Into all variants yeast energizer VitaFerm® Ultra F3 (Erbslöh, Geisenheim, Germany) was added in amount of 0.1 g·L^{-1}. Next, commercial yeast Vulcaferm (Vulcascot, Wien, Austria), a specifically selected dry yeast strain of *Saccharomyces cerevisiae*, was rehydrated in distilled water at 35–40°C during 30 min and added in the amount of 0.6 g·L^{-1} of wort. The process of alcoholic fermentation was conducted at 25°C for 10 days. All fermentations were carried out in triplicate using a system that consisted of 250 mL flasks containing 180 mL of wort mixture and fitted with an airlock used to release CO_2 produced during fermentation. Dynamics of the fermentation process were controlled based on weighing the flasks in time on a scale every 24 h throughout the alcoholic fermentation.

General oenological parameters. At the end of fermentations, oenological parameters of mead: dry matter content, pH, volatile acidity and ethanol content

Table 1 Physicochemical parameters of honey

Tested parameter	Bosnia and Herzegovina legislation [12]	Honey
Moisture content, %	Not more than 20	19.40 ± 0.10
Diastase activity, Schade scale	Not less than 8	27.00 ± 0.50
HMF content, mg·kg^{-1}	Not more than 40	1.795 ± 0.005
Acidity, mmol·L^{-1}	Not more than 50	41.50 ± 0.50
Reducing sugars, g·100 g^{-1}	Not less than 60	74.80 ± 0.26
Saccharose, g·100 g^{-1}	Not more than 5	1.410 ± 0.003
Electrical conductivity, mS·cm^{-1}	Not more than 0.8	0.354 ± 0.005
Ash content, g·100 g^{-1}	Not more than 0.6	0.15 ± 0.01
pH	n.r.*	3.57 ± 0.01

*n.r. – not regulated

were determined according to standard methods and reducing sugars by 3,5-dinitrosalicylic acid method [14, 15].

Determination of total phenolic content. The total phenolic content in meads was measured spectrophotometrically according to the Folin-Ciocalteau method, as described by Wolfe and Liu [16]. Gallic acid standard in different concentrations (0–500 μg·mL^{-1}) was used to obtain a standard calibration curve. Results were expressed as total phenolics equivalent to gallic acid (μgGAE·mL^{-1}).

Determination of total flavonol content. The total flavonol content in meads was measured using the method of Kumaran and Karunakaran [17]. Results were expressed as flavones equivalent to quercetin (μgQE·mL^{-1}).

Determination of total flavonoid content. The total flavonoid in meads was measured using the method of Ordoñez *et al.* [18]. Results were expressed as flavonoids equivalent to quercetin (μgQE·mL^{-1}).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The antioxidant activity of meads against stable DPPH radical was determined by the method of the Liyana-Pathiranana and Shahidi [19]. Trolox (1–10 μg·mL^{-1}) was used as reference standard. The results were expressed in μgTrolox Equivalent·mL^{-1} (μgTE·mL^{-1}).

2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging assay. The test was performed as described by Re *et al.* [20]. Trolox (0.1–5 μg·mL^{-1}) was used as reference standard. The results were expressed in μgTrolox Equivalent·mL^{-1} (μgTE·mL^{-1}).

Table 2 Kinetics of the fermentation process of meads (g-cumulative mass of produced ethanol)

Sample	Day				
	3	6	8	9	10
CW	3.085	7.040	8.296	8.767	9.168
B5W	5.314	9.723	10.440	10.857	11.014
B10W	5.697	9.966	10.405	10.752	10.875
B20W	5.593	9.723	10.053	10.404	10.457

Statistical analysis. All tests were performed in triplicate and the results were expressed as means ± standard deviation. Variance analysis (ANOVA) was applied to test significant differences between mead samples. Tukey's test was used to identify differences between mean values obtained in meads ($P \leq 0.05$). Characteristic kinetic parameters of alcoholic fermentation were obtained by fitting the measured values of ethanol production into a modified Gompertz equation, performing nonlinear regression analysis. The statistical analysis of the developed mathematical relations was done applying linear regression analysis and Fisher's statistical tests.

RESULTS AND DISCUSSION

Based on the results presented in Table 1, the characteristics and quality of the honey were in agreement with the requirements established by Bosnia and Herzegovina legislation, and that the tested sample represents a good starting raw material for the production of mead [12].

The pH of honey was 3.57. This parameter is useful in quality evaluations because it influences the HMF formation rate, honey texture, stability and shelf-life, and may indicate honey fermentation or adulteration caused by the bees themselves. The fermentation of the analysed meads spanned for 10 days (Table 2).

Kinetics of the fermentation process of meads were determined based on changes in the weight of fermentation samples in time and expressed as the cumulative mass (g) of produced ethanol per day. The kinetics of ethanol production during fermentation of analysed samples were presented in Fig. 1.

The graphs in Fig. 1 show quite well overlapping of fitted curves with experimentally determined data. The correlation coefficient (R^2), as measurement of the fit's goodness, is 0.980 for CW sample and 0.997 for all other samples (BW5, BW1 and BW20), i.e. the samples with added blackberry juice. As it can be seen from the graphs, mass of produced ethanol rapidly increases after lag phase of fermentation process, corresponding to the exponential growth of the yeast cells.

The characteristic kinetic parameters of developed equations are summarized in Table 3, and compared to the corresponding parameters calculated from the experimental data (Table 2). The predicted values of the maximum mass of ethanol produced (P_m) correspond quite well to the actual values. Maximum rates of ethanol production ($r_{p,m}$), calculated from the experimental data, were being lower than predicted values, which can be explained by the fact that conducted experiments did not involve measurements of ethanol production during the lag phase. According to the developed equations this phase last from 1.6 to 1.9 days. Cuenca *et al.*, reported the lag phase in the mead fermentation being from 5 to 65 h [10].

According to the data in Table 3 and curves in Fig. 1, samples with the blackberry juice added (BW5, BW10 and BW20) show almost identical mathematical dependence of the ethanol production on time. Therefore, one can use single equation, with mean values of characteristic parameters, to describe production of ethanol during fermentation of mead with added blackberry juice in different amounts. Also, addition of the blackberry juice in the mead-making process had a positive effect on fermentation kinetic giving a rise to almost 25% higher rate of fermentation than in control samples (CW). The rate of fermentation depends on concentration of different inhibitors such as: ethanol, acetic acid, fatty acids (hexanoic, octanoic, decanoic acid), proteins (enzymes), furfural, hydroxymethylfurfural etc. [21]. The inhibitors interact synergistically with high osmotic pressure and the increasing concentration of ethanol during fermentation. Adamenko *et al.* investigated the influence of added juices from Cornelian cherry cultivars on mead fermentation and showed that juices addition caused CO_2 emission to be 10% higher than in control samples (without added juice) after 5 days of the fermentation process [3].

Based on the results shown in Table 4, it can be noticed that introduction of blackberry juice in mead fermentation improved all physicochemical characteristics and composition of resultant meads. Resultant dry matter contents were not too high, and among different samples were small differences. In sample CW, the dry matter content was the highest and in samples B5W and B10W the lowest (4.88 and 3.85%, respectively) which was an indicator, together with the reducing sugar content, that the fermentation process was almost at the end. According to the results of the content of residual sugar, it was obvious that the residual dry matter comes from other substances such as disaccharides (saccharose, maltose, isomaltose), trisaccharides, tetrasaccharides, glycerol, etc. [22]. Monitoring pH and acidity after honey-must

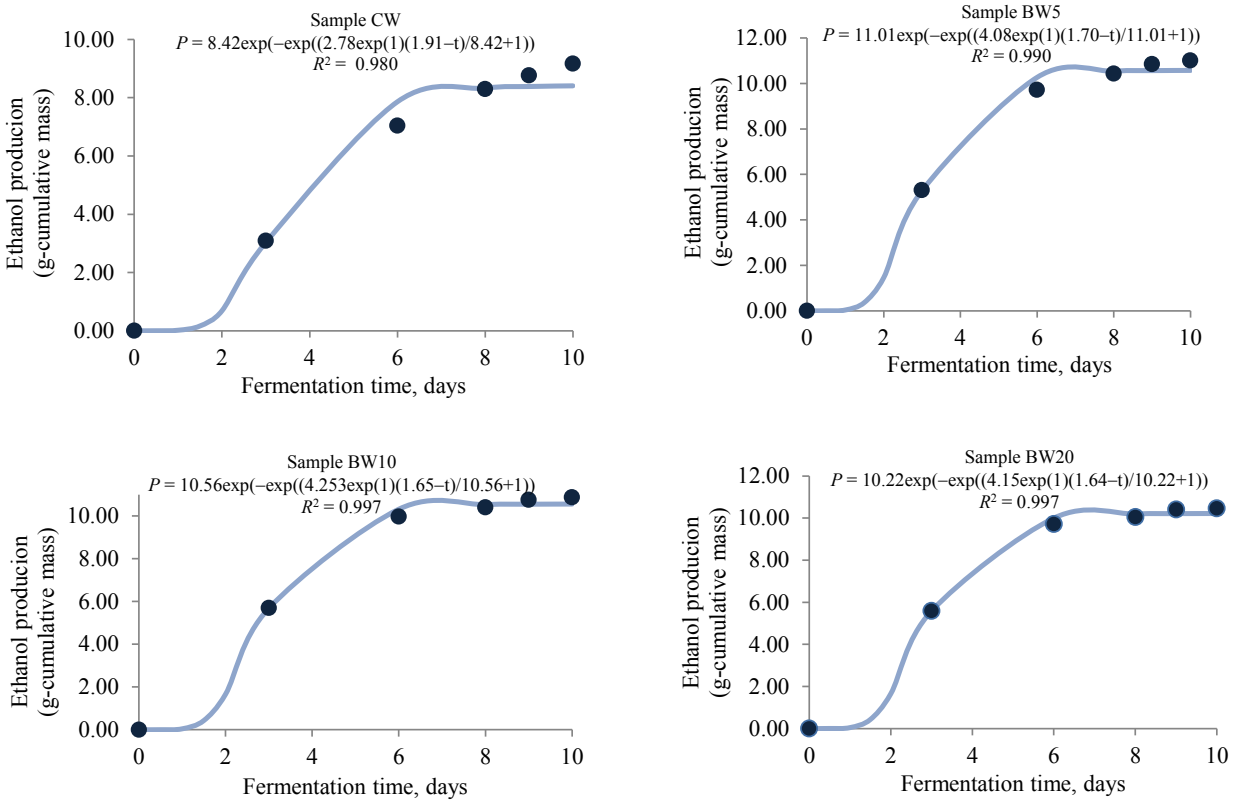

Figure 1 Ethanol production kinetic and results from fitting the experimental data into a modified Gompertz equation (solid line – fitted curve, symbol ● – experimental data)

Table 3 Kinetic parameters of the developed mathematical models and corresponding experimental data

Sample	P_m, g-cummulative mass	$r_{p,m}$, g·day^{-1}	t_l, day	R^2
CW	8.42	2.78	1.91	0.980
	9.17*	1.33*	–	
BW5	10.58	4.08	1.70	0.990
	11.01*	1.77*	–	
BW10	10.56	4.25	1.65	0.997
	10.88*	1.90*	–	
BW20	10.22	4.15	1.64	0.997
	10.46*	1.86*	–	

* measured values

preparation and during fermentation were important issues to prevent premature fermentation arrest and incomplete sugar breakdown. Acetic acid and succinic acid produced during fermentation by yeasts lead to an increase in the content of unsaturated fatty acids, which can cause slowing or even stopping fermentation. The lowest pH value and acidity were measured in sample B20W and the highest in sample CW (3.07 and 3.30, respectively), indicating that the samples with added blackberry juice had lower pH values compared to the control sample (CW), which could be due to the naturally low pH value of added blackberry juice. Acidity plays a significant role in alcohol beverages as it has a direct impact on their taste and stability [8]. In addition, excessive decrease in the pH value may contribute to reduced fermentation performance yield of a yeast strain [1]. The pH values of meads analyzed in this study were similar to those reported by Adamenko *et al.* and Akalin *et al.* but lower than Kawa-Rygielska *et al.* [3, 8, 23].

The titrable acidity increased during fermentation from 3.5 g·L^{-1} in the honey-must (data not shown), to 5.39 g·L^{-1} (B20W) in the final mead. Samples with added blackberry juice had higher titrable acidity than CW which could be due to the naturally high titrable acidity of added blackberry juice. Our results were pretty low compared to other authors [8, 22, 24]. Another important indicator of mead quality is volatile acidity. The production of acetic acid, by far the most abundant volatile acid, can have a dramatic effect on the quality of the final product. In addition to undesirable aromas, high levels of acetic acid were toxic to yeast and can led to stuck alcoholic fermentations. The volatile acidity in our study ranged from 400 to 660 mg·L^{-1} of acetic acid and was lower than the values reported by other authors [1, 3, 8, 23]. Reduction of volatile acidity in samples with blackberry juice was probably due to decrease of acetic acid synthesis by cells of *Saccharomyces cerevisiae* due to change in conditions or by a limitation of stress factors [24]. In our study the addition of blackberry juice improved the concentrations of ethyl alcohol from 6.93% vol. in CW to 7.98% vol. in B10W. The effect of meads supplementation on ethanol content, was studied by various authors. Pereira *et al.* reported the highest ethanol concentration in the samples with coupled addition of vitamins and mineral salts [1]. Kawa-Rygielska *et al.* determined the effect of fruit additives such as syrup from chokeberry or grape seeds, and herbal in the form of a dandelion syrup on the course of the fermentation process of "trójniak" before and after aging and reported the highest ethanol content in samples with added grape seeds powder or sugar syrup from chokeberry fruits, but lower in sample with added dandelion syrup [23]. In study conducted by Adamenko *et al.* it was demonstrated that ethanol production is affected by both the type of Cornelian cherry juice and the yeast strain used in mead manufacture: alcohol production by the SF yeast was 20 g·L^{-1} higher compared with the SM yeast, and more ethyl alcohol was produced in the sample with juice from red-fruit Cornelian cherry (MR) [3].

Study results demonstrate that the meads with the addition of blackberry juice had a significantly higher total phenolic, total flavonoid and total flavonol content and significantly stronger antioxidant activity compared to the control meads without juice addition (CW). The highest total phenolic, total flavonoid and total flavonol content was determined in the mead with the maximum addition of blackberry juice (B20W): it reached 490.88, 50.34 and 62.57 μgQE·mL^{-1}, respectively, and was 6-fold higher for total phenolic and total flavonoid content, and 10-fold higher for total flavonol content than in the mead without juice addition (CW) due to highest

Table 4 Composition of obtained meads

Parameter	Sample			
	CW	B5W	B10W	B20W
Dry matter content, %	4.88 ± 0.03^a	3.85 ± 0.00^b	3.85 ± 0.05^b	4.05 ± 0.05^c
pH	3.30 ± 0.06^a	3.10 ± 0.02^b	3.08 ± 0.02^b	3.07 ± 0.06^b
Acidity, g·L^{-1}	3.84 ± 0.03^a	4.59 ± 0.03^b	5.00 ± 0.02^c	5.39 ± 0.03^d
Volatile acidity, mg·L^{-1}	660.00 ± 60.00^a	560.00 ± 34.64^b	440.00 ± 34.64^c	400.00 ± 17.42^c
Ethanol content, % vol.	6.93 ± 0.03^a	7.55 ± 0.03^b	7.98 ± 0.08^c	7.84 ± 0.02^d
Reducing sugar content, g·L^{-1}	26.10	7.83	3.30	3.11

^{a,b,c,d,e,f} = significantly different ($P \leq 0.05$)

Table 5 Results of total polyphenols content and antioxidative activity of meads

	Total phenolic content, μgGAE·mL^{-1}	Total flavonol content, μgQE·mL^{-1}	Total flavonoid content,μgQE·mL^{-1}	ABTS, μgTE·mL^{-1}	DPPH, μgTE·mL^{-1}
Blackberry juice (raw)	498.71 ± 31.74	243.49 ± 4.72	209.11 ± 0.77	8.89±0.04	2.50±0.02
CW	79.83^{a} ± 10.16	6.06^{a} ± 0.21	8.87^{a} ± 0.30	0.40^{a} ± 0.06	2.29^{a} ± 0.15
B5W	160.56^{b} ± 4.36	22.15^{b} ± 1.09	21.74^{b} ± 0.35	0.63^{b} ± 0.00	6.89^{b} ± 0.03
B10W	261.68^{c} ± 5.33	34.77^{c} ± 0.29	29.66^{c} ± 2.26	0.65^{b} ± 0.01	6.98^{b} ± 0.03
B20W	490.88^{d} ± 13.35	62.57^{d} ± 0.57	50.34^{d} ± 1.11	0.54^{c} ± 0.00	5.18^{c} ± 0.17

^{a,b,c,d,e,f} = significantly different ($P \leq 0.05$)

amount of added blackberry juice. Altough the highest content of phenolic compounds was measured in B20W, the strongest antioxidant activity was determined in the B10W mead; it accounted for 6.98 μgTE·mL^{-1} (DPPH assay) and for 0.65 μgTE·mL^{-1} when analyzed with the ABTS assay, what was 1.5-fold and 3-fold higher, respectively, than in the mead without juice addition (CW). All honeys are rich sources of secondary metabolites with antioxidant activity, especially polyphenols. The main polyphenols are the flavonoids, and during fermentation process they are modified through polymerization and complexation with proteins [25]. This might be the answer why B20W had higher content of polyphenols but lower antioxidant activity than B5W and B10W. The differences in the antioxidant activity of meads assayed with DPPH and ABTS tests may result from differences in the kinetics of these tests and in the concentration of substrates (e.g. ABTS method is used for lipophilic and hydrophobic antioxidants, contrary to DPPH method) [23]. The literature provides data on the total concentration of polyphenols and antioxidant properties measured with the DPPH and ABTS assays in different types of meads. Socha *et al.* determined the highest concentration of total polyphenols in the mead with juice from rowanberry what was 45-fold lower compared to that measured in the mead with juice from red fruits of Cornelian cherry [3, 7]. In other studies on the antioxidant properties of mead, much lower antioxidant activity was obtained in meads obtained from different kinds of honey and in meads that differ in their production technology [9].

CONCLUSION

The conducted study demonstrated that the use of blackberry juice influenced the course of fermentation of meads as well as their physicochemical and antioxidative properties (positive effect on fermentation dynamics – almost 25% higher rate of fermentation than in control samples, and improvement of all physicochemical characteristics and composition of resultant meads). The strongest antioxidative activity and the most beneficial chemical composition were determined in the B10W mead. The graphs in Fig. 1. showed quite well overlapping of fitted curves with experimentally determined data.

CONTRIBUTION

A. Savić, A. Velemir, S. Papuga, M. Stojković conceived and designed the experiments; performed the experiments; analyzed the data; contributed reagents, materials and analytical tools; wrote the paper.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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Secondary metabolites in *in vitro* cultures of Siberian medicinal plants: Content, antioxidant properties, and antimicrobial characteristics

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Abstract:
Introduction. Wild-crafting leads to the local extinction of many medicinal plants that are rich in phenolic substances. *In vitro* cultivation of cells and organs of higher plants can be the optimal solution to this problem. The research objective was to study the biosynthetic activity of *in vitro* extracts of wild Siberian plants.
Study objects and methods. The study featured callus, cell suspension, and hairy root extracts of such Siberian medicinal plants as *Eleutherococcus senticosus*, *Codonopsis pilosula*, *Platanthera bifolia*, and *Saposhnikovia divaricata*. They were obtained by *in vitro* cultivation using modified nutrient media of Murashige and Skoog and Gamborg. The content of secondary metabolites was studied using the methods of thin-layer and high-performance liquid chromatography. A set of *in vitro* experiments tested the antioxidant and antimicrobial activity of the extracts.
Results and discussion. All the samples demonstrated a high content of secondary metabolites of phenolic nature. Flavonoglycosides, apigenin, and rutin were found to be the predominant biologically active substances in the callus extracts. Flavonoglycosides dominated in the suspension extracts. The root extracts contained more caffeic acid, rutin, ecdysteroids, quercetin, apigenin, cardiofolin, and coleofolide than the callus and suspension cultures. The list of prevailing secondary metabolites in the root extracts included rutin, apigenin, coleofolide, and quercetin. All the extracts showed antimicrobial and antioxidant activity.
Conclusion. All the extracts demonstrated good antioxidant and antimicrobial properties. Therefore, they can be used for the production of pharmaceuticals and biologically active food supplements as they can be helpful against infectious diseases, as well as oncological, cardiovascular, and neurodegenerative diseases linked to oxidative stress.

Keywords: Callus culture, cell suspension culture, hairy roots, medicinal plant, secondary metabolite, phenolic substances, antioxidant, oxidative stress, antimicrobial properties, extraction

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INTRODUCTION

According to World Health Organization (WHO), medicinal herbs receive a lot of attention in medicine worldwide. Currently, more than 50 000 plant species are used in herbal and allopathic medicine [1]. About 60% of medicinal plants are harvested from their natural habitat, the proportion of cultivated pharmaceutical plants being negligible [2–4]. Many medicinal plant species become extinct as a result of environmental degradation [5]. From 4000 to 10 000 species of medicinal plants have become endangered in the recent decades [3, 6].

In vitro cultivation of cells (callus, suspension cultures) and organs (hairy roots) of higher plants can be a good alternative to wild-crafting [7, 8]. *In vitro* methods have a lot of advantages in terms of secondary metabolites production. First, climatic chambers with their controlled environment do not depend on the weather conditions. Second, these methods allow for a

greater control over production of biologically active substances (BAS) in sterile conditions [9].

Polyphenols are the best known and most numerous metabolites with more than 8000 identified compounds, including phenolic acids, flavonoids, anthocyanins, and stilbenes. Plant polyphenols have excellent biotechnological prospects as they possess anticarcinogenic, antioxidant, antimicrobial, and anti-inflammatory properties [10].

Phenolic BAS with antimicrobial and antioxidant properties can be obtained from many plants [11]. The present research featured secondary metabolites obtained from *in vitro* cultures of wild medicinal plants growing in the Siberian Federal District, namely spiny eleuterococcus (*Eleutherococcus senticosus*), Asian bell (*Codonopsis pilosula*), butterfly orchid (*Platanthera bifolia*), and siler (*Saposhnikovia divaricate).*

The rhizomes and roots of *E. senticosus* owe their pharmacological properties to eleutherosides, which are special glycosides, conventionally marked as A, B, B_1, C, E, F, and G. In addition, *E. senticosus* contains polysaccharides, lipids, essential oils, tannins, and flavonoids, which make it a popular immunomodulatory agent. This plant is described in the Russian Pharmacopoeia [12].

C. pilosula has a general tonic and immunomodulatory effect [13]. *C. pilosula* proved to be a source of several neutral and acidic polysaccharides with immunomodulatory and antitumor properties [14].

The chemical composition of *P. bifolia* remains understudied. Its young tubers are known to contain mucus (up to 50%), which consists mainly of proteins (≤ 15%), sugar (≤ 1%), starch (≈ 27%), coumarin, mineral salts, traces of essential oil and alkaloids, and a small amount of calcium oxalate [15]. Salep possesses anti-inflammatory, antiseptic, tonic, tonic, and anticonvulsant properties [16].

S. divaricata (Turcz.) Schischk. owes its antipyretic, analgesic, hypotensive, antimicrobial, and antitumor properties to various useful substances in their roots. The list includes chromones, triterpenoids of cimifugine and *β*-glycosylcymiosyl sitosterol, steroids *β*-D-glycoside and *β*-sitosterol, coumarins, e.g. emperorin, scopoletin, psoralen, deltoin, bergapten, felloperin, and xanthotoxin [17].

The research objective was to study the biosynthetic activity of callus, cell suspension, and hairy root *in vitro* cultures of *E. senticosus*, *C. pilosula*, *P. bifolia*, and *S. divaricate.* The study also featured the antimicrobial and antioxidant properties of the biologically active substances produced by their cell cultures.

STUDY OBJECTS AND METHODS

The callus, cell suspension, and hairy root cultures of spiny eleuterococcus (*Eleutherococcus senticosus* L.), Asian bell (*Codonopsis pilosula* L.), butterfly orchid (*Platanthera bifolia* L.), and siler (*Saposhnikovia divaricate* L.) were obtained from their seeds. According to aseptic regulations, the seeds were washed in a surfactant solution and sterilized for 1 min in a 0.1% $HgCl_2$ solution. After being rinsed three times in distilled sterile water, the seeds were planted on agar nutrient media in 60 mm Petri dishes in order to obtain sterile seedlings.

The callus cultures of *E. senticosus* were grown on a nutrient medium which consisted of 50.00 mL of MS (Murashige and Skoog) macrosalts (20×), 1.00 mL of MS microsalts, 30.00 g of sucrose, 0.10 g of meso-inositol, 0.50 mg of vitamin B_6, 0.50 mg of nicotinic acid, 0.10 mg of vitamin B_1, 1.00 mg of kinetin, 0.50 mg of 2,4-dichlorophenoxyacetic acid (2,4-D), 0.07 g of jasmonic acid, and 20.00 g of agar (per 1 liter of distilled water) [18]. The callus cultures of *E. senticosus* were grown on a nutrient medium which consisted of 50.00 mL MS (Murashige and Skoog) macrosalts (20×), 1.00 mL of MS microsalts, 30.00 g of sucrose, 0.10 g of meso-inositol, 0.50 mg of vitamin B_6, 0.50 mg of nicotinic acid, 0.10 mg of vitamin B_1, 1.00 mg of kinetin, 0.50 mg of 2,4-dichlorophenoxyacetic acid (2,4-D), 0.07 g of jasmonic acid, and 20.00 g of agar (per 1 liter of distilled water) [18].

The callus cultures of *C. pilosula* were grown on a nutrient medium which consisted of 50.00 mL of MS macrosalts (20×), 1.00 mL of MS microsalts, 30.00 g of sucrose, 0.10 g of meso-inositol, 0.50 mg of vitamin B_6, 0.50 mg of nicotinic acid, 0.10 mg of vitamin B_1, 1.00 mg of kinetin, 0.50 mg of 2,4-dichlorophenoxyacetic acid (2,4-D), 2.00 g of Tween 80, and 20.00 g of agar (per 1 liter of distilled water).

Callus cultures of *P. bifolia* were grown on a nutrient medium which consisted of 50.00 mL of MS macrosalts (20×), 1.00 mL of MS microsalts, 30.00 g of sucrose, 0.10 g of meso-inositol, 0.50 mg of vitamin B_6, 0.50 mg of nicotinic acid, 0.10 mg of vitamin B_1, 1.00 mg of kinetin, 0.50 mg of 2,4-dichlorophenoxyacetic acid (2,4-D), 1.00 g of chitosan, and 20.00 g of agar (per 1 liter of distilled water).

The callus cultures of *S. divaricata* were grown on a nutrient medium of the following composition which consisted of 50.00 mL of MS macrosalts, 1.00 mL of MS microsalts, 30.00 g of sucrose, 0.10 g of meso-inositol, 0.50 mg of vitamin B_6, 0.50 mg of nicotinic acid, 0.10 mg of vitamin B_1, 1.00 mg of kinetin, 0.50 mg of 2,4-dichlorophenoxyacetic acid (2,4-D), and 20.00 g of agar (per 1 liter of distilled water).

The first seedlings appeared after 6–8 weeks. The callus cultures were induced to eight-week-old sterile seedlings with 2–4 leaves: the leaves were cut into pieces and planted on agar medium in 60 mm Petri dishes. The first calli formed on days 7–14. The callus cultures were allowed to grow for 28 days.

The cell suspension cultures were grown in 250 mL flasks (30–40 mL of suspension per flask) in a shaker (100 rpm): 300–400 mg of callus cultures were placed in 25–30 mL of liquid nutrient media.

The cell suspensions of *E. senticosus* were grown on a nutrient medium which consisted of 50.00 mL of MS macrosalts (20×), 1.00 mL of MS microsalts, 30.00 g

of sucrose, 0.10 g of meso-inositol, 0.50 mg of vitamin B_6, 0.50 mg of nicotinic acid, 0.10 mg of vitamin B_1, 1.00 mg of kinetin, 0.50 mg of 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.07 g of jasmonic acid (per 1 liter of distilled water).

The cell suspensions of *C. pilosula* were grown on a nutrient medium which consisted of 50.00 mL of MS macrosalts, 1.00 mL of MS microsalts, 30.00 g of sucrose, 0.10 g of meso-inositol, 0.50 mg of vitamin B_6, 0.50 mg of nicotinic acid, 0.10 mg of vitamin B_1, 1.00 mg of kinetin, 0.50 mg of 2,4-dichlorophenoxyacetic acid (2,4-D), 2.00 g of Tween 80 (per 1 liter of distilled water).

The cell suspensions of *P. bifolia* were grown on a nutrient medium which consisted of 50.00 mL of MS macrosalts, 1.00 mL of MS microsalts, 30.00 g of sucrose, 0.10 g of meso-inositol, 0.50 mg of vitamin B_6, 0.50 mg of nicotinic acid, 0.10 mg of vitamin B_1, 1.00 mg of kinetin, 0.50 mg of 2,4-dichlorophenoxyacetic acid (2,4-D), 1.00 g of chitosan (per 1 liter of distilled water).

The cell suspensions of *S. divaricata* were grown on a nutrient medium which consisted of 50.00 mL of MS macrosalts, 1.00 mL of MS microsalts, 30.00 g of sucrose, 0.10 g of meso-inositol, 0.50 mg of vitamin B_6, 0.50 mg of nicotinic acid, 0.10 mg of vitamin B_1, 1.00 mg of kinetin, and 0.50 mg of 2,4-dichlorophenoxyacetic acid (2,4-D) (per 1 liter of distilled water).

The cell suspension cultures were maintained under 16 h of light and 8 h of dark for 14–21 days.

The root cultures (hairy roots) were obtained from the leaves of 14–28-day-old seedlings. The seedling explants were inoculated with a wild strain of *Agrobacterium rhizogenes* A4 grown on a YEB nutrient medium for 48 h in the dark at 26°C in a shaker that performed circular motions with an amplitude of 5–10 cm and rotation speed of 90 rpm. The medium consisted of 5 g/L of peptone, 1 g/L of yeast extract, 5 g/L of sucrose, and 0.5 g/L of $MgCl_2$ [19].

The transformation was conducted according to the following pattern. After a pair of leaves appeared, the aerial part of the seedlings was separated from the roots, and the leaves, the caulicle, and the hypocotyl were cut into 1.0–1.5 cm segments. After that, the leaf rib was carefully pricked with an insulin syringe needle along the epicotyl and hypocotyls, attempting to reach the vascular system in the center and of the plant. The explants were subsequently transferred onto the YEB medium and kept in a magnetic bath for 10–100 for a more efficient transformation. The incubation time was 48 h. After the incubation of the explants with agrobacterium, the plant material was rinsed in sterile water and transferred onto solid Gamborg B5 medium. To eliminate *A. rhizogenes*, the medium contained 500 mg/L of claforan [20]. The Petri dishes with the explants were placed in a light chamber, where they stayed until they developed transformed roots.

After the roots reached a certain size, they were transplanted onto a fresh hormone-free B-5 nutrient medium to eliminate *A. rhizogenes* completely. The roots were cultivated in the dark at 23°C for 35 days using a shaker (100 rpm). They were subsequently transplanted into a fresh medium as the contamination with the agrobacterium increased.

Secondary metabolites were extracted from the biomass of callus, cell suspension, and hairy root cultures with 70% ethanol by placing 3.0 g of dried biomass of callus, cell suspension, and hairy root cultures in a 50 mL plastic test tube. Together with an appropriate amount of ethyl alcohol, the portion was placed in a shaker and stirred for 60 min. Table 1 demonstrates the extraction parameters.

The resulting extracts were filtered, and the filtrates were centrifuged at 3900 rpm. The solvent was then removed from the extracts by evaporation under reduced pressure in a rotary evaporator. The flask was weighed to measure the extract yield. The dry extract was dissolved in a suitable solvent, which underwent thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) to study the composition of biologically active substances.

The TLC was conducted according to the standard specified in the Russian Pharmacopoeia, Chapter 1.2.1.2.0003.15.

The HPLC was performed using a Shimadzu LC-20 Prominence chromatograph (Shimadzu, Japan) with a Shimadzu SPD20MA diode array detector and a Zorbax C-18 column (150×4.6 mm, phase particle size = 5 μm). The mobile phase included acetonitrile (solvent A) and 0.1% trifluoroacetic acid (B). The HPLC involved gradient and isocratic separation; the wavelength during detection was 276 nm.

The biologically active substances were identified in two ways. First, the UV spectra and retention times of the peaks in the chromatograms were compared with the corresponding parameters in the chromatographically

Table 1 Extraction of secondary metabolites from the biomass of callus, cell suspension, and hairy root cultures with 70% ethyl alcohol

Plant	Water duty	Time, min	Temperature, °C
Callus cultures			
Eleutherococcus senticosus	1:10	60	60
Codonopsis pilosula	1:10	60	40
Platanthera bifolia	1:10	60	40
Saposhnikovia divaricata	1:10	60	40
Suspension cultures			
Eleutherococcus senticosus	1:10	60	60
Codonopsis pilosula	1:10	30	40
Platanthera bifolia	1:5	30	40
Saposhnikovia divaricata	1:10	60	40
Root cultures			
Eleutherococcus senticosus	1:10	60	60
Codonopsis pilosula	1:10	60	40
Platanthera bifolia	1:10	60	40
Saposhnikovia divaricata	1:10	60	40

pure samples. The chromatograms were processed in the LabSolutions. Second, the biologically active substances were identified using high performance liquid chromatography combined with tandem mass spectrometry (HPLC-MS).

The DPPH method made it possible to assess the antioxidant activity of the extracts as stated in [21]. First, the optical absorption of a 2,2-diphenyl-1-picryl-hydrazyl (DPPH) in methanol solution was measured at 515 nm. The DPPH solution and the antioxidant solution were mixed, and the optical density was measured again after 10 min. The antioxidant activity was calculated by the formula:

$$AA = \left(E_{DPHH} - \frac{E_{ex}}{E_{DPPH}}\right) \cdot 100\% \quad (1)$$

where E_{DPPH} and E_{ex} – the optical density of the DPPH solution and the antioxidant solution, respectively.

The antimicrobial properties of the extracts were determined in relation to the opportunistic and pathogenic test strains on a solid nutrient medium (diffusion method) and in a liquid nutrient medium. The test strains involved *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Proteus vulgaris* ATCC 63, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* EMTC (Russian Collection of Extremophilic Microorganisms and Type Cultures) 34, *Leuconostoc mesenteroides* EMTC 1865, *Shigella flexneri* ATCC 12022, and *Shigella sonnei* ATCC 25931.

The diffusion method determined the antimicrobial activity of the extracts according to the following pattern. The test strain was inoculated on beef-extract agar using the spread plate technique. A paper disc with the nutrient medium served as control, and a disc with an antibiotic ciprofloxacin served as reference. The Petri dishes were incubated at 35–37°C for 24 h. The results depended on the presence and size (mm) of the microorganism-free transparent zone around the disc. The diameter of the inhibition zones was measured with an accuracy of 1 mm with a vernier caliper.

The second method involved incubating the test strains in 96-well culture plates. Overnight broth cultures were re-suspended in a Mueller Hinton plate (*C. albicans* – in Sabouraud's medium) until the number of microorganisms reached the seed dose of ~10^5 CFU/mL. The cell suspension and the extracts were simultaneously introduced into the wells in an amount of 1/10 of the total volume. MRS medium served as control, ciprofloxacin (10 μg/mL) – as reference; the total suspension volume in each well was 200 μL, the test was performed in duplicate. The wells were incubated at 35°C in a shaker (580 rpm). After 24 h, the optical density was measured using a multi-reader at 595 nm. Bactericidal activity was assessed by the change in the optical density in comparison with the control. In the wells where cell growth stopped or slowed down, the optical density was lower than in the wells with normal microbial growth. Ciprofloxacin served as reference because it is known as a standard for this group of antibacterial medications. It is also effective against Gram-negative microorganisms and staphylococci, including some strains that are resistant to other antibiotics.

Statistical data were processed using Microsoft Office Excel 2007 and the paired Student's t-test. Differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

The resulting callus, cell suspension, and hairy root cultures of spiny eleuterococcus (*Eleutherococcus senticosus* L.), Asian bell (*Codonopsis pilosula* L.), butterfly orchid (*Platanthera bifolia* L.), and siler (*Saposhnikovia divaricate* L.) were dried *in vitro* and extracted with ethyl alcohol (see Table 1 for extraction parameters).

Tables 2–4 demonstrate the content of secondary metabolites in the callus, suspension, and root extracts.

The chromatographic tests showed that the biomass of callus, suspension, and root cultures of *E. senticosus*, *C. pilosula*, *P. bifolia*, and *S. divaricata* accumulated such secondary metabolites as phenolic acids, flavistonoids, ecdysanthonoids, and ecdysanthonoids.

Table 2 shows that the callus extracts proved rich in flavonoglycosides, apigenin, and rutin. Codonopsin, cardiofolin, and coleofolide were less abundant. The

Table 2 Content of secondary metabolites in callus extracts

Secondary metabolite	Content of secondary metabolites, mg/kg			
	E. senticosus	*C. pilosula*	*P. bifolia*	*S. divaricata*
Caffeic acid	2.430 ± 0.120	0.400 ± 0.020	0.800 ± 0.040	4.200 ± 0.210
Rutin	3.080 ± 0.150	3.440 ± 0.170	3.550 ± 0.180	0.650 ± 0.030
Total ecdysteroids	0.030 ± 0.002	0.100 ± 0.010	0.070 ± 0.010	0.470 ± 0.020
Mangiferin	0.180 ± 0.010	0.360 ± 0.020	7.370 ± 0.370	0.140 ± 0.010
Quercetin	1.170 ± 0.060	0.650 ± 0.030	0.120 ± 0.010	0.780 ± 0.040
Total flavonoglycosides	8.070 ± 0.400	7.560 ± 0.380	6.680 ± 0.330	4.310 ± 0.220
Apigenin	2.740 ± 0.140	4.710 ± 0.240	5.230 ± 0.260	4.210 ± 0.210
Codonopsin	0.760 ± 0.040	0.390 ± 0.020	0.440 ± 0.020	0.510 ± 0.030
Cardiofolin	0.950 ± 0.050	0.810 ± 0.040	0.350 ± 0.020	0.540 ± 0.030
Coleofolide	0.810 ± 0.040	0.470 ± 0.020	0.210 ± 0.010	3.910 ± 0.200

Table 3 Content of secondary metabolites in suspension extracts

Secondary metabolite	Content of secondary metabolites, mg/kg			
	E. senticosus	*C. pilosula*	*P. bifolia*	*S. divaricata*
Caffeic acid	2.43 ± 0.12	0.41 ± 0.02	0.82 ± 0.04	4.29 ± 0.21
Rutin	3.24 ± 0.16	3.21 ± 0.16	3.31 ± 0.17	0.34 ± 0.02
Total ecdysteroids	0.13 ± 0.01	0.13 ± 0.01	0.77 ± 0.04	0.67 ± 0.03
Mangiferin	0.38 ± 0.02	0.96 ± 0.05	8.71 ± 0.44	0.44 ± 0.02
Quercetin	1.18 ± 0.06	0.41 ± 0.02	0.27 ± 0.01	0.17 ± 0.01
Total flavonoglycosides	8.27 ± 0.41	6.21 ± 0.31	1.48 ± 0.07	2.37 ± 0.12
Apigenin	1.74 ± 0.09	1.71 ± 0.09	1.23 ± 0.06	1.12 ± 0.06
Codonopsin	0.76 ± 0.04	4.39 ± 0.22	2.44 ± 0.12	5.51 ± 0.28
Cardiofolin	0.35 ± 0.02	0.31 ± 0.02	0.95 ± 0.05	0.21 ± 0.01
Coleofolide	4.81 ± 0.24	5.47 ± 0.27	6.21 ± 0.31	3.91 ± 0.20

Table 4 Content of secondary metabolites in root extracts

Secondary metabolite	Content of secondary metabolites, mg/kg			
	E. senticosus	*C. pilosula*	*P. bifolia*	*S. divaricata*
Caffeic acid	9.43 ± 0.47	8.40 ± 0.42	8.80 ± 0.44	7.20 ± 0.36
Rutin	63.08 ± 3.15	13.44 ± 0.67	53.55 ± 2.68	30.65 ± 1.53
Total ecdysteroids	6.03 ± 0.30	7.10 ± 0.36	6.07 ± 0.30	5.47 ± 0.27
Mangiferin	7.18 ± 0.36	8.36 ± 0.42	3.37 ± 0.17	5.14 ± 0.26
Quercetin	12.17 ± 0.61	14.65 ± 0.73	17.12 ± 0.86	12.78 ± 0.64
Total flavonoglycosides	2.07 ± 0.10	5.56 ± 0.28	6.68 ± 0.33	6.31 ± 0.32
Apigenin	32.74 ± 1.64	14.71 ± 0.74	75.23 ± 3.76	12.12 ± 0.61
Codonopsin	1.76 ± 0.09	0.59 ± 0.03	0.54 ± 0.03	0.31 ± 0.02
Cardiofolin	3.95 ± 0.20	11.81 ± 0.59	10.35 ± 0.52	7.61 ± 0.38
Coleofolide	17.81 ± 0.89	20.47 ± 1.02	40.21 ± 2.01	63.91 ± 3.20

callus extract of *P. bifolia* had the maximal amount of mangiferin. The extracts of *S. divaricata* and *E. senticosus* demonstrated the biggest amount of caffeic acid, while *E. senticosus* had the highest content of quercetin.

According to Table 3, flavonoglycosides appeared to be the predominant secondary metabolites in the suspension extracts. However, their content was much lower in the suspension extracts of *C. pilosula*, *P. bifolia*, and *S. divaricata* by 17.8, 77.8, and 45.0%, respectively, in comparison with callus extracts. The suspension extract of *P. bifolia* had the highest content of mangiferin: its content increased by 18.2% in comparison with the callus extract. The contents of caffeic acid, rutin, total ecdysteroids, and quercetin followed the same pattern as in the callus extracts. As for apigenin, its content in the suspension extracts of *E. senticosus*, *C. pilosula*, *P. bifolia*, and *S. divaricata* decreased by 36.5, 63.7, 76.5, and 73.4%, respectively. The content of codonopsin in the suspension extracts of *C. pilosula*, *P. bifolia*, and *S. divaricata* increased by 10.2, 4.5, and 9.8 times, respectively. The suspension extracts of *E. senticosus*, *C. pilosula*, and *P. bifolia* demonstrated a higher biosynthesis of coleofolide in comparison with callus extracts.

Table 4 shows that the content of caffeic acid, rutin, ecdysteroids, quercetin, apigenin, cardiofolin, and coleofolide in the root extracts was higher than in callus and suspension extracts. Rutin, apigenin, coleofolide, and quercetin were found to be the dominant biologically active substances in the root cultures.

Secondary metabolites of medicinal plants often demonstrate various types of biological activity, e.g. antimicrobial or antioxidant. Experiments *in vitro* proved that caffeic acid possesses antimicrobial, antimycotic, and immunomodulatory properties, as well as the ability to absorb free radicals [22]. Other studies [23, 24] also revealed its antibacterial properties.

Rutin is known for its antioxidant properties, which were found superior to those of vitamins C and E [25–27]. The antioxidant action of this flavanoid can be explained by its ability to activate antioxidant enzymes [25]. Quercetin is one of the most powerful antioxidative polyphenols [28, 29]. It also possesses anti-inflammatory, antimicrobial, anticarcinogenic, and antiviral properties [30]. Mangiferin is also known worldwide for its experimentally confirmed antioxidant, radioprotective, and immunomodulatory properties [31].

The obtained results made it possible to study the antioxidant activity of the callus, suspension, and root extracts (Fig. 1).

Figure 1 shows that all the samples exhibited antioxidant properties. The root extracts demonstrated the maximal antioxidant activity. The antioxidant activity of the extracts obtained from the biomass of hairy roots was 4.2–10.1 times (depending on the

species) higher than in callus extracts and 4.0–4.9 times higher than in suspension extracts. The root extract of *E. senticosus* had the best antioxidant properties. The revealed pattern is consistent with that for phenolic biologically active substances, where the root extracts also demonstrated the greatest accumulation (Tables 2–4).

Table 5 shows the antimicrobial activity by the diffusion method, while Figs. 2–4 show the results of the optical density method.

According to Table 5, all the extracts possessed antimicrobial activity against the tested strains. The best antimicrobial properties belonged to root extracts. The diameter of the lysis zone was 18.0–23.0 mm: in the callus and suspension extracts, this value did not exceed 17.5 mm. These results correlate with the results obtained for the antimicrobial properties of extracts in a liquid nutrient medium (Figs. 2–4).

CONCLUSION

The present research featured callus, cell suspension, and hairy root cultures of spiny eleuterococcus (*Eleutherococcus senticosus* L.), Asian bell (*Codonopsis pilosula* L.), butterfly orchid (*Platanthera bifolia* L.), and siler (*Saposhnikovia divaricate* L.). The TLC and HPLH tests showed a high content of secondary metabolites belonging to phenolic acids, flavonoids, ecdysteroids, and xanthones.

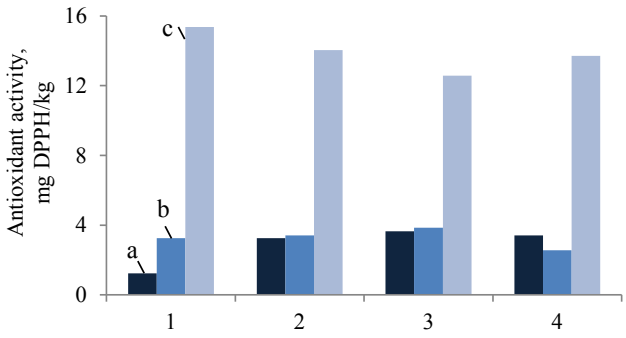

Figure 1 Antioxidant activity of callus (a), suspension (b), and root (c) extracts obtained from *Eleutherococcus senticosus* (1), *Codonopsis pilosula* (2), *Platanthera bifolia* (3), and *Saposhnikovia divaricate* (4)

For the callus extracts, the list of prevailing biologically active substances included flavonoglycosides apigenin, and rutin. Their total content depended on the plant species and varied from 4.31 to 8.07 mg/g for flavonoglycosides, from 2.74 to 5.23 mg/kg for apigenin, and from 3.08 to 3.55 mg/kg for rutin. The callus extract of *P. bifolia* appeared to have the highest content of mangiferin (7.37 mg/kg).

In case of all suspension extracts, flavonoglycosides dominated. The suspension extract of *P. bifolia* had the highest content of mangiferin: the concentration

Table 5 Antimicrobial activity of callus, suspension, and root extracts of *Eleutherococcus senticosus*, *Codonopsis pilosula*, *Platanthera bifolia*, and *Saposhnikovia divaricate* (diffusion method)

Test strain	Diameter of inhibition zone of test strains, mm, for callus, suspension, and root extracts					
	E. senticosus	*C. pilosula*	*P. bifolia*	*S. divaricata*	Control	Ciprofloxacin
E. coli ATCC 25922	15.0 ± 0.8	13.0 ± 0.7	14.0 ± 0.7	12.0 ± 0.6	0	23.0 ± 1.2
	13.0 ± 0.7	15.0 ± 0.8	17.0 ± 0.9	14.0 ± 0.7		
	22.0 ± 1.1*	23.0 ± 1.2	22.0 ± 1.1	20.0 ± 1.0		
S. aureus ATCC 25923	13.0 ± 0.7	12.0 ± 0.6	10.0 ± 1.0	13.0 ± 0.7	0	21.0 ± 1.1
	15.0 ± 0.8	13.0 ± 0.7	13.0 ± 0.7	12.0 ± 0.6		
	19.0 ± 1.0	19.0 ± 1.0	18.0 ± 0.9	20.0 ± 1.0		
P. vulgaris ATCC 63	12.0 ± 0.6	12.0 ± 0.6	16.0 ± 0.8	12.0 ± 0.6	0	22.0 ± 1.1
	11.0 ± 0.6	12.0 ± 0.6	13.0 ± 0.7	14.0 ± 0.7		
	20.0 ± 1.0	21.0 ± 1.1	21.0 ± 1.1	19.0 ± 1.0		
P. aeruginosa ATCC 9027	17.0 ± 0.9	11.0 ± 0.6	15.0 ± 0.8	12.0 ± 0.6	0	24.0 ± 1.2
	17.0 ± 0.9	15.0 ± 0.8	14.0 ± 0.7	14.0 ± 0.7		
	21.0 ± 1.1	23.0 ± 1.2	22.0 ± 1.1	19.0 ± 1.0		
C. albicans EMTC 34	15.0 ± 0.8	11.0 ± 0.6	12.0 ± 0.6	14.0 ± 0.7	0	23.0 ± 1.2
	12.0 ± 0.6	14.0 ± 0.7	11.0 ± 0.6	14.0 ± 0.7		
	21.0 ± 1.1	21.0 ± 1.1	20.0 ± 1.0	21.0 ± 1.1		
L. mesenteroides EMTC 1865	15.0 ± 0.8	14.0 ± 0.7	13.0 ± 0.7	12.0 ± 0.6	0	23.0 ± 1.2
	14.0 ± 0.7	16.0 ± 0.8	15.0 ± 0.8	16.0 ± 0.8		
	22.0 ± 1.1	20.0 ± 1.0	21.0 ± 1.1	22.0 ± 1.1		
Sh. flexneri ATCC12022	16.0 ± 0.8	15.0 ± 0.8	15.0 ± 0.8	12.0 ± 0.6	0	22.0 ± 1.1
	16.0 ± 0.8	17.5 ± 0.9	16.0 ± 0.8	16.0 ± 0.8		
	20.0 ± 1.0	21.5 ± 1.1	21.0 ± 1.1	22.0 ± 1.1		
Sh. sonnei ATCC 25931	15.5 ± 0.8	15.0 ± 0.8	12.0 ± 0.6	14.0 ± 0.7	0	20.0 ± 1.0
	17.5 ± 0.9	17.0 ± 0.9	14.5 ± 0.8	16.5 ± 0.9		
	20.0 ± 1.0	18.0 ± 0.9	19.5 ± 1.0	19.0 ± 1.0		

*line 1 – callus cultures, line 2 – suspension cultures, line 3 – root cultures

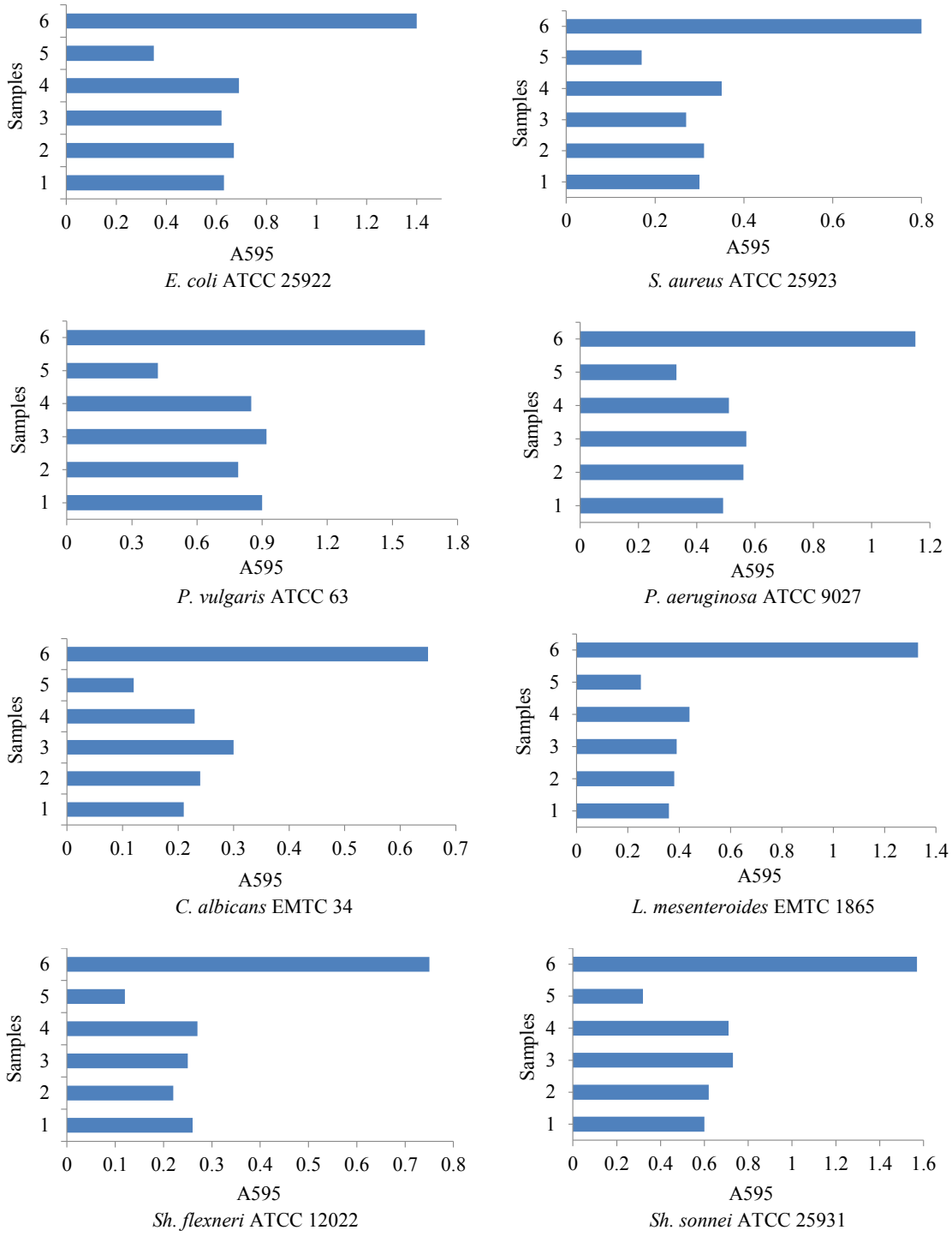

Figure 2 Antimicrobial activity of callus extracts (optical density method): 1 – *Eleutherococcus senticosus*; 2 – *Codonopsis pilosula*; 3 – *Platanthera bifolia*; 4 – *Saposhnikovia divaricata*; 5 – ciprofloxacin; 6 – control

of this xanthone glycoside increased by 18.2% in comparison with the callus extract. All the suspension extracts demonstrated the same pattern as the callus ones in relation to caffeic acid, rutin, ecdysteroids, and quercetine. The content of apigenin was lower than in the callus extracts, while that of codonopsin increased. The suspension extracts of *E. senticosus*, *C. pilosula*, and *P. bifolia* also demonstrated a higher biosynthesis of coleofolide than the callus extracts.

All the root extracts had an even higher content of caffeic acid, rutin, ecdysteroids, quercetine, apigenin, cardiofolin, and coleofolide. The list of prevailing biologically active substances included rutin (13.44–63.08 mg/kg), apigenin (12.12–75.23 mg/kg), coleofolide (17.81–63.91 mg/kg), and quercetin (12.17–17.12 mg/kg).

The experiments *in vitro* revealed antioxidant activity in all the samples. The maximal antioxidant activity belonged to the hairy root extracts.

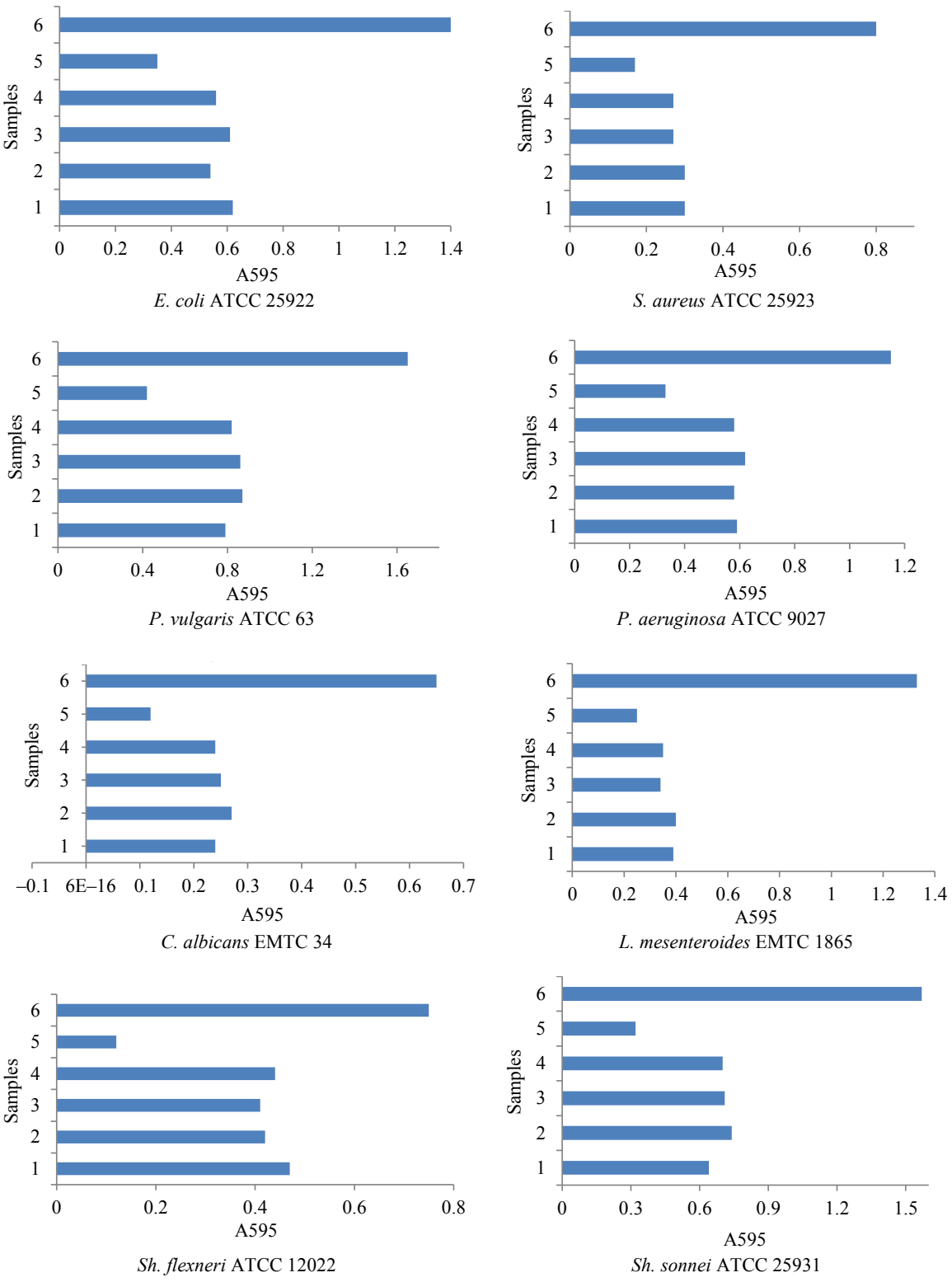

Figure 3 Antimicrobial activity of suspension extracts (optical density method): 1 – *Eleutherococcus senticosus*; 2 – *Codonopsis pilosula*; 3 – *Platanthera bifolia*; 4 – *Saposhnikovia divaricata*; 5 – ciprofloxacin; 6 – control

All the samples demonstrated antimicrobial activity against test strains of *Escherichia coli*, *Staphylococcus aureus*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Candida albicans*, *Leuconostoc mesenteroides*, *Shigella flexneri*, and *Shigella sonnei*. The root extracts demonstrated the maximal antimicrobial properties.

Further research could cover such issues as isolation of individual phenolic substances from extracts of medicinal plants *in vitro*. This raw material can serve

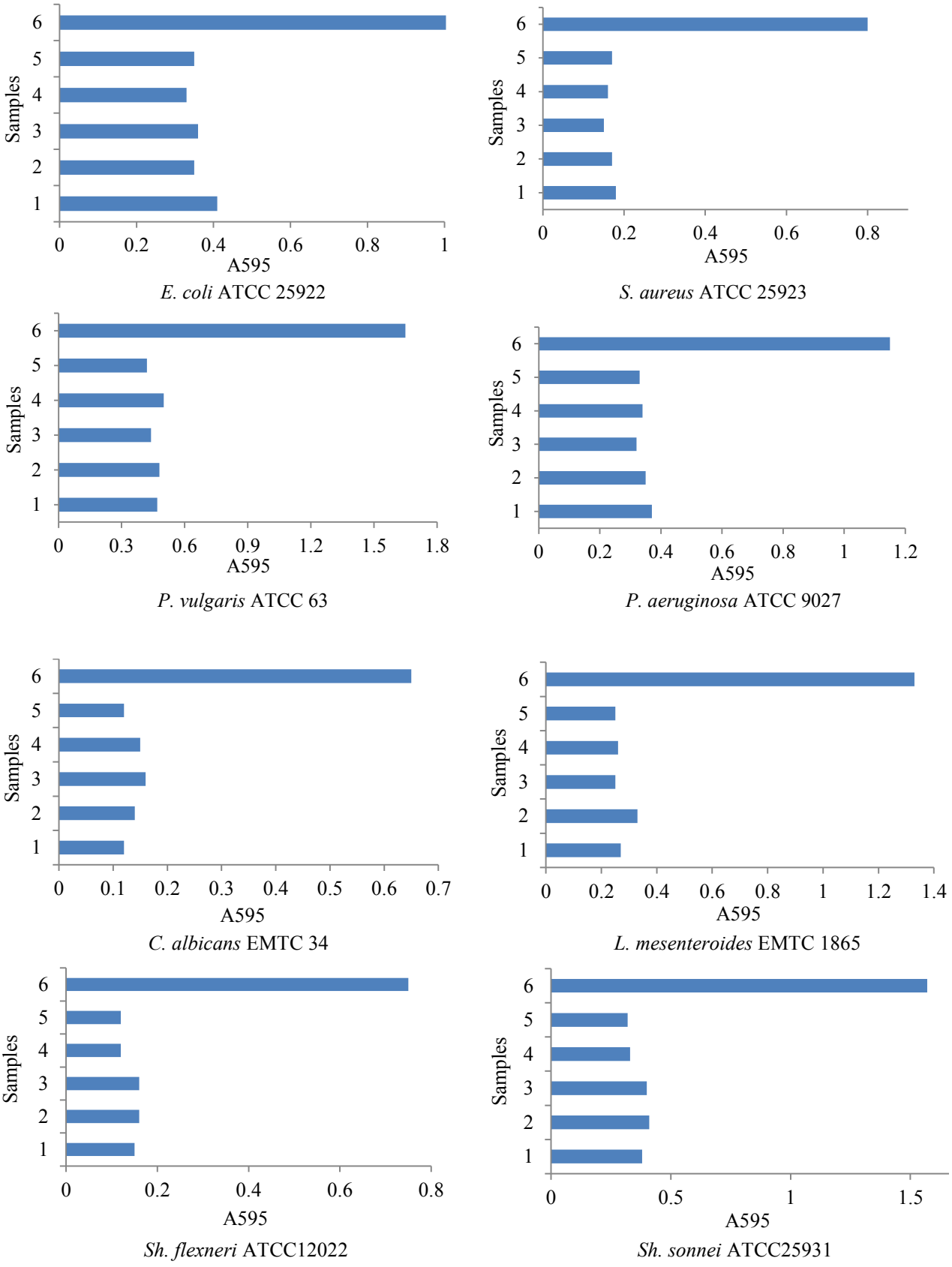

Figure 4 Antimicrobial activity of root extracts (optical density method): 1 – *Eleutherococcus senticosus*; 2 – *Codonopsis pilosula*; 3 – *Platanthera bifolia*; 4 – *Saposhnikovia divaricata*; 5 – ciprofloxacin; 6 – control

as basis for medications and biologically active food supplements for the prevention and treatment of infectious diseases or conditions linked to oxidative stress.

CONTRIBUTION

I.S. Milentyeva prepared the test samples and described the content, antioxidant activity, and antimicrobial properties of the secondary metabolites. V.M. Le studied the content of secondary metabolites in the callus extracts. O.V. Kozlova wrote the introduction. N.S. Velichkovich studied the antioxidant properties of callus, suspension, and root extracts. A.M. Fedorova researched the antimicrobial properties of the root extracts in liquid growth medium. A.I. Loseva described the research results.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of the present article.

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Egyptian kishk as a fortificant: Impact on the quality of biscuit

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Abstract:
Introduction. Biscuit is a mass-consumption product worldwide. As biscuit contains low amount of protein, it can be fortified with protein-containing raw materials. In the present work, we fortified biscuit with kishk, an Egyptian dairy product, and evaluated changes in its physicochemical properties and volatile compounds.
Study objects and methods. We analyzed biscuit from wheat flour (control sample) and biscuit form wheat flour with 10, 20, 30, 40, and 50% of kishk (experimental samples). The experiments were carried out by using standard methods.
Results and discussion. The fortified biscuit samples showed higher lightness (L^*) values than control. The antioxidant activity in the biscuit increased with the increasing amount of kishk. Aldehydes were the main volatile compounds in all the biscuit samples, followed by sulfur-containing compounds and alcohols. The predominant aldehyde was benzldehyde. However, kishk in amounts more than 20% affected adversely the aroma, taste and texture, as well as volatile compounds of the biscuits. Both the control and fortified with kishk biscuits contained an increased amount of total phenolic compounds. Based on sensory evaluation and volatile analysis, the most acceptable amount of kishk for fortification of biscuit was 10%.
Conclusion. Fortification of biscuit with Egyptian kishk enhanced its protein, fat and fiber, as well as antioxidant activity at all levels of fortification with no significant effect on appearance and color. Further studies are needed to evaluate storage conditions and shelf life of biscuits with kishk.

Keywords: Kishk, biscuit, flour, hardness, antioxidant properties, volatile compounds, physicochemical properties

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INTRODUCTION

Today, nearly all levels of society consume biscuit as snacks or weaning for infants. It has pleasant aroma, various taste, low cost, and high stability during storage at ambient temperature [1]. Recently, the consumption of biscuit has increased, which encourages manufacturers to develop new formulations of biscuit with non-traditional raw materials to enrich the flavor of biscuit [2]. In addition, the increasing demand for functional and nutraceutical food products makes it possible to increase consumption of bakery products such as biscuit by fortifying it with protein, fiber and other essential constituents from various sources.

The fortification process provides several advantages, namely it can predict heart, coronary and diabetic diseases, as well as improve body functions. Generally, the main ingredients used in biscuit production include wheat flour, fat, sugar, eggs, emulsifiers, salt, etc. The chemical composition of these ingredients lacks for vitamins and protein (8–10%), which reduces the nutritional properties of bakery products [3–5]. Therefore, manufacturers have made efforts to replace the main ingredients of biscuit with more nutritional and healthy ones.

Egyptian kishk is characterized by high nutritive value and good stability during all year seasons. The chemical composition of Egyptian kishk includes a high content of essential fatty acids, especially oleic and linoleic, as well as a high concentration of essential amino acids, especially lysine, tyrosine, and proline [6]. The main ingredients of kishk are parboiled wheat and milk, which make kishk an available and rich source for vitamins, minerals and fiber [7, 8].

We found no research on improvement of the nutritional properties and volatile content of snacks like biscuit by replacing wheat flour with Egyptian kishk. Therefore, the current study aimed to reveal effects of various proportions of Egyptian kishk on the nutritional

Table 1 Formulation of biscuit fortified with Egyptian kishk

Ingredients	Control	Kishk 10%	Kishk 20%	Kishk 30%	Kishk 40%	Kishk 50%
Wheat flour, g	100.00	90.00	80.00	70.00	60.00	50.00
Kishk, g	–	10.00	20.00	30.00	40.00	50.00
Shortening, g	15.00	15.00	15.00	15.00	15.00	15.00
Baking powder, g	1.00	1.00	1.00	1.00	1.00	1.00
Salt, g	2.85	0.57	1.14	1.71	2.28	2.85
Milk, mL	20.00	20.00	20.00	20.00	20.00	20.00

Salt percentage in kishk = 5.7 ± 0.82%

composition, sensory characteristics, antioxidant activity, and volatile content of biscuit.

STUDY OBJECTS AND METHODS

The objectives of the study were biscuit without kishk (control samples) and biscuit with 10–50% of kishk (experimental samples). Kishk, wheat flour (72% extraction), sugar, shortening, and baking powder were purchased from the local market (Dokki, Egypt). Folin-Ciocalteu's reagent, ABST and DPPH were purchased from Sigma-Aldrich Canada (Ontario, Canada). All chemicals were of analytical grade.

The biscuit formulation was developed at the National Research Center (Dokki, Egypt) as described by Hussein *et al.* [9]. Control and experimental formulations are shown in Table 1.

Proximate composition. The biscuit samples and Egyptian kishk were analyzed for moisture, ash, crude fat, and crude protein contents using Association of Official Analytical Chemist' Approved Methods 925.10, 920.87, 920.85, 923.03, and 963.09, respectively [10]. The analyses were conducted in triplicate.

Rheological properties. Rheological properties of dough samples with different amounts of kashk were evaluated using a farinograph according to [11].

Physical characteristics of fortified biscuits. The volume and weight of three biscuit samples of each treatment were recorded. Specific volume was calculated by dividing of the volume to weight. The diameter, height and spread ratio (diameter/height) of the biscuits were determined according to the method described in [11]. The thickness and diameter of the biscuits were directly measured using STECO (Germany) as mentioned by Mc-Watters *et al.* [12]. The spread ratio was calculated as quotient of diameter and thickness. The biscuits were weighed (Precisa 105 A, Swiss) and the weight was expressed in grams. A total of ten biscuits were used for the measurement.

Color parameters. Surface color characteristics of biscuit (intact) were measured in terms of Hunter color parameters, namely L^*, a^*, and b^* values as mentioned by Dauda *et al.* For this, we used a spectrocolorimeter (Tristimulus Color Machine) with the CIE Lab color scale (Hunter, Lab Scan XE-Reston VA, USA) in the reflection mode [13]. The instrument was standardized with the white tile of Hunter Lab Color Standard (LX No. 16379): X = 72.26, Y = 81.94, and Z = 88.14 (L^* = 92.46; a^* = –0.86; b^* = –0.16). Color difference (ΔE) was calculated from a^*, b^* and L^* parameters using the Hunter-Scotfield's equation: $\Delta E = (\Delta a^2 + \Delta b^2 + \Delta L^2)^{0.5}$. A total of ten biscuits were involved in the experiment.

Texture analysis. The texture of the baked biscuit samples was determined by a texturometer (Brookfield, CT3-10 kg, USA) equipped with a cylinder probe (TA-AACC36). Texture profile analysis was conducted to determine hardness. The analyzer was set to perform two cycle measurements to give a two bite texture profile curve. Trigger load and test speed were 9.00 N and 2.5 mm/sec, respectively.

Sensory evaluation. The sensory properties were evaluated using the 9-point Hedonic scale where 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 [14]. The analysis was carried out by 10 trained panelists for four sensory descriptors, namely appearance, color, odor, taste, and texture.

Total phenolic and antioxidant activity. Total phenolic content. The biscuit and Egyptian kishk samples were finely powdered and extracted with petroleum ether (40–60°C) to remove fats and resinous materials. The residues were thoroughly separately extracted with 500 mL of 70% methanol. The extract was filtrated through Whatman No. 1 filter papers and the filtrates were evaporated to dryness under reduced pressure on a rotary evaporator at 40°C.

Total phenols were evaluated using the Folin-Ciocalteu method described in [15]. The results were expressed as milligram gallic equivalents (GAE) per gram of biscuit.

Antioxidant activity. The methanol extract (25, 50, and 75 uL) was used for antioxidant activity determination. DPPH-radical scavenging activity and ABST assay were determined as described by [16, 17], respectively.

Volatile compound analysis. Extraction of volatile compounds. Volatile compounds of the biscuits under study were isolated by headspace solid phase microextraction (HS-SPME) sampling and analyzed by gas chromatography coupled with mass spectrometry (GC/MS) [18]. Ten grams of grinded biscuits were put

into a 20-mL headspace vial fitted with a Teflon-lined septum. Volatiles were sampled for 30 min at 50°C from the headspace of the vial using DVB/CAR/PDMS fiber (Divinylbenzene/Carboxene/Polydimethylsiloxane, Supelco, Bellefonte, Pa., U.S.A.). The fiber was then immediately inserted into the injection port of the gas chromatograph for 5 min at 260°C.

Chromatographic conditions. A Hewlett-Packard HP5890II gas chromatograph coupled to a 5971 MSD quadrupole mass spectrometer was used. Chromatograph was equipped with a MDN-5 (60 m×0.25 mm×0.25 μm, Supelco) column. Operating conditions for GC/MS were the following: helium flow 0.6 mL/min, initial oven temperature 40°C (3 min), then 8°C/min to 200°C, and 20°C/min to 280°C (3 min).

Identification of volatile compounds. Volatile compounds were identified by comparison of their retention indices and mass spectra with standards, or, in some cases tentatively only by NBS 75K mass spectra library search and retention indices. Retention indices were calculated for each compound using homologous series of C_6–C_{22} *n*-alkanes [19].

Statistical analysis. Statistical analysis was conducted using SPSS software version 16 (SPSS Inc., Chicago, USA). All analyses were performed in triplicate and data reported as means ± standard deviation (SD). Data were subjected to analysis of variance (ANOVA). The confidence limits used in this study were based on 95% ($P < 0.05$) [20].

RESULTS AND DISCUSSION

Chemical composition of biscuit. Table 2 demonstrates the proximate chemical composition of biscuits without Egyptian kishk (control sample) and biscuits with 10–50% of Egyptian kishk (experimental samples). An increased amount of kishk from 10 to 50% resulted in an increased content of protein, fat, ash as well as fiber. The 50% kishk biscuit had the highest content of protein (9.38%) compared to the control sample (8.29%). A similar trend was found by Eneche, who revealed that biscuit fortified with pigeon pea flour showed an increase in protein content [21].

Values are the mean ± SD of three replicates. Mean followed by different letters in the same column differs significantly ($P \leq 0.05$)

In general, the increase in fat, fiber and ash of kishk supplemented biscuit can be explained by the high content of those ingredients in kishk. Similar findings were also obtained by Nazni *et al*. and Pastuszka *et al*. [22, 23]. On the other hand, total carbohydrates decreased with an increase in the amount of Egyptian kishk (Table 2).

In the current investigation, we also determined the total phenolic content of methanolic extract in kishk, as well as in the control and experimental biscuits. The obtained data were expressed as mg GAE/g. The results showed that the biscuits with kishk had increased total phenolic content (9.56–12.04 mg GAE/g) compared to kishk and the control biscuit (1.57 and 8.23 mg GAE/g, respectively). The highest phenolic content was revealed in the 50% kishk biscuits (12.04 mg GAE/g).

Rheological properties. Table 3 shows the rheological properties of wheat flour (control) and its mixtures with Egyptian kishk in different amounts (10, 20, 30, 40, and 50%). The water absorption, arrival time, dough development time (DDT) and weakening of dough increased with an amount of kishk. That may be due to high fiber and protein amounts containing in kishk, while fibers in wheat flour tend to bind more water**.** On the other hand, interaction of kishk fibers with wheat flour constituents and the added water may cause a decreased stability of dough.

In this context, Kim *et al*. reported that an increased proportion of rice grain dietary fibers in the recipe of bread increased the water absorption of dough [24]. Other researchers reported that an increasing amount of barley flour caused a progressive increase in water absorption, arrival time, and dough stability, with addition of gelatinized corn flour to wheat flour [24–26]. Whereas in our work, dough stability decreased with increasing Egyptian kishk content (Table 3).

Physical characteristics of biscuit. The studied physical characteristics (weight, volume, specific volume, diameter, thickness, and spread ratio) exhibited a significant effect ($P < 0.05$) of Egyptian kishk on the quality of biscuits (Table 4).

The weight and volume of the experimental biscuits were significantly lower than those of the control sample ($P < 0.05$). This effect may be due to higher

Table 2 Nutritional composition of Egyptian kishk and biscuit fortified with kishk

Samples	Moisture, %	Protein, %	Fat, %	Ash, %	Fiber, %	Carbohydrates, %	Total phenols, mg GAE/100 g
Kishk	8.41 ± 0.01	12.10 ± 0.24	3.92 ± 0.02	8.39 ± 0.20	2.59 ± 0.09	72.13 ± 0.51^a	1.57 ± 0.12
Control	4.31 ± 0.06^a	8.29 ± 0.04^a	17.25 ± 0.01	2.58 ± 0.07	0.53 ± 0.01	71.35 ± 0.07^a	8.23 ± 0.18
Kishk 10%	4.16 ± 0.06^b	8.56 ± 0.05^a	17.63 ± 0.06^a	3.05 ± 0.08^a	1.19 ± 0.03	69.56 ± 0.08	9.56 ± 0.32
Kishk 20%	4.19 ± 0.11^b	8.79 ± 0.09^b	17.90 ± 0.11^a	3.64 ± 0.05^a	1.84 ± 0.05^a	67.83 ± 0.09^b	10.35 ± 0.26^a
Kishk 30%	4.49 ± 0.31	8.92 ± 0.03^b	18.19 ± 0.17	4.31 ± 0.06^b	1.93 ± 0.02^a	66.65 ± 0.12^b	10.82 ± 0.15^a
Kishk 40%	4.58 ± 0.37	9.15 ± 0.07	18.42 ± 0.17^b	4.89 ± 0.10^b	2.08 ± 0.13	65.46 ± 0.18^c	11.75 ± 0.16^b
Kishk 50%	4.36 ± 0.22^a	9.38 ± 0.16	18.53 ± 0.09^b	5.31 ± 0.26	2.19 ± 0.02	64.59 ± 0.15^c	12.04 ± 0.25^b

Table 3 Rheological properties of dough with and without kishk

Samples	Water absorption, %	Arrival time, min	Dough stability, min	DDT, min	Dough weakening (B.U)
Control	58.00	1.50	7.50	3.00	70
Kishk 10%	59.50	1.50	4.50	2.00	100
Kishk 20%	61.00	1.50	2.50	3.50	115
Kishk 30%	62.00	1.50	3.50	2.00	120
Kishk 40%	63.50	2.00	2.00	3.50	170
Kishk 50%	65.00	2.00	1.50	2.50	185

Table 4 Physical characteristics of biscuit fortified with Egyptian kishk

Sample	Weight, g	Volume, cm^3	Specific volume, $cm^3 \times 100\ g^{-1}$	Diameter, cm	Thickness, cm	Spread ratio (W/T)
Control	7.23 ± 0.41^a	3.50 ± 0.08^a	0.48 ± 0.05^b	4.50 ± 0.12^a	0.33 ± 0.02^a	13.63 ± 0.19^a
Kishk 10%	7.26 ± 0.29^a	3.50 ± 0.11^a	0.48 ± 0.03^b	4.50 ± 0.09^a	0.33 ± 0.06a	13.63 ± 0.14^a
Kishk 20%	6.32 ± 0.31^c	3.50 ± 0.13^a	0.55 ± 0.09^a	4.53 ± 0.14^a	0.30 ± 0.07^b	15.11 ± 0.13^a
Kishk 30%	6.84 ± 0.15^b	3.36 ± 0.07^a	0.49 ± 0.02^a	4.50 ± 0.06^a	0.33 ± 0.05^a	13.63 ± 0.15^a
Kishk 40%	6.10 ± 0.17^c	3.33 ± 0.05^b	0.55 ± 0.06^a	4.07 ± 0.19^b	0.30 ± 0.09^b	13.53 ± 0.17^a
Kishk 50%	6.98 ± 0.62^b	3.37 ± 0.14^b	0.48 ± 0.07^b	4.03 ± 0.07^b	0.30 ± 0.04^b	13.43 ± 0.16^a

Values are the mean ± SD of three replicates. Mean followed by different letters in the same column differs significantly ($P \leq 0.05$)

Table 5 Effect of Egyptian kishk on hardness and color determinants of biscuits

Sample	Surface				Back				Hardness, g force
	*L**	*a**	*b**	Δ*E*	*L**	*a**	*b**	Δ*E*	
Control	60.16 ± 0.58^a	8.48 ± 0.62^a	32.44 ± 0.57^a	–	56.72 ± 0.99	11.25 ± 1.09^a	33.77 ± 0.65^a	–	39.28 ± 1.32^a
Kishk 10%	64.39 ± 0.76^b	8.71 ± 0.46^a	31.85 ± 0.59^a	4.29 ± 0.17	65.75 ± 3.05	9.27 ± 1.84^b	34.67 ± 1.32^b	9.36 ± 2.14	27.43 ± 5.22
Kishk 20%	63.53 ± 0.27	9.42 ± 0.92^b	30.93 ± 0.68^b	3.82 ± 0.35^a	60.29 ± 2.68^a	11.48 ± 1.41^a	33.70 ± 1.10^a	3.02 ± 0.50^a	34.24 ± 5.70^b
Kishk 30%	60.80 ± 0.95^a	9.05 ± 0.40^b	30.78 ± 0.45^b	2.05 ± 0.20^b	61.17 ± 1.56^a	10.24 ± 0.57	33.13 ± 0.87^b	4.74 ± 0.43^a	34.81 ± 7.38^b
Kishk 40%	61.51 ± 0.23^c	7.73 ± 0.23^c	29.92 ± 0.27^c	2.99 ± 0.38^b	62.55 ± 0.58^b	8.05 ± 1.24^b	31.01 ± 1.42^c	6.14 ± 2.93^b	40.32 ± 3.75^a
Kishk 50%	61.65 ± 0.34^c	7.79 ± 0.09^c	29.60 ± 0.39^c	3.32 ± 1.09^a	61.41 ± 0.47^b	7.96 ± 0.29	30.54 ± 0.06^c	6.58 ± 1.24^b	47.76 ± 6.82

Values are the mean ± SD of three replicates. Mean followed by different letters in the same column differs significantly ($P \leq 0.05$)

fiber and protein contents in kishk. Moreover, kishk also affected the diameter, thickness, and spread ratio of the biscuit. The results obtained by Brito *et al*. [27] showed a decrease of volume in cookies with higher amount of quinoa flour compared to that in cookies from corn starch.

In our work, due to the decrease in diameter in biscuit with Egyptian kishk, its surface also decreased compared to control. As we can see from Table 4, high amounts of Egyptian kishk showed a remarkable correlation with the decrease in physical properties of the biscuits. In other study carried out by Mofasser *et al*., the authors found an increase in diameter of gluten-free biscuit compared to traditional biscuit [28].

Color values and hardness evaluation. The main criteria for the consumer to purchase the food product are its appearance and color. Table 5 demonstrates the color characteristics of the biscuits under study, namely *L**, *a**, *b**, and ΔE.

The lightness values (*L**) in the biscuits fortified with kishk were higher (60.80–64.39) than that in the control sample (60.16). Yellowness values (*b**) were found to be lower in the samples with Egyptian kishk (29.60–31.85) than in the control biscuit (32.44). A similar trend for color values in both surface and back of biscuit was observed. Redness (*a**) showed no significant difference between the control and fortified sample with 10% of kishk, as well as between 40 and 50% kishk samples in the surface analysis (Table 5). An opposite trend was found in back analysis, which had significant variation between the samples.

The main color characteristics of biscuit depends on the degree of caramelization, or Maillard reaction, and are affected by various factors such as protein, carbohydrates, water activity, pH, etc. Therefore, the processing conditions during baking should be controlled [29, 30].

We also analyzed the effect of kishk in biscuit on its textural properties, namely hardness (Table 5). The obtained results indicated that the increasing amount of kishk increased the hardness of biscuit from 27.43 to 47.76 g (for the 10 and for 50% kishk samples, respectively). The same trend was observed by Drisya *et al.* who added dehydrated *Murraya koenigii* powder to cookies [31]. Our results are also consistent with Parul *et al.*, who found that spirulina and sorghum flours incorporated in dough increased the hardness of biscuit [32].

Table 6 Sensory scores of biscuits fortified with Egyptian kishk

Samples	Appearance	Color	Aroma	Taste	Texture
Control	7.85 ± 0.67	8.00 ± 1.39	8.15 ± 1.23	8.30 ± 0.97	8.05 ± 1.02
Kishk 10%	8.85 ± 0.41	8.75 ± 0.62	8.80 ± 0.61[a]	8.70 ± 0.48[a]	8.70 ± 0.48[a]
Kishk 20%	8.50 ± 0.76[a]	8.35 ± 0.54[a]	8.25 ± 0.49[a]	8.30 ± 0.39[a]	8.15 ± 0.53[a]
Kishk 30%	8.30 ± 0.47[a]	8.10 ± 0.67[b]	8.05 ± 0.35	8.10 ± 0.48[b]	8.15 ± 0.53[a]
Kishk 40%	8.00 ± 0.75[b]	8.30 ± 0.58[a]	7.80 ± 0.59[b]	8.05 ± 0.52[b]	7.90 ± 0.70[b]
Kishk 50%	8.00 ± 0.79[b]	8.20 ± 0.59[b]	7.65 ± 0.75[b]	7.80 ± 0.73	7.75 ± 0.81[b]

Values are the mean ± SD of three replicates. Mean followed by different letters in the same column differs significantly ($P \leq 0.05$)

The highest hardness of the 50% kishk biscuit compared to the other samples may be explained by its high protein content [33]. A gradual increase in hardness of biscuits with increasing the Egyptian kishk amount may be due to water absorption by kishk. Therefore, it is important to control baking conditions during addition of kishk with the view to replace wheat flour. Nandeesh *et al.* also reported that 30% of differently treated wheat brans increased the hardness of biscuit dough and decreased its cohesiveness, springiness, and adhesiveness [34].

Sensory evaluation. Table 6 demonstrates the effect of replacing wheat flour with Egyptian kishk in biscuit on its sensory properties. We evaluated appearance, color, aroma, taste, and texture. All the attributes showed a lower score in the fortified samples compared to control. Our results are similar to those reported by Drisya *et al.* who used *Murraya koenigii* leaf powder in the amount of 10% to fortify cookies, and this amount was acceptable [31]. Bajerska *et al.*, who used green tea extract in bread, mentioned its adverse effect on the sensory properties of the bread [35]. These data may contrast due to significant differences in processing conditions for biscuit and bread.

The obtained results are in agreement with those reported by Patel and Rao, who fortified biscuit with black gram flour [4]. Another study carried out by Ezeagu *et al.*, who substituted wheat flour with 15% of *Muccuna* seed flour in biscuit, supported our findings [36]. The results of sensory evaluation were in parallel with the color metric measurements.

Antioxidant activity. Figure 1 shows antioxidant activity in the control and fortified biscuits determined by DPPH and ABST assays. Both assays revealed a pronounced increase in antioxidant activity with the increase of kishk amount in the dough. However, the DPPH assay showed better results compared to ABST. Our results are in good agreement with Galla *et al.*, who supplemented biscuit with spinach leaves extract in the amount of 5–15% [37].

The correlation coefficient (R^2) between total phenolic and antioxidant activity by DPPH and ABST methods were 0.973 and 0.984, respectively, which revealed that phenolic compounds are responsible for antioxidant activity in the biscuit samples. Phenolic compounds form in bakery products as a result of Maillard reaction and caramelization [38, 39].

With increasing amounts of kishk in biscuit, its antioxidant activity rose (Fig. 1). Biscuit fortified with marine collagen peptide, as reported by Kumar *et al.*, demonstrated a weak antioxidant activity due to low content of phenolic compounds, which is responsible for antioxidant activity [40]. Therefore, the improvement in the antioxidant activity of the biscuit fortified with Egyptian kishk in the present study may be due to formation of Maillard reaction products during bakery processing as reported by Krystyjan *et al.* and

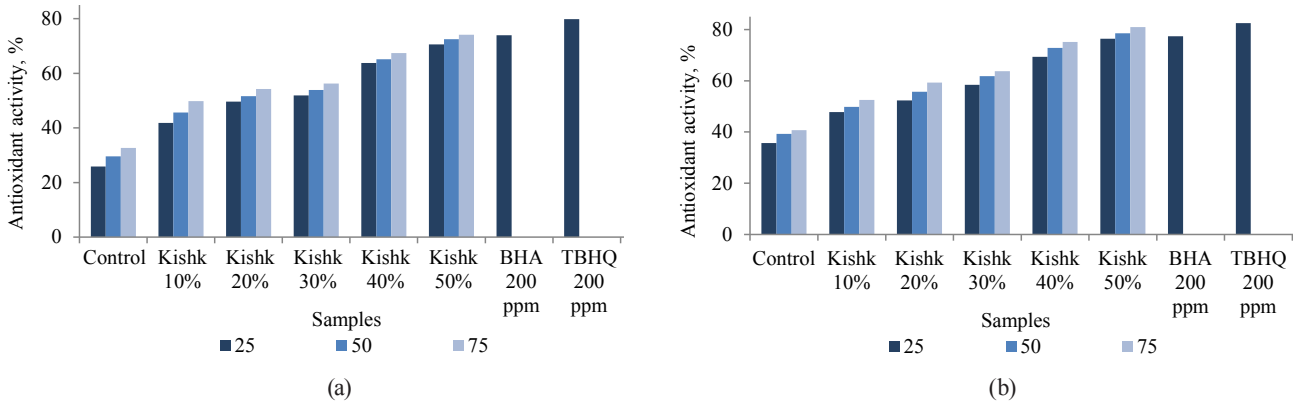

Figure 1 Antioxidant activity of biscuit fortified with Egyptian kishk determined by using DPPH (a) and ABST (b) assays. The same letters are not significant ($P \leq 0.05$)

Antoniewska *et al*. [41, 42]. The authors mentioned that antioxidant activity in biscuit and muffins was due to Maillard reaction products rather than polyphenols compounds. Therefore, a more detailed investigation on isolation and identification of Maillard reaction products formed in the fortified biscuit is needed.

Volatile compounds. Table 7 shows volatile constituents detected in the headspace of the biscuit samples fortified with Egyptian kishk. A total of 28 volatile compounds were identified and quantified. They were alcohols (4), aldehydes (9), ketones (2), carboxylic acids (2), esters (2) terpenes (1), and sulfur-containing compounds (8).

Aldehydes were the main group of volatile compounds in the biscuit samples, especially in the control biscuits, followed by sulfur-containing compounds and alcohols. The predominant aldehyde was benzldehyde (16.52%). The volatile compounds differed significantly among the control and fortified biscuits. The kishk-fortified biscuits showed higher levels of aldehydes, especially hexanal, heptanal and nonanal, which exhibited about a 2–10-fold increase compared to the control sample due to their presence in the raw material.

Iranmanesh *et al.* analyzed volatile compounds in dried kishk obtained from different animals. The authors

Table 7 Volatile compounds of biscuit fortified with Egyptian kishk

Volatile compounds	RI	Control	Kishk 10%	Kishk 20%	Kishk 30%	Kishk 40%	Kishk 50%	Organoleptics [48, 52]
				Alcohols				
1-Hexanol	863	1.93[b]	0.82	0.56	0.39	0.25	0.08	Resin, flower, green
Oct-1-en-ol	991	2.01	1.95	1.27	1.06	0.59	0.37	Mushroom, herbal earthy
1-Butanol	1182	1.46	0.19	0.83	0.57	0.18	n.d	Medicine, fruit
2-Furanmethanol	1665	7.12	5.23	4.19	0.45	2.93	1.58	
				Aldehydes				
2-Methylbutanal	647	2.75	2.04	1.62	1.29	0.78	0.38	Cocao, almond
3-Methylbutanal	668	4.18	3.51	2.78	2.55	1.78	1.49	Malt
Hexanal	805	6.37	7.12	8.53	10.16	11.35	11.96	Grass, tallow, fat
2-Furfural	841	6.83	4.53	3.91	2.87	1.09	0.87	
Propanal	847	0.15	3.92	0.86	0.59	0.37	0.34	Solvent, pungent
Heptanal	906	1.29	4.59	6.52	7.83	8.26	10.43	Fat, citrus, rancid
Octanal	1013	0.42	5.03	7.19	8.24	9.18	11.25	Fat, soap, lemon, green
Nonanal	1105	5.18	6.14	8.53	10.17	10.04	13.12	Fat, citrus, green
Benzldehyde	1521	16.52	12.93	10.92	9.84	9.76	8.23	
				Ketones				
2,3-Butanedione	695	1.95	1.57	0.98	0.73	0.54	n.d	
2,3-Pentanedione	702	0.72	0.93	0.62	0.85	0.39	n.d	
				Carboxylic acids				
Hexanoicsalysilate	1825	2.38	3.51	5.09	6.87	7.12	8.93	
Dodecanoicpalmitate	2516	0.95	n.d	3.62	5.64	5.29	6.78	
				Esters				
Ethyl acetate	1194	0.25	n.d	0.42	0.53	0.57	0.69	Pineapple
Ethyl hexanoate	1923	1.83	1.93	1.27	2.06	2.48	2.83	
				Terpenic compounds				
D-Limonene	1191	7.02	13.78	13.94	14.07	14.61	15.19	Lemon, orange
				Sulphur-containing compounds				
Pyrazine	721	2.19	1.52	0.93	0.75	0.62	0.49	
2-Methyl pyrazine	829	4.26	2.76	1.48	1.29	0.53	0.18	
2,6-Dimethyl pyrazine	927	0.98	n.d	0.65	0.42	0.18	0.07	
2-Ethyl-5-methyl pyrazine	998	1.35	1.72	1.28	1.09	0.95	2.13	
2-Penyl furan	1234	5.37	1.52	0.73	0.44	0.17	n.d	
2-Ethyl pyrazine	1332	6.59	5.98	4.18	n.d	3.54	0.16	
Acetyl pyrazine	1621	2.07	1.32	1.59	2.14	1.07	1.64	
2-Acetyl pyrrol	1972	4.52	2.06	2.86	3.76	1.49	0.58	

RI – retention indices

[b] Values are expressed as relative area percentage

n.d – not detected

detected aldehydes in all samples, with significant amounts in those samples containing flour during preparation [43]. Another explanation for the high levels of aldehydes in the fortified samples is thermal treatment of food products [44]. Among Strecker aldehydes forming via Maillard reaction, 2-Methylbutanal and 3-Methylbutanal were present both in the control and the fortified biscuit samples. However, significant amounts of hexanal, octanal, and heptanal were determined in the fortified samples compared to the control biscuit. This fact clarifies why the sensory properties the biscuits with high amounts of kishk had low scores. All the biscuit samples included alcohol. These compounds were identified in biscuit with purple wheat by Pasqualone *et al*. [45].

The furan compounds were mostly represented by furfural, a caramel-like odorant deriving by Maillard reaction, significantly more abundant in the control and 5% kishk samples, compared to the other fortified samples. Furfural is typically present in biscuits, which was reported in several studies [45, 47, 48]. Little amounts of pyrazines, produced by Maillard reaction, were also detected in the current research.

Mohsen *et al*. found three Strecker aldehydes in wheat cookies: 2-methylbutanal, 3-methylbutanal, and benzaldehyde. Those could have been derived from some amino acids, e.g. leucine, isoleucine or phenylalanine [45, 47]. These aldehydes are in good correlation with biscuit flavor which formed during preparation from Maillard reaction especially Strecker degradation [49–51]. In our study, we found that the biscuit fortified with kishk contained low amounts of these aldehydes (Table 7), which explains its low score of sensory evaluation. On the other hand, pentanal, 2-hexenal and alcohols, such as 1-pentanol, have an adverse effect on biscuit flavor due to linoleic acid oxidation [47, 52]. Low amounts of these aldehydes and alcohol make off-flavor with the increasing of kishk in biscuit (Table 7).

CONCLUSION

The current study found that fortification of biscuit with Egyptian kishk (10–50%) improved its nutritional composition such as protein, fiber, etc. The water absorption, arrival time, dough development time, and weakening of dough increased in the kishk-fortified biscuit. The fortified biscuit, especially the sample with 50% of kishk, also had higher lightness values (L^*) compared to the control sample. In addition, both the control and fortified with kishk biscuit contained an increased amount of total phenolic compounds. The antioxidant activity increased with increasing kishk amounts.

However, kishk had a negative effect on the sensory characteristics, physical properties, as well as volatile compounds of the biscuit. The kishk-fortified biscuits showed higher amounts of aldehydes, especially hexanal, heptanal, and nonanal. Further studies are needed to reveal effects of storage conditions on the physicochemical attributes and volatile compounds of kishk biscuits and to isolate and identify Maillard reaction products formed in the biscuits.

CONTRIBUTION

All the authors equally contributed to the study and are equal responsible for plagiarism.

CONFLICT OF INTEREST

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

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Mycotoxin-contaminated food and feed in Saudi Arabia: review of occurrence and toxicity

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Abstract:

Introduction. Mycotoxins are reported to have a considerable impact on the health of consumers. There has been plentiful research into the effects of mycotoxins, fungi, and bacteria on the safety and quality of food and feed. This research paper reviews the literature on mycotoxins.

Study objects and methods. The study focused on mycotoxins in food and feed produced in Saudi Arabia. We reviewed literature on the occurrence and health impacts of foodborne mycotoxins. We also studied the presence of mycotoxins in herbs, nuts, cereals, dried fruits and vegetables, infant formulas and baby foods, as well as dairy products. Finally, the paper offers a review of mycotoxin analysis methods.

Results and discussion. The findings showed that mycotoxins attract a lot of scientific interest in Saudi Arabia. Certain types of mycotoxins (zearalenone, aflatoxins) and fungi (*Aspergillus flavus*, *Penicillium chrysogenum*, and *Aspergillus niger*) were common in the samples (isolates) of products tested in the Saudi regions. Furthermore, the researchers used different techniques of analysis such as the HPLC method, dilution plate method, thin layer chromatography, total plate count method, and seed-plate method to detect, identify, and isolate mycotoxins.

Conclusion. Most importantly, the results showed that mycotoxins have serious health impacts on consumers and most of the contamination cases are caused by improper storage conditions and/or inappropriate handling and harvesting practices.

Keywords: Toxins, fungi, herbs, nuts, cereals, dried fruits, vegetables, baby foods, dairy products, health, food contamination

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INTRODUCTION

Mycotoxins are toxic substances produced by certain molds (filamentous fungi). The most common of such molds include *Aspergillus*, *Penicillium*, and *Fusarium*. Different molds produce different mycotoxins, with over 400 mycobacterial toxins identified so far and more to come [1]. There are many fungi that grow on foods, especially those stored under certain temperatures and humidity favorable to their growth. Most mycotoxins produced by fungi are carcinogenic to the cells and cause nervous disorders. Mycotoxins have been investigated all over the world and reported to have serious health impacts on humans [2]. These toxins can be produced in the field before or after harvest and during storage of various materials. As a result of growth, fungi perform metabolic processes and produce mycotoxins. The growth of fungi requires specific environmental conditions such as increased humidity, poor storage, abundance of oxygen, contamination with fungi and mechanical damage [3].

Statement of the problem. The storage of grains and products is accompanied by many microorganisms, such as fungi, yeasts and bacteria. They multiply when conditions are appropriate for their growth, causing damage to stored materials. This, in turn, leads to lower product quality, as well as chemical changes in the product. Fungi play a dangerous role, especially during storage, compared to other microorganisms. Mycotoxins have significant economic impacts on many agricultural crops, especially wheat, yellow corn, pistachios, nuts, cotton seeds, and tea. As reported by the Food and Agriculture Organization of the United Nations, 25% of the world's crops are contaminated with fungal toxins, with annual losses of about 1 billion metric tons of foods not suitable for consumption [4].

Mycotoxins can be found in all products that are affected by mold and can be seen with the naked eye where the mold is invisible or long gone. Therefore, a high quality product may contain hazardous substances. To prevent mycotoxins from entering food products, special government programs have been developed to control the production and storage of cereal crops, food raw materials, and animal feed. There are standards for the maximum permissible concentration of mycotoxins per group in different raw materials. During quality certification, each batch is checked for compliance with the standards [5].

The purpose of this article was to review the occurrence and health impacts of food-borne mycotoxins in Saudi Arabia, to elicit the advanced trends in mycotoxin analysis methods in Saudi Arabia, and to identify the effect of mycotoxins on different foods in Saudi Arabia.

STUDY OBJECTS AND METHODS

The work was performed at the Faculty of Medical Science, Department of Toxicology, University of Jeddah, Saudi Arabia. The study objects included scientific and methodological publications, articles in scientific periodicals, conference materials, intellectual property items, regulatory documents, and Internet resources. The methods employed to analyze theoretical data included registration, filing, grouping, classification, comparative analysis, and consolidation of scientific materials.

RESULTS AND DISCUSSION

History and occurrence of mycotoxins. Aflatoxin is the most common of all mycotoxins and one of the most carcinogenic compounds of natural origin. In 1952, the consumption of rotting yellow corn by pigs intended for breeding caused poisoning in the south of the United States. In 1960, so called X-Disease resulted in the death of 100 000 turkeys in England. Aflatoxins are produced by *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* fungi. The symptoms of aflatoxin-contaminated food poisoning include loss of appetite, lethargy, muscle weakness, liver bleeding and necrosis, kidney injections, and liver cancer. Aflatoxin of Type B1 is the most dangerous of all mycotoxins. This poison is produced by most types of the *Aspergillus* genus, especially *A. flavus*, which grows on a number of nutrients such as corn and peanut seeds [5].

Mycotoxins are among the strongest known toxins which can cause serious diseases in concentrations of even $< 1\times10^{-6}$. One cause is that they are heat resistant to a degree that is difficult to destroy by traditional thermal treatments used in manufacturing and cooking processes. Another cause is that they spread quickly from mold colonies to food [6].

Mycotoxins can affect several products that consumers eat on a daily basis, including bread, coffee, and spices, as well as milk and its derivatives [6]. In addition to health hazards, they lead to huge material losses when contaminating raw materials [7]. Mycotoxins represent key health risks to consumers. They are commonly present in foods, varying to certain degrees in each type of products.

In Saudi Arabia, mycotoxins occur in many local and imported products due to storage conditions, geographical area, and harvesting techniques [8]. Therefore, it is important that local agencies adhere to the applicable standards to prevent contamination of products. Several studies have examined the toxicity of mycotoxins in different individual food and feed products, focusing on the type and occurrence of mycotoxins in Saudi Arabia. This review aims to identify the occurrence and health impacts of food-borne mycotoxins in food and feed, as well as identify the effect of mycotoxins on different foods in Saudi Arabia.

Occurrence and health impacts of food-borne mycotoxins in Saudi Arabia. Humans and animals can be exposed to, and poisoned by, mycotoxins in direct and indirect ways. Direct exposure can be through contact with the skin, inhalation, or consumption of mycotoxin-contaminated food. Indirectly, humans can be exposed through the consumption of animal products contaminated with residual fungal toxins or their derivatives, such as milk, eggs or meat from animals that were previously fed on toxin-contaminated feed. In general, mycotoxins reach human and animal food through its contamination with the fungus that produces these toxins as the food material encourages fungal growth during different stages of production, transportation or storage [9].

The presence of mycotoxins in foods and tissues is of enormous importance in the field of public health since they are capable of causing acute and chronic effects in humans and animals. These effects range from death to the disruption of the central nervous system, heart, pulmonary and intestinal blood vessels. Mycotoxins are the greatest source of concern as they can cause cancer. Cases of food contamination with aflatoxin were recorded as deaths. Mycotoxins are divided according

Table 1 Ranges of mycotoxins in food and feed in Saudi Arabia

Reference	Mycotoxins	Food & Feed	Range
Alwakeel and Nasser [16]	Aflatoxin B1	peanuts	8.4 μg·mL^{-1}
Alwakeel and Nasser [16]	Aflatoxin B2	sunflower	1.6 μg·mL^{-1}
Aly *et al*. [17]	Aflatoxin G1	white cheese	8 to 14 ppm
El Tawila *et al*. [18]	AFT	nuts	1.0 to 109.0 μg/kg
Abdel-Gawad and Zohri [19]	fungi	nuts	1966–7703 and 1949–7432 colonies/g
Al Husnain and AlKahtani [20]	Fusarium sp	maize and rice	(2000 μg/kg)
Al Khalifa and Ahmad [21]	Pb and Cd	baby foods and infant formulas	5 Ug cd/L and 5 ppm
Al Khalifa and Ahmad [21]	Sb and Sn	baby foods and infant formulas	0.04 and 0.054 ppm
Alghuthaymi and Bahkali [22]	Fusarium	banana fruits	over 1 μg·mL^{-1}
Al-Kahtarii [23]	aflatoxin	Wheat	1.7 μg·mL^{-1}

Prepared based on the findings of this study

to their regions of influence in mammals (hepatotoxins, renal toxins, neurotoxins, muscular toxins, cardiac toxins, skin toxins, reproductive toxins, gastrointestinal toxins), and there is no doubt that higher concentrations of mycotoxins in food increase the resulting damage in the organism [10].

Mycotoxins were reported to constitute a major health risk to consumers. In Saudi Arabia, several studies have been conducted to analyze the risks of food-borne mycotoxins and all of them have confirmed their health risks [2]. In a study by Al-Hindi *et al*., the agar wood was tested in Jeddah city, with mycotoxins isolated and storage conditions analyzed for their impact on the growth of fungi. The researchers used the dilution plate method for the isolation of mycotoxins [7]. Their findings revealed *Rhizopus*, *Fusarium*, and *Penicillium* to be the common types of mycotoxins found in the analyzed samples. The study concluded that the direct exposure to these fungi jeopardizes consumers' health.

Alwakeel, in another study, tested the fungi in fruits growing in Saudi Arabia in order to identify their most common types and health impacts [11]. Out of 34 isolates, 14 were found to be contaminated by *Penicillium chrysogenum*, *Adametzii*, and *Aspergillus oryzae*. According to the author, these fungi are mostly useful and scarcely pathogenic. However, they still cause diseases in people with weak immunity. The study recommended evaluating all products for mycotoxins in order to know the types of fungi present in them and the associated health risks.

In a further study by Bokhari, the fodders of wheat bran, corn, and barley were evaluated from seven Saudi Arabia regions [12]. The analysis showed that most of the samples were contaminated with fungi and toxins. Furthermore, zearalenone, ochratoxin, and aflatoxins were found in the samples and reported to have negative health impacts.

The contamination by mycotoxins can occur both directly or indirectly [13]. Direct contamination is the result of eating food contaminated with these toxins, especially peanuts, peanut butter, and nuts such as pistachios and almonds. Mycotoxin contamination most commonly occurs in the field before harvest and also during some stages of production such as storage and handling, or oil extraction. Equipment can also be a source of contamination, leading to poisoning [5].

Indirect contamination, on the other hand, is caused by using substances (or processed foods) contaminated with mycotoxins. An individual can be exposed to low concentrations of mycotoxins as a result of consuming animal products that contain residues of mycotoxins originating from livestock fed on contaminated feed [9].

Researchers have been able to discover aflatoxin in some types of infected foods such as nuts (peanuts, pistachios, cashews, and almonds), crops (corn, wheat, rice, barley, soybeans, cocoa, and cotton seed), and fodder made from fish, potatoes, and seeds [14]. Olives and legumes can also be contaminated with mycotoxins. Aflatoxins are mostly common in nuts, especially peanuts from which peanut butter is made and used in many food industries, e.g., to make chocolate and biscuits. Also, breakfast cereal flakes prepared from wheat or corn may be contaminated with aflatoxin [15].

Table 1 shows concentration ranges of mycotoxins in food and feed in Saudi Arabia.

Advanced trends in methods of mycotoxin analysis. Analytical chemistry has advanced in recent years and introduced new methods for mycotoxin analysis and titration. Identifying the amount of any chemical substance requires certain steps including sampling, extraction, cleaning up, and analysis [24].

Chromatography is considered one of the most common and effective methods for separating and estimating chemicals in terms of quantity and quality. It is performed in several ways, including thin layer chromatography, gas chromatography, and high performance liquid chromatography [4].

High performance liquid chromatography (HPLC) is among the most important methods for chromatographic separation of liquids. This method uses the static medium in the form of small-sized particles that is pushed by the moving medium (liquid) through the column using a pump at pressures of up to 8000 Psi. The column has velocities between 50 and 50 mL/min

although the particles in the medium are only several micrometers in diameter. It has already been possible to obtain flow velocities of 1–4 mL/min using columns filled with 5 μm particles. In this way, compounds can be separated that are not volatile or those that are not resistant to high temperatures. Also, it is possible to use fine particles of solid material with adsorption properties, materials with ion substitution properties, gels with specific pores, as well as liquid loaded on fine particles of solid material [5].

The relationship between certain materials and a specific mycotoxin contamination profile has motivated the researchers to focus on certain materials to study and investigate. Also, researchers use a variety of methods to detect mycotoxins. Eshelli *et al*. reported that various approaches have been used to assess and control mycotoxin contamination [25]. Critical challenges still exist due to the diverse and complicated nature of food. Different methods, such as genomics, proteomics, transcriptomics, and metabolomics, are used to detect fungal contamination and to identify and isolate mycotoxins, whether before or after harvest. Also, multi-omics techniques are used with developed technical approaches and chemometrics to deliver an explanation of these metabolites yielded before or during crop contamination.

Taheur *et al*. discussed the removal of mycotoxins and detoxification in order to biologically control these subjects [1]. The literature on mycotoxins biotransformation showed the ability of fungi, bacteria, and enzymes to control and minimize the level of mycotoxins. This, in turn, enhances the quality of food and feed, especially meat, eggs, and milk. On the other hand, microbial detoxification was reported to be a trending strategy that does not leave any poisons or less poisonous compounds.

Furthermore, Bokhari used polar solvents such as acetone, water, methanol, and others to investigate the presence of mycotoxins in camel diseases [12]. The results showed that most of the mycotoxins can be dissolved in polar solvents and cannot be solved in non-polar solvents. Also, Bokhari used a similar technique to investigate mycotoxins in anise seeds and black pepper fruits, with *Fusarium*, *Aspergillus* and *Penicillium*, reported as widely spread [6].

Mycotoxin analyses in food and feed in Saudi Arabia. Various techniques of analysis have been employed to identify mycotoxin in different products in Saudi Arabia, as well as in animal feed and food (maize, wheat).

Almoammar *et al*. examined fungal infestation and mycotoxin contamination in camel feedstuffs in Saudi Arabia [26]. The results showed that many samples were contaminated by moulds relating to *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium*, *Setosphaeria*, and *Nuersopra*. *Fusarium verticillioides* was the biggest generator of fumonisin, zearalenone, and deoxynivalenol (1050, 245, and 640 μg/kg, respectively). *Alternaria alternata* was the biggest generator of altenuene and alternariol (52 and 29 μg/kg, respectively). The Charm ROSA quantitative lateral flow assay method was employed to measure overall aflatoxin. The aflatoxin B1 was found between 1.0–3.5 ppb in the gathered samples.

A study of sorghum grains conducted by Yassin *et al*. found 31 species from 17 fungal genera [27]. They used an agar plate method to check 24 samples gathered from the markets in Riyadh city, Saudi Arabia. According to the findings, *Rhizopus stolonifer*, *Aspergillus niger*, and *Penicillium funiculesum* were the most common isolated species of fungi. Also, the use of HPLC to examine mycotoxin showed that the mycotoxin-producing isolates differed in kind and concentrations of yielded toxins.

In another study, Alghuthaymi and Bahkali examined the fumonisin B1, deoxynivalenol, and zearalenone in 19 *Fusarium* isolates from bananas brought to Saudi Arabia in 2012 [22]. The trinucleotide repeat analysis was utilized to identify these isolates. The simple matching coefficient was used to determine the similarity degree between the isolates. The fumonisin B1 was reported as common in the imported fruits. Furthermore, Bokhari examined the existence of mycotoxins in camel diseases. In this study, 20 samples of fodders were investigated in seven regions of Saudi Arabia to identify zearalenone, ochratoxin, aflatoxins, and fungi [12]. The analysis of isolates from natural feed showed ten genera and 38 kinds of fungi. The level of toxigenic fungi ranged from 6 to 30 percent in the natural feed and from 5 to 20% in the compound feed.

Effects of mycotoxins on different foods. Mycotoxins are toxic metabolites that are produced by many strains of fungi and are found in food products and livestock feed. These toxins are often invisible, tasteless and chemically stable at high temperatures and during long storage periods. Since some mycotoxins are highly toxic, they may have an adverse effect on health and, thus, products need to be reliably tested. Exposure to mycotoxins occurs either directly, by consuming food contaminated with mycotoxins, or indirectly, by consuming animal-based products, especially milk [9]. The effects of some mycotoxins transmitted by food are severe. Mycotoxins cause serious pathological symptoms that occur immediately after consuming contaminated foods. Other fungal toxins that appear in foods have long-term effects on health, including cancer and immune deficiency [10].

Mold is not the only way for mycotoxins to reach nutrients – they can be found in milk, meat and eggs that were not previously contaminated with mold [13]. Mycotoxins are produced by different groups of molds, namely three genera: *Aspergillus*, *Penicillium*, and *Fusarium*. Despite their harmful effects (plant diseases, food rot and spoilage, production of toxins), fungi play a distinctive role in many food and therapeutic industries such as the production of dairy products,

bread, antibiotics, vitamins, enzymes, cholesterol, fats, and ferments for livestock feed. The growth of fungi, and consequently their production of mycotoxins in agricultural products, is influenced by a set of natural factors, including the fungus strain, nutrient nature, relative humidity, temperature and time, ventilation, spoilage, growth and maturity [14].

The risks caused by mycotoxins poisoning are either through direct ingestion of food contaminated with poison or through the consumption of food such as milk, eggs and liver from animals whose feed was contaminated with poison. There are two types of exposure to aflatoxin, depending on the dose taken. The first is acute exposure – eating a large quantity of poison in food or fodder in a short period of time, which sometimes leads to death. The second is chronic exposure – eating poison in small quantities for a long period of time, leading to tissue changes and then to infection. Several symptoms and conditions caused this way include weakness and lethargy, loss of appetite, weakness of muscles, liver and kidney bleeding, liver necrosis and cirrhosis, bile cell division, liver cancer, acquired immunodeficiency, arteriosclerosis, biliary cirrhosis, diabetes, brain damage, heart failure, hyperactivity syndrome, high blood pressure, infertility, inflammation of the intestine, corneal stiffness, and Alzheimer [15].

Aflatoxin is found in grains such as corn, wheat, barley, rice, and pistachio in addition to its presence in poultry meat and eggs. The toxicity of these substances depends on the nature of poison, its quantity, period of consumption, and food composition. The general effects of aflatoxin on bird health include liver and kidney cancer, hormonal imbalance, lower immunity, proneness to disease, and reduced egg production. In particular, 10 ppb of aflatoxin B1 reduces egg production by half [5].

Also, it is reported that feeding livestock, such as cows, sheep and goats, on feed contaminated with aflatoxin for long periods leads to symptoms and diseases similar to those in birds, negatively affecting milk production, the quality of milk, and muscle tissue [15]. Since aflatoxins are very persistent compounds during the manufacturing process, they cannot be destroyed by normal biochemical methods based on laboratory experiments. However, it is possible to reduce the risks associated with aflatoxin-poisoned food by using special processing procedures to remove the toxins from food. The choice of these procedures depends on the chemical stability of aflatoxin, the nature of food treatment, and the type of their interaction with the contaminated food [9].

Mycotoxins in herbs. In Saudi Arabia, many studies have reported the existence of mycotoxins in herbs [28]. For example, Hashem and Alamri tested the presence of contamination by molds in a number of herbs in Riyadh city, Saudi Arabia [29]. They studied 520 fungal isolates, which represented 57 species, from dried and ground spice samples on three different media using the standard dilution plate method. The study revealed the presence of *Aspergillus*, *Penicillium*, and *Rhizopus* in the species. Also, they found *A. flavus* in ginger. The study indicated that *A. flavus* occurs in herbs due improper storage conditions and, unless they are properly managed, these herbs pose serious health risks.

On the other hand, Bokhari examined mycotoxins in 50 samples of spices such as marjoram, ginger, cumin, pepper, red pepper, and anise. The samples were collected from the retail markets in Jeddah city, Saudi Arabia [6]. The results showed that *Aspergillus*, *Penicillium*, and *Fusarium* were the most widespread fungi in the selected samples. Furthermore, *A. flavus* was found in most of the tested spices. These findings showed that the degree of fungal contamination did not exceed the limits set by the international agencies.

Also, Bokhari tested the existence of aflatoxin and ochratoxin in coffee beans in Jeddah governorate, Saudi Arabia. The researcher used HPLC to test the samples and found that aflatoxin B and ochratoxin were the most common mycotoxins found in the coffee beans [8]. The study indicated improper storage conditions as the key factor of contamination. Also, the coffee beans contamination was found to be a health risk to the consumers and most of the contamination cases occured during the treatment process.

Bokhari and Aly investigated fumonisin B1 in herbal tea consumed by Saudis [30]. Fumonisin B1 (0–266 μg/kg) was detected via HPLC with fluorescence detection. The samples of herbal tea were checked for mycotoxins. The researchers found that the most common mycoflora were shown in 13 types representing 25 species, the most important being *Aspergillus*, *Penicillium*, and *Fusarium*.

Mycotoxins in dried fruits and vegetables. Mycotoxins are naturally occurring contaminates of dried fruits and vegetables. The factors that influence their existence include the type of fruits and vegetables, the geographical region, treatments before and after harvest, harvesting techniques, and storage conditions. Dried fruits are fruits in which most water has been removed naturally by sun-drying or using special dryers. Fruit drying is also a traditional method of long-term preservation and storage [16]. Dried fruits include raisins, peaches, figs, and apricots. About half of their production is sold in the local markets of many countries.

Dried fruits are a source of basic mineral elements (calcium, iron, potassium, copper, boron), vitamins (A, C, K) and plant fibers, which are sometimes low in daily diets. Dried fruits are exposed to many microorganisms, such as bacteria and fungi, which play a large role in the processes of corruption. This condition occurs at different stages before and after harvest, as well as during storage. Several studies have confirmed the

contamination of dried fruits with many species of fungi such as *Aspergillus*, *Rhizopus*, *Alternaria*, *Penicillium Scopulariopsis*, and *Ulocladium*, dominated by *Aspergillus* and *Penicillium* [31].

Dried fruits are food pillars that support the growth of fungi and their production of compounds and metabolic products, including mycotoxins. Thus, mycotoxin contamination is a major concern in many countries of the world. The most common mycotoxins in dried fruits are *Strigmatocystin*, *Patuline*, *Ochratoxin*, and *Aflatoxin cyclopiazonic*. These toxins are produced by certain types of *Aspergillus ochraceus*, *A. parasiticus*, and *A. flavus* [5].

Gherbawy *et al*. analyzed mycotoxins in dried fruits in a number of retail markets in Taif city, Saudi Arabia [32]. The researchers checked the existence of both aflatoxins and ochratoxin A in the dried fruits, isolating 22 fungi from 50 kinds of dates. Using the thin layer chromatography, they found *R. stolonifer*, *A. flavus*, *A. niger*, and *P. chrysogenum* to be the most common mycotoxins in the samples. Their toxicity was measured using Artimia larvae and 9 of 36 isolates were found to contain *A. niger*.

In a study by Alwakeel and Nasser, 40 samples of dried seeds were analyzed in Riyadh city, Saudi Arabia. The researchers used the seed-plate method and the sucrose/glucose-Czapek's agar [16]. Mycotoxins were detected with the thin layer chromatography. Also, the total plate count method was employed to find bacteria. The results showed that *A. flavus* and *A. niger* were dominant in all types of media. Aflatoxin B1 was found in the peanuts (8.4 $\mu g \cdot mL^{-1}$) and aflatoxin B2, in the sunflower (1.6 $\mu g \cdot mL^{-1}$).

In another study, Al-Hazmi analyzed the existence of patulin and ochratoxin A in apple juice produced in Jeddah city, Saudi Arabia [31]. The contamination with fungi was tested on a potato dextrose agar. Also, HPLC was used together with the UV detector in order to determine patulin. The findings showed that the whole samples of apple juice were free of yeasts and fungi. In addition, out of 17 samples, only one contained patulin in a concentration higher than 153 ppb. Also, ochratoxin A was found only in five types. According to Ouf *et al*., patulin is among the most common mycotoxins in fruits, accounting for 84%, especially in the damaged parts of apples, pears, and grapes [33]. The juices and jams produced from these fruits are also infected with fungi secreted by this toxin. The researchers also found aflatoxins B1 and C1 in cherries and carrots, which were then passed on to the juice. Finally, the study revealed concentrations of aflatoxin in dried apricots and figs [33].

Mycotoxins in infant formulas and baby foods. Baby foods are among the key products due to their importance to infants and toddlers. Different studies have examined mycotoxins in baby foods and formulas, especially in the Saudi context. In a study by AlFaris *et al*., the presence of aflatoxins in baby foods was tested using the liquid-liquid extraction method, as well as the immunoaffinity column cleanup [34]. The samples were taken from the markets of Riyadh city, Saudi Arabia. The findings showed that the levels of aflatoxins in baby foods were in line with the European Standards.

Aly *et al*. analyzed the presence of minerals and microbes in white cheese for children and infants in Jeddah city, Saudi Arabia [17]. The researchers found aflatoxin G1 (8–14 ppm) in three samples of cheese using the immune-adsorbent column chromatography. On the other hand, the study showed that yeast and fungi relied on the products that contained Potato Dextrose and Sabouraud. The authors concluded that the cheese products for babies were risk-free as they were not contaminated with a large amount of microbes. However, the removal of affected parts of food does not lead to the complete elimination of fungal toxins formed in these foods.

Therefore, consumers should avoid the growth of fungi responsible for the production of most important toxins. These toxins have a cumulative effect that can manifest 10–20 years after eating contaminated food. Another problem is that the detection of these toxins does not stimulate the immune system and there are no drugs to reduce their impact. As a result, they constitute a health disaster worldwide [10].

Mycotoxins in dairy products. Mycotoxins in dairy products have interested researchers all over the world, including Saudi Arabia, due to the importance of these products to public health, especially to that of children. Dairy products are especially vulnerable to contamination by aflatoxins due to highly complicated processing procedures they undergo. Unless the processing is well controlled, dairy products are more likely to be contaminated, compared to other products. Also, since cow milk is one of the most important dairy products, it has been well studied and analyzed to identify the types of mycotoxins found in it and reasons for contamination [13].

For example, Rahmani *et al*. reviewed some studies that investigated the existence of aflatoxin M1 (AFM1) in milk in a number of countries in the Middle East [35]. They ranked the countries according to the presence of AFM1 in raw milk. The findings showed that ultrahigh temperature milk in Saudi Arabia had lower concentrations of AFM1 than that in Iran and Turkey. The study also indicated that AFM1 in raw milk was higher in Palestine, Lebanon, and Egypt than in Iran, Turkey, and Syria. Finally, the authors concluded that children in the Middle East, compared to other countries, are at a higher risk since they eat raw milk, which may contain high AFM1.

Abdallah *et al*. analyzed ultrahigh cow milk in Najran, Saudi Arabia, to detect the presence of aflatoxin M1 [36]. For the experiment, 96 samples were tested using the enzyme-linked immunosorbent assay. AFM1

was found in about 79 samples. Also, they found that AFM1 was below the permissible levels in the positive samples. This means that the existence of AFM1 in the milk products in Najran does not seem to constitute a severe health problem.

Nasser examined white cheese in Saudi Arabia to detect whether it was contaminated by fungi [3]. For the experiment, 13 kinds of microorganisms belonging to four types of mold and yeast were separated from 20 samples of cheese. The malt extract was utilized. The results showed that most of the cheese samples were free of fungi. Also, *Penicillium* members and *Aspergillus* A were reported to be common in cheese.

Mycotoxins in nuts. There have been studies into the occurrence of aflatoxin contamination in different nuts in Saudi Arabia. For example, El Tawila *et al*. examined 264 samples of nuts gathered from the markets of Makkah city, using the Aflatest immunoaffinity column technique [18]. The findings showed that 70% of the samples were contaminated with aflatoxin (1.0–109.0 μg/kg). In particular, aflatoxin was found in 18% of groundnuts, 18% of almond, 33% of pistachio, 49% of walnuts, 16% of cashew, and 44% of hazelnut samples. About 23% of the samples were within the tolerable limits set by the European Union. Only two products exceeded the limits, namely pistachio and peanut samples.

In another study, Abdel-Gawad and Zohri examined seeds of walnut, pistachio, hazelnut, chestnut, cashew, and almond [19]. The samples were collected from the markets of Ar'Ar city, Saudi Arabia. The study revealed 50 species and three kinds of fungi, namely *A. flavus*, *P. chrysogenum*, and *A. niger*. Their volume ranged between 1966–7703 and 1949–7432 colonies/g of dry seeds.

The permissible ranges of aflatoxins for nuts in Saudi Arabia have been established in line with the standard developed by the GCC Standardization Organization (GSO), as shown in Table 2.

Alhussaini examined 12 samples of edible nuts and dried fruits purchased from the markets in Mekka and Al-Dawadmy regions, Saudi Arabia [38]. Using two methods, dichloran rosebengal chloramphenicol (DRBC) and dichloran 18% glycerol (DG18), the researcher separated 23 fungal species relating to 12 genera. The most important fungi were *A. flavus*, *A. niger*, and *Penicillium citrinum*. Other kinds, found in moderate amounts, included *Aspergillus fumigatus*, *Aspergillus terreus*, *Aspergillus sydowii*, *Eurotium amstelodami*, *Paecilomyces variotii*, and *Trichoderma harzianum*. Out of 40 *Aspergillus* strains, 16 (40%) were reported to generate mycotoxins. Aflatoxins B1 and B2 were generated by eight out of 20 *A. flavus* strains (100–600 μg/L of culture medium). Aflatoxins G1 and G2 were also produced by one isolate of *A. parasiticus* (200 μg/L). Ochratoxin A was produced by *A. niger*, *Aspergillus brasiliensis*, *Aspergillus aculeatus* and *Aspergillus sclerotioniger* strains (100–200 μg/L).

Table 2 Permissible level of aflatoxins in nuts in GCC countries

Commodity/Product Name	Maximum level, μg/kg
Almonds	10
nuts	10
Hazelnuts	10
Peanuts	15
Pistachios	10
Pistachios	15
Dried figs	10

GCC Standardization Organization [37]

In another study, Alwakeel and Nasser analyzed edible nuts using 40 samples of dried seeds picked up from various places in Riyadh city, Saudi Arabia [16]. The seed-plate and dilutions plate approaches were used. Also, the thin layer chromatography was employed to detect mycotoxins. Furthermore, the presence of bacteria was analyzed by the total plate count technique. The study showed that *A. niger* and *A. flavus* existed in all kinds of medium. Also, Aflatoxin B was found in peanuts and sunflower seeds, where it was dominant. Four samples of nuts demonstrated a contamination with bacteria.

Mycotoxins in cereals and cereal-based foods. Cereals have been among the most common objects of study with regard to mycotoxins, especially in the Saudi context. For example, Al-Kahtarii examined mycotoxins in grain and wheat materials [23]. In their study, wheat and other types of grains were analyzed independently for the presence of any types of mycotoxins. About 16 samples were collected from the retail markets in Riyadh city. With the HPLC technique, the study showed that *Alternaria* (69%) and *Aspergillus* (24%) were the most noticeable genera in the samples. Also, the samples contained *Aspergillus* (24%) and *Fusarium* (7%).

In another study, Al Husnain and AlKahtani investigated the presence of fungi in the seeds of maize and rice in certain locations of Saudi Arabia [20]. They also aimed to solve the diversity in 18s rDNA gene found in a number of mycotoxins. The isolated fungi were classified as *Fusarium* sp. and *Alternaria* sp. based on the morphological characters and spores. According to the results, *Fusarium* sp. was the most highly detected fungus, with more than 2000 μg/kg fumonisin, particularly in the 2011 cereal season. The study also showed that the cereals with the highest level of mycotoxins were the ones taken from Dammam.

Mahmoud *et al*. examined four types of cereals, including white and yellow corn and red sorghum seeds, imported to and produced in Saudi Arabia [39]. The experiment was conducted on 80 samples of sorghum and corn grains using the direct plating method. The analysis showed that the white and yellow grains

contained *Aspergillus* spp. and *Fusarium* spp. to a higher degree and *Alternaria* spp. to a lesser degree.

Abudabos *et al.* examined the presence of mycotoxins in dried grains in Saudi Arabia [15]. The study showed that the grains contained higher levels of fumonisin B2, aflatoxin B1, aflatoxin G1, and deoxynivalenol. However, the samples showed a lesser presence of other aflatoxins and ochratoxin A.

Mycotoxins in animal feeds. In Saudi Arabia, natural ingredients such as wheat bran and corn are the most common kinds of animal feed. Bokhary, in his study on camels in Saudi Arabia, reported ochratoxin, zearalenone, and aflatoxins among common fungi found in 20 samples of camel fodder [12]. Some of the tested samples had extra amounts of zearalenone, aflatoxins, and ochratoxin A, as well as other fungi. The study concluded that these fungi can cause health problems to camels.

In a study by Al-Julaifi and Al-Falih, 843 commercial animal feed and foodstuff samples were gathered from all over Saudi Arabia in 1997–2000. They were analyzed for type A and type B trichothecenes (diacetoxyscirpenol, neosolaniol, HT-2 toxin, T-2 toxin, nivalenol, fusarenon-x, deoxyniva-lenol) [40]. The levels of mycotoxins ranged from < 2 to 4000 µg/kg deoxynivalenol, 3.25 to 500 µg/kg fusarenon-x, 3.13 to 600 µg/kg nivalenol, 3.13 to 50 µg/kg diacetoxyscirpenol, 6.25 to 200 µg/kg neosolaniol, 3.13 to 18.75 µg/kg HT-2 toxin, and 6.25 µg/kg T-2 toxin.

All animal feed – whether prepared in the field, imported or stored in silos – may contain mycotoxins. Under certain moisture and heat conditions, these mycotoxins lead to the growth of fungi. Some of these fungi are toxic, affecting animal health and productivity. The symptoms of poisoning with mycotoxins in milk cattle and fattening calves are mostly general and non-specialized, overlapping with other diseases [2]. The diagnosis mainly depends on the exclusion of similar diseases through the examination and analysis of animal feed for mycotoxins. Also, 90% of the problems caused by mycotoxins are subclinical. They include lower productivity and food intake, diarrhea that can be dark or bloody, and increased rates of pathological problems in the herd (placenta retention, displacement of rennet, ketosis, and udder infections) [41].

Mycotoxins accumulate in the tissues of animal flesh or products when animals consume feeds contaminated with aflatoxin. They can pass on to humans and cause liver cancer, even through very small amounts consumed for a period of time. Animal fungal contamination has also been proven harmful to the health of people who eat meat or other animal products, especially children who rely on milk as a staple food.

Some studies have demonstrated a relationship between increased liver cancer and the daily consumption of foods contaminated with aflatoxin. Moreover, laboratory experiments have shown that eating contaminated food may lead to fetal abnormalities, decreased growth and damage to the immune system in animals [40].

The best way to avoid aflatoxins is to eat food from reliable sources (especially peanuts, almonds and nuts). Also, food producers must harvest, manufacture, store, and trade products under appropriate sanitary conditions. The risk of food contamination with aflatoxin increases in countries that suffer from high temperatures and humidity leading to poor storage [14].

CONCLUSION

The review has shown that mycotoxins can occur in all kinds of products with relative differences between the samples tested. Attention should be paid to storage conditions, harvesting techniques, and handling processes. Also, the regulatory agencies should monitor standard specifications that include maximum permissible limits for toxins in foods in micrograms/kilograms. Strict monitoring of mycotoxins should be regularly implemented to ensure that their amounts in foods are within the allowed limits.

There are many factors that increase the production of mycotoxins in foods. One of them is poor storage since storing food at high temperatures and humidity leads to the release of many mycotoxins into food. Post-harvest stages, such as drying and storage, are among the most important stages of production. Food can become vulnerable to mycotoxins if storage conditions are not subject to strict control. Usually, the presence of *Aspergillus*, *Fusarium* and *Penicillium*, as well as their fungal toxins, can lead to food contamination during storage and handling under inappropriate conditions.

CONTRIBUTION

Alisraa Mohammed Althagafi collected the data, contributed data and analysis tools, performed the analysis and wrote the draft. Hamad Majob Alshegifi collected the data, contributed data, performed the analysis, and wrote the draft. Thamer Salem Qussyier collected the data, contributed data, performed the analysis, and wrote the paper draft. Abdalbasit Mariod contributed data, revised the paper, and submitted the paper. Mansour Tobaiqy performed the analysis and revised the paper.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Lead exposure through eggs in Iran: health risk assessment

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Abstract:

Introduction. Contamination of food, including animal protein sources, with heavy metals is a major threat to humans. The aim of this research was to determine lead concentrations in eggs from different Iranian regions and assess risks to human health.

Study objects and methods. In this study, lead concentrations in eggs produced at laying hen farms in Qom, Isfahan (Kashan city), and Khorasan Razavi (Mashhad city) provinces were measured by an atomic absorption device. Health risk was estimated using the Human Health Risk Assessment (HHRA) model.

Results and discussion. The levels of lead in eggs were significantly different ($P \leq 0.05$) among the three regions. They were lower than the permissible limit (0.1 mg/kg) for Kashan (0.0756 mg/kg) and Mashhad (0.0633 mg/kg), but eggs from Qom contained 0.1163 mg/kg of lead. In all the three regions, the estimated daily intake (EDI) of lead was lower than the maximum tolerable daily intake (MTDI), indicating no health risk for lead through egg consumption among Iranian consumers. Also, no risks were detected for adults in terms of non-cancer risk, or target hazard quotients (THQ), and carcinogenic risk (CR) of lead ($THQ < 1$ and $CR < 10^{-6}$).

Conclusion. The results of this study indicated that lead health risk through egg consumption is within safe limits. However, the nutritional importance and high consumption of eggs among households necessitate a more careful monitoring of lead concentrations to meet public health requirements.

Keywords: Heavy metals, laying hen farm, estimated daily intake (EDI), carcinogenic risk (CR), lead, eggs

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INTRODUCTION

Environmental pollution caused by the development of livestock and poultry production has raised concerns about food safety, in particular about possible residues of heavy metals in feed additives or poultry feed and products, including eggs [1]. In addition to being non-biodegradable, heavy metals have a long biological half-life and can conceivably accumulate in different parts of the human and animal body due to the lack of sufficient mechanisms for their elimination [2].

Heavy metals can be transmitted from poultry to eggs through environmental pollution or via the food chain [3]. Thus, female poultry can absorb heavy metals from different sources in the environment and transmit them into their eggs [4].

Studies have shown that feed is one of the most important ways of absorbing heavy metals by laying hens [5, 6]. Several parameters influence the bioaccumulation of heavy metals by laying hens, including the chemical and physical properties of heavy metals, season, location, and chicken qualities (nutritional behaviors, metabolic cycle, and age) [5].

Since lead is one of the most common heavy metals in the environment, its low concentrations can be found in many organisms. Sources of lead pollution of soil, air, and water include lead gasoline, industrial effluents, lead pipes, lead-based paints, as well as alloy and oil processing plants [3, 7]. Exposure to lead can also result in the consumption of contaminated animal tissues or plants and seeds grown in contaminated soil [7].

Lead can enter the body through the skin, respiration, or, more importantly, via the digestive tract. In case of continued contamination, it can accumulate in the body, causing acute or chronic toxicity. Lead

poisoning is a cause of disease in humans, animals, and birds. Nutrition and climate are the factors that influence this complication [8]. Symptoms of heavy metal poisoning include dizziness, nausea, vomiting, diarrhea, sleep disorders, loss of appetite, and decreased consciousness. Therefore, it is essential to monitor and estimate heavy metal levels due to their negative effects at different levels, from a biochemical response to population-level changes [9].

Currently, food safety is viewed as a significant worldwide concern, chiefly because more than 90% of human exposure to heavy metals is through food consumption [2]. In addition, food safety concerns are growing because of the accumulation of heavy metals in the environment and the consequent risks to human health [2]. One of the most important aspects of environmental quality control and food safety is monitoring the level of toxic and potentially toxic elements. Human health risk assessment is one of the most extensive systematic processes to estimate the potential impacts of health hazards on ecological systems or human populations within a specified timeframe.

There are several models for health risk assessment that require multiple contents to assess health risk. Modeling can provide a non-descriptive method for assessing exposure risks rather than damage to organisms [10–12]. The Provisional Tolerable Daily Intake (PTDI) rate is a reference value set by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) that indicates a safe daily pollutant intake [13]. The amount of PTDI is based on the daily intake of such pollutants as heavy metals that should not accumulate in human body throughout lifetime. It is used as a primary health indicator for both food and non-food sources to determine the "total exposure level to a given contaminant" [5].

Since chickens and other birds are exposed to heavy metals, such as lead, they are highly suitable for monitoring the presence and effects of environmental pollutants. In addition, studies indicate that birds in many cases are more sensitive to environmental pollutants than other vertebrates [14, 15].

Lead has been identified in chickens and its public exposure is a potential health concern. The lack of clear clinical signs in chickens that cause lead exposure or toxicity makes early detection difficult [7]. Heavy metals accumulate in various bodily tissues and organs of chickens, hence the contamination of resultant products, depending on the type of feeding and breeding conditions, which has not yet been studied comprehensively in Iran [16]. The likely entry of heavy metals into the body of a chicken may result in the contamination of eggs from farms, in addition to tissue contamination. Heavy metals can be transferred to edible parts of the egg [17]. A global egg lead concentration of 0.01–0.97 mmol/kg has been reported considering seasonal effects, which highlights the importance of health and safety assessment of this product [18].

Eggs are highly nutritious foods that benefit human health [4]. They are a rich source of essential amino acids, vitamins (e.g., vitamin E), beneficial fats (e.g., ω3 fatty acids), and trace elements (e.g., Fe, Zn, and Se) [17, 19]. Over 80% of individuals believe that eating eggs is useful for their health [20]. According to recent research on egg consumption in adults, consuming one egg per day can diminish a risk of stroke by 12% [20]. The Food and Agriculture Organization (FAO) predicts that egg consumption will increase from 6.5 to 8.9 kg per year in developing countries and from 13.5 to 13.8 kg per year in industrialized countries between 1999 and 2030 [21].

Eggs may contain high levels of heavy metals, mainly derived from food and water consumed by laying hens, which are affected by the environment. Toxic heavy metals can affect the quality of consumable eggs. Lead accumulates in the shell, yolk, and albumin. Yet, higher lead deposits in the shell than in egg contents were reported in most studies, including [22]. There are a number of methods evaluate lead concentrations in birds and their products. Adapted from previous studies, the atomic absorption spectrophotometry with graphite oven is currently used as a rapid and accessible method for this experiment [23]. Exposure of hens to lead may be chronic, with variable individual clinical symptoms resulting from different concentrations of lead [24].

Currently, industrial and other wastes enter the environment without any treatment. It is, therefore, necessary to control the amounts of heavy metals in diets and ultimately in poultry products to ensure consumer health [25]. In connection with the above, our study aimed to estimate a lead exposure risk through eggs, determine lead concentrations in three provinces of Iran, and compare lead concentrations and exposure assessments between the three provinces of Iran.

STUDY OBJECTS AND METHODS

Egg preparation and analysis. For this study, 10 samples of consumable eggs were randomly collected from 25 industrial laying-hen farms (totally 250 samples) during 4 months and transferred to the laboratory. Since Qom province has a relatively higher production volume, 15 laying farms were selected from this province. In Khorasan Razavi and Isfahan provinces, five farms were sampled from their two industrial cities, Mashhad and Kashan, respectively. The samples from each farm were then homogenized completely for the experiment.

The samples were prepared for acid digestion and reading by an atomic absorption device (Varian SpectrAA-20 plus, Australia) according to the AOAC standard method [26]. It should be noted that a standard lead solution at a concentration of 1000 μg/mL was prepared from the stoke standard (Spex CertiPrep®,

Table1 Parameters for graphite atomic absorption spectrophotometry

Metal	Wave-length, (nm)	Temperature, °C				D2 Lamp
		Drying	Ashing	Atomi-zation	Cleaning	
Lead	283.3	130 10[a] 30[b]	650 5[a] 10[b]	1900 0[a] 2[b]	2500 2[a] 2[b]	on

a: Ramp; b: Hold

USA). Lead concentrations in egg samples were determined directly in the final prepared solution using an oven atomic absorption (Varian SpectrAA-20 plus, Australia) according to the AOAC standard method [26]. The temperature program of the oven is shown in Table 1. Nitric acid (10% v/v) (68%, Darmstadt, Germany) was used as the blank.

A homogenate sample (1 g) was weighed, transferred into a crucible, and placed on a hot plate (C-MAG HP, Germany) for the ashing process. After the end of smoke from the crucible, it was placed in an oven at 200–250°C, and the oven temperature was increased gradually to 500 ± 50°C with a rate of about 50°C/h within 8 h. The process of cooling the crucible, moistening with nitric acid, and re-incubating in the oven was repeated until the sample was completely transformed to ash (grayish-white). Five milliliters of 6 M chloridyric acid (37%, Darmstadt, Germany) was then added to the crucible so that all the ash was impregnated with the acid. The residual content in the crucible was dissolved with 5 mL of 0.1 M nitric acid, covered with a watch glass, and left for 30 min. The crucible content was passed through a Whatman filter paper (0.45 μm), transferred to a 100 mL volumetric flask, and made into volume [26].

Health risk assessment. Problem formulae. In this study, the human health risk assessment (HHRA) model was used to describe the potential risk of heavy metals through the consumption of poultry eggs collected from commercial strains of laying hens in three provinces of Iran. The HHRA model was proposed by the US Environmental Protection Agency (USEPA) to calculate health risk needs (estimated daily intake, target hazard quotient, and carcinogenic risks) [27, 28].

Daily intake assessment. Estimated daily intake (EDI) of heavy metal contaminants through egg consumption depends on heavy metal concentrations in the egg content, daily egg consumption, and consumer body weight [29, 30], which is obtained using the following formula:

$$\mathrm{EDI} = \frac{C \times IR_d}{\mathrm{BW}} \quad (1)$$

where C is a heavy metal concentration (mg/kg wet weight) in the egg content, IR_d is a daily egg intake (16.95 g per day), and BW is body weight (70 kg for an adult) [31, 32].

Non-carcinogenic and carcinogenic risks. In this study, non-carcinogenic health risks associated with egg consumption were investigated using the target hazard quotient (THQ). The calculations were based on the guidelines of the United States Environmental Protection Agency [33] as follows:

$$\mathrm{THQ} = \frac{\mathrm{EFr} \times \mathrm{ED} \times FIR \times C}{\mathrm{RfD} \times \mathrm{BW} \times \mathrm{AT}} \times 10^{-3} \quad (2)$$

where EFr represents exposure frequency (365 days/year), ED denotes exposure duration (70 years for adults), FIR indicates the egg consumption rate (g for person daily), AT symbolizes the average exposure time for non-carcinogenic risk (365 days/year×70 years), and RfD shows the reference food dose (1 mg/kg BW/day) for lead [34].

Carcinogenic risks (CR) are estimated as an incremental likelihood of developing cancer in an individual throughout lifetime as a result of exposure to potentially carcinogenic factors [28]. The cancer slope factor (CSF) provided by the USEPA's Integrated Risk Information System (IRIS) is 0.001 mg/kg/day^{-1} for lead. Acceptable levels of risk for carcinogens vary from 4^{-10} (1 out of 10 000 risk of cancer development during human lifetime) to 6^{-10} (1 out of 1 000 000 risk of cancer development in human lifetime). The following equation was used to estimate lifetime CR [27, 29]:

$$\mathrm{CR} = \frac{\mathrm{EFr} \times \mathrm{ED} \times FIR \times C \times \mathrm{CSF}}{\mathrm{BW} \times \mathrm{AT}} \times 10^{-3} \quad (3)$$

Statistical analysis. Data were analyzed by descriptive statistics using SPSS 17 software (SPSS, Chicago, IL).

RESULTS AND DISCUSSION

Lead concentrations. Figure 1 compares lead levels in eggs sampled from 25 laying hen farms with the maximum permissible level (0.1 mg/kg) [35].

According to Fig. 1, the lead concentration in eggs from Qom exceeded the international standard, while eggs from Kashan and Mashhad demonstrated lower values.

Lead is a pollutant generally found in surface artifacts, but industrial emissions are a chief cause of lead exposure in animals and humans. In this research, high levels of lead in all the farms can be related to such factors as industrialization of the studied cities and high environmental contamination, including contaminated feed consumed by laying hens (Fig. 1). In addition, Islam *et al.* found high concentrations of lead in grains and vegetables, which are likely because of leads melting, heavy traffic, and other industrial activities near commercial farms [36].

After oral administration, lead or lead salts are merely partially absorbed (roughly 10% of the oral dose). Fortunately, absorption is limited due to the low solubility of many lead compounds, the deposition of lead ions with bile acids, and the formation of lead

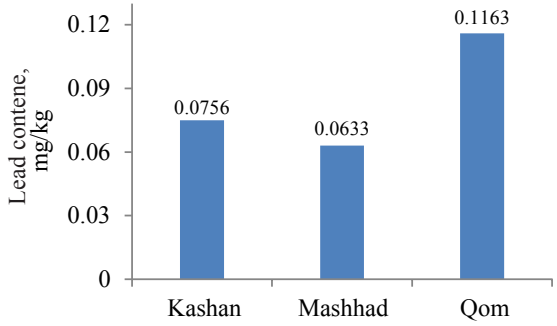

Figure 1 Comparison of international lead standard with lead contents in the egg samples under study (mean ± standard deviation)

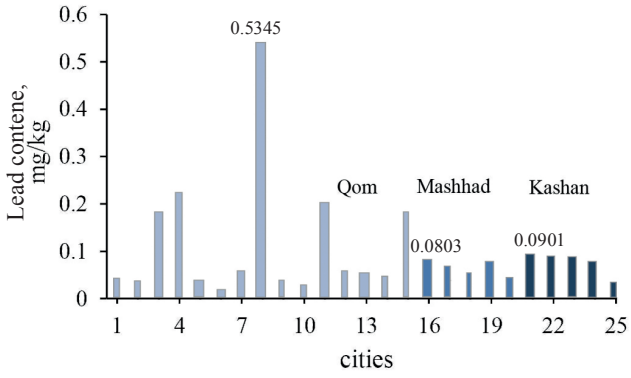

Figure 2 Comparison of lead concentration in eggs from studied regions

sulfide by Enterobacteria. It has previously been shown that interactions with other elements can affect the absorption of lead from the intestine [37].

An acute type of poisoning causes central neurological signs such as epileptic seizures or cramps, whereas chronic poisoning is often remarkably difficult to diagnose due to the lack of pathognomonic signs. Signs occur more in lead levels greater than 500 mg/kg feed. Therefore, this amount is several times higher than the maximum limit (10 mg/kg) for a separate feed of plant roots. Lead levels in laying hen feeds are higher than in broiler feeds, which may be due to the provision of calcium sources to increase the quality of eggshell, usually made from oyster or limestone shells.

It may well be contended that various kinds of calcium sources may be added to moderately higher measures of minor components in mixed feeds for laying hens, but a large scale calcium production seems to be negligible [37, 38]. Lead is naturally present in the environment as a pollutant derived from agricultural and industrial activities. Food is the principle source of human exposure to this element, which accumulates in the body and affects the central nervous system [39].

On the other hand, the number of pollutants can vary, depending on the type of industry (poultry versus livestock farming; freshwater or marine fisheries versus terrestrial animals; extensive system versus severe animal husbandry) and the geopedological attributes of soils where crops are cultivated. Also, the principal food source for laying hens in all poultry farms is grain or commercial layer pellets, and commercial feed exposure to heavy metals in storage is comparatively rare. The main route of exposure to heavy metals is actually food [37].

Our study agrees with other researchers who have shown that high concentrations of heavy metals, such as lead, in commercial eggs can be associated with the use of dietary supplements and polluted water sources, as well as pesticides to discard insects [4, 39, 40].

In Qom eggs, sample 8 showed the highest concentration of lead (0.5345), with was much higher than the international limit (Fig. 2). This could result from the proximity of farms to industrial plants. Subsequently, the highest lead levels in eggs from Mashhad and Kashan were 0.0901 and 0.0803, respectively.

Lead concentrations in the eggs under study revealed no statistically significant differences between the studied regions (Table 2, $P \geq 0.05$). A significant difference ($P \leq 0.05$) was observed between the mean concentration (0.097 mg/kg) of the studied samples and the international standard lead level in eggs (0.1 mg/kg).

Zariff *et al.* studied lead uptake rates through eggs in children from one of southern Australian cities. The authors found that eggs produced from farms near industrial areas contained the highest lead concentrations, leading to increased lead levels in the blood of those children who consumed the products of these farms [41]. Their reported mean lead concentration (0.09) is in agreement with our study results.

Farahani *et al.* also reported a lead concentration of 0.75 mg/kg in 32 laying hen farms from Markazi province, which is significantly higher than the permissible level and our results [42]. Uluozlu *et al.* and Kirkpatrick and Coffin reported lead levels of 0.05 and 0.01 mg/kg in eggs, respectively, which are lower than those in our study [43, 44]. Further, egg lead concentrations of 0.51, 0.27, and 0.13 mg/kg were detected in the studies of Basha *et al.*, Abdulkhaliq *et al.*, and Khan *et al.*, respectively, which are higher than our results [45–47]. Basha *et al.* also found a mean lead of

Table 2 Concentration of lead in eggs from different cities ($P \leq 0.05$)

Metal	Lead permissible level, mg/kg	Samples	Mean lead concentration, mg/kg	Concentration of lead, mg/kg		
				Kashan	Mashhad	Qom
Lead	0.1	125	0.0970	0.0756	0.0633	0.1163

Table 3 EDI, THQ, and CR of lead for national consumers of eggs in different regions

	Region	EDI, μ/kg^{-1} BW/day	THQ	CR
	Kashan	0.018306000	0.004577	1.55601E-07
	Mashhad	0.015327643	0.003832	1.30285E-07
	Qom	0.028161214	0.007040	2.3937E-07
PTDI		3.57	3.57	3.57
TDI		3/1.5	3/1.5	3/1.5

0.02 mg/kg obtained by consuming one egg per day [45]. Grace and MacFarlane found that increased contents of heavy metals resulted from high uptake of these elements in birds through feed and water, along with possible effects of such factors as differences in their age, species, and laying cycle [14].

Contrary to our research, a study on eggs consumed in California and another study on eggs produced in New York local orchards reported lead concentrations above 0.97 and 0.167 mg/kg, respectively [16, 18]. In Saudi Arabia, feeds used in 74 poultry farms were reported to be heavily contaminated with heavy metals [46]. Relatively higher concentrations of lead in environmentally contaminated areas detected in the previous studies confirm our findings and also indicate that environmental concerns necessitate an increasing need to determine toxic metals in eggs [48].

In order to explain the effects of contamination on poultry products, many studies have been conducted on the fetal effects of metal contaminations. In a study by Surai, hens could control high metal deposition in eggs by avoiding mineral deposition [49]. However, protective layers that may be sufficient for such minerals as chromium and manganese may not be appropriate for other metals such as lead in eggs [50].

A matter of concern is that lead disrupts the bodily enzymatic reactions, particularly the synthesis of molecules that form an important part of hemoglobin and are an essential component of oxygen transfer in the body. An increased lead content was observed in blood groups of Arak city, which was attributed to elevated environmental pollution in the industrial areas [51].

In a study on metal contents in eggs in Nigeria, an average total concentration of 0.59 μg/g was found for lead, with a strong positive correlation between metal contents in feed and the corresponding levels in eggs [52]. In Pakistan, a research on the elemental composition of eggs revealed an average lead concentration of 0.52–0.62 mg/kg [22]. Hui detected high concentrations of lead in eggs similar to those reported in eggs collected from California cities. Although the author did not specify the exact concentrations, the highest concentration was shown to exceed the estimated safe concentrations [50]. However, the need for monitoring lead concentrations has been emphasized in all of these studies.

Health risk assessment. In the present study (Table 3), lead EDI (mg/kg body weight per day) was estimated based on per capita egg consumption (16.95 g/day) in adults (70 kg body weight) [28]. Our study also compared the EDI values with those of the Provisional Tolerable Daily Intake (PTDI) and the respective Tolerable Daily Intake (TDI). The EDI values were lower than the PTDI and the TDI, indicating no lead-related health risks through egg consumption. Qom accounted for the highest value among the three provinces in this study.

Risk assessment determines the potential health effects of doses that a human receives from a contaminant through one or more exposure routes. Non-cancer risk (THQ) and CR of egg consumption for Iranians are shown in Table 3. The THQ is a ratio of the dose determined from a contaminant to the level of a reference dose. If the ratio is greater than one, the exposed population is likely to experience conspicuous adverse effects [53]. We also recorded a THQ value lower than one for lead in eggs, suggesting that lead uptake through eggs does not pose a significant non-carcinogenic risk, in line with Hashemi *et al.* [5]. Finally, the level of non-carcinogenic risk by egg consumption did not exceed the USEPA risk management criterion.

For people who consume eggs exposed to lead, there is a potential risk to their health. Although most families consume eggs three times per week, continuous exposure to high concentrations of lead can prompt adverse health impacts in children. These impacts include attention-deficit hyperactivity disorder, behavioral disorders, IQ deficiencies, and diminished brain volume [54, 55]. In fact, there are no clearly defined thresholds for lead adverse health impacts [55]. Subsequently, laying hen owners should know about the risk of repeated consumption of lead-polluted eggs, even at concentrations that they see to be inconsequential.

Carcinogenic risks. Based on animal studies, lead has been classified among carcinogenic agents [29]. In general, cancer risk at the lead concentration of less than 10^{-6} is insignificant, above 10^{-4} is considered unacceptable, and between 10^{-4} and 10^{-6} is regarded as acceptable [34]. In our study, carcinogenic risks from lead were in negligible ranges in all the three regions, indicating no carcinogenic risk for Iranian consumers through the consumption of egg-borne lead, which is consistent with other studies in Iran [32, 56]. Ki *et al.* examined cancer development through the consumption of eggs containing heavy metals in Tehran, indicating safe metal concentrations for Iranian consumers [56].

CONCLUSION

Our study investigated the presence of lead, whose potential toxicity is properly known, in eggs produced in three Iranian regions. We found that lead health risks through egg consumption are within a safe range. Despite the relatively low lead concentrations in eggs,

except for Qom province, and also the acceptable range of health risk for consumers determined here, the eggs produced in these regions contribute to a portion of daily lead consumption sources. Regular consumption of contaminated eggs in these areas is a source of lead, particularly for children and pregnant women. Given the importance of eggs in the daily diet, tolerable human intake increases economically and nutritionally, along with the availability of other food and environmental sources of this metal. Thus, more attention should be paid to this issue to consider strategies for the control and reduction of lead intake by humans. Also, control programs are recommended to reduce lead concentrations.

Therefore, a safer option is to raise awareness among poultry proprietors, public health offices, and veterinarians of the problem and potential resources. In higher risk environments (e.g., farms in industrial cities), testing environmental samples before placing poultry is recommended, and monitoring poultry and eggs after placing them ought to be considered.

CONTRIBUTION

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

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Functional and sensory properties of jam with different proportions of pineapple, cucumber, and *Jatropha* leaf

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Abstract:
Introduction. Fruits and vegetables are vital for healthy food consumption. Conservation is the only option to prolong their shelf life. Nigeria is currently experiencing an increase in production of fruit jams that incorporate vegetables. Cucumbers, *Jatropha tanjorensis* L. leaf, and pineapples have a lot of health benefits, which makes them very promising for jam making. The present research featured the effect of cucumber, pineapple, and *Jatropha* leaf in different proportions on the functional and sensory properties of composite jam.
Study objects and methods. The technology of jam making followed standard procedures. Pineapple jam without cucumbers and *Jatropha* leaf served as control (pineapple:cucumber:*Jatropha* leaf = 100:0:0). The experimental jam samples had increasing amounts of *Jatropha* leaf (J), decreasing amounts of pineapple pulps (P), and a constant amount of cucumber (C), i.e. P:C:J = 85:10:5, 80:10:10, 75:10:15, and 70:10:20. The functional analysis involved chemical and proximate aspects, whereas the sensory evaluation involved appearance, aroma, taste, spreadability, and overall liking.
Results and discussion. The experimental samples showed a significant difference ($P < 0.05$) in vitamins, minerals, total titratable acidity, pH, Brix, and total soluble solids. The control sample (P:C:J = 100:0:0) had significantly lower ($P < 0.05$) contents of moisture, protein, ash, fat, and fiber than the experimental ones. However, the pH and total titratable acidity of the experimental samples 85:10:5 and 80:10:10 appeared to be quite similar ($P > 0.05$). Compared to the control sample, the sensory properties of the experimental samples differed significantly ($P < 0.05$) by appearance, aroma, and spreadability but were of similar ($P > 0.05$) taste and overall liking.
Conclusion. The obtained functional and sensory data proved that the new pineapple jam with cucumber and *Jatropha* leaf is a promising functional product.

Keywords: Composite jam, fruits, vegetables, quality characteristics, sensory properties, proximate analysis

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INTRODUCTION

Nowadays, fruits and vegetables are increasingly vital for healthy food consumption. Not only are they highly recommended aspects of health-promoting diets, but they also contain minerals, phytochemicals, and vitamins [1–3]. Public awareness of the beneficial properties of fruits and vegetables continues to increase due to recommendations of dieticians and physicians, educational programs, and media [3, 4]. However, poor availability and considerable post-harvest losses challenge both consumption and processing of fruits and vegetables [3]. Moreover, fruits and vegetables are not always accessible at the same time due to peculiarities of location and seasonality [3].

For long-term consumer benefits, robust harvesting of fruits and vegetables requires effective and efficient

storage facilities and techniques. To preserve their freshness for a long period of time, they have to be transformed into a more stable product able to retain the initial nutritional and mineral contents [3]. Jam is an effective method of product conversion as it retains health and nutritional benefits of the raw material, especially of seasonal products [3]. Jams exist in diverse forms, e.g. chutney, fruit butter, jelly, marmalade, fruit spread, etc. [5]. To make jams, fruits and vegetables have to be cut, crushed, and/or ground until they reach required consistency [6]. Jam making includes the following major steps: thermal processing, adding sugar for pectin activation, mix formation, and packaging. Pectin helps to preserve the raw material as it substantiates the gelling agent, sugar or honey [6, 7].

Cucumbers (*Cucumis sativus* L.) possess a considerable therapeutic potential. They are an excellent source of beta-carotene, manganese, phytochemicals (alkanoids, flavonoids, tannins, phlabotannins, steroids, and saponins), and vitamin C [3, 8, 9]. Cucumbers possess anti-bacterial, antifungal, cytotoxic, antacid, but carminative properties [10], with ample amounts of water and little calories, fat, cholesterol, and sodium [11]. Cucumbers are reported to demonstrate such useful attributes as antioxidant activity, blood pressure and body weight management, cancer prevention, cholesterol reduction, and diabetes control [12].

Despite being widely cultivated in various parts of Nigeria, *Jatropha tanjorensis* still remains an exotic plant of *Euphobiacea* family [13]. In Nigeria, people call it "*Hospital is too far*". Due to their high water content, its leaves are a popular ingredient for vegetable soup with a mild, soothing taste. This dish is especially popular in the southwestern regions, where the plant is used to treat cardiovascular ailments, anemia, i.e. as a hematinic agent, and diabetes, given its hypoglycaemic properties [14–16]. Indeed, the anti-oxidant mechanism of *J. tanjorensis* gives it preventive and protective capacity to exert some anti-anemic potential [17, 18]. A phytochemical analysis of *J. tanjorensis* leaf extract revealed terpenoids, saponins, cardiac glycosides, flavonoids, and tannins [19]. Due to its antioxidant properties, *J. tanjorensis* leaf could effectively ameliorate oxidative stress, if administered in the right doses. However, its excessive consumption may impair bioavailability due to the high phytate content [20].

As for pineapple (*Ananas comusus* L. Merr.), this tropical/sub-tropical fruit can be consumed fresh, cooked, or juiced [21, 22]. Pineapple fruit comprise many individual berries that fuse around a central core [23]. Despite being highly perishable and seasonal, pineapples contain carbohydrates, calcium, crude fiber, potassium, vitamin C, water, and various minerals, which can contribute to a balanced nutrition. Ripe pineapples contain citric acid, malic acid, vitamins A and B, sugar, protein digesting enzyme, and bromelin [21]. Pineapple pulp is a complex multicomponent system, but insoluble solids can make it look opalescent or turbid [24].

When combined, the above-mentioned health benefits of cucumber, pineapple, and *J. tanjorensis* leaf should be very promising for jam making [3, 8–11, 14–18, 21]. In fact, pineapple combinations with other products proved quite successful [3, 25]. Pineapple pulp provides both sweet taste and succulent effect when incorporated into confectionary products [25]. In addition, fruit and vegetable mixes are gaining more and more popularity in the Nigerian cuisine, especially in the east of the country. The cucumber, pineapple, and *Jatropha* leaf composite jam appears to be part of local diet, which has not been reported in scientific literature, to the best of our knowledge. Considering the rapidly growing population of Nigeria, the functional role of fruits and vegetables in enhancing food security can hardly be overestimated.

This research featured the effect of blend variations on the functional and sensory properties of cucumber, pineapple, and *Jatropha* leaf composite jam. The functional analysis involved chemical and proximate aspects, whereas sensory evaluation involved such attributes as appearance, aroma, taste, spreadability, and overall liking. The research objective was to produce a jam wherein blended fruits and vegetables complement each other to actualize a composite product of higher nutritional quality and appealing sensory properties.

STUDY OBJECTS AND METHODS

Chemicals and reagents. The chemicals and reagents were obtained from reputable sources and were of analytical grade standard.

Collection of samples. Healthy and mature cucumbers and pineapples were purchased from the North Bank market situated in Makurdi, Benue State, Nigeria. The fruits and vegetables can be considered consumer safe because the North Bank market, which serves the local community, is supervised by the local government and adheres to good hygiene (GHP) and good storage (GSP) practices. The fruit samples were selected according to shape, size, uniformity, color, and integrity. Fruits with signs of damage and diseases were discarded. Fresh *Jatropha* leaves were harvested from the vicinity of University of Agriculture in Makurdi, where other people of the community usually pluck them.

Making of jam. The technology of jam making involved three steps, namely: a) preparations and processing of the selected raw materials; b) formulation of cucumber, pineapple, and *Jatropha* leaf jam samples; and c) mixing of the pulp and sugar to make the jam.

The preparations and processing involved sorting and washing to remove soil and dirt from the fruit skin. The selected fruits were peeled, cut into small pieces,

Table 1 Concentrations of cucumber fruit, pineapple, and *Jatropha* leaf in jam, %

Samples	Pineapple	Cucumber	*Jatropha*	Total
Control sample	100	0	0	100
Experimental sample A	85	10	5	100
Experimental sample B	80	10	10	100
Experimental sample C	75	10	15	100
Experimental sample D	70	10	20	100

and then crushed/blended to obtain pulp. The *Jatropha* leaf was identified by the representatives of College of Agronomy, Federal University of Agriculture, Makurdi, Nigeria. The preparation of *Jatropha* leaf followed the method used by local artisans and underwent blending to obtain pulp.

Table 1 shows amounts of cucumber, pineapple, and *Jatropha* leaf in the jam. The ingredients were combined to formulate a blend for the jam based on the following percentage ratios:

a) control sample = 100% pineapple (P:C:J = 100:0:0);
b) experimental sample A = 85% pineapple; 10% cucumber; 5% *Jatropha* (P:C:J = 85:10:5);
c) experimental sample B = 80% pineapple; 10% cucumber; 10% *Jatropha* (P:C:J = 80:10:10);
d) experimental sample C = 75% pineapple; 10% cucumber; 15% *Jatropha* (P:C:J= 75:10:15); and
e) experimental sample D = 70% pineapple; 10% cucumber; 20% *Jatropha* (P:C:J = 70:10:20).

The pineapple jam with neither cucumber nor *Jatropha* leaf served as the control sample. In the subsequent blends, the amounts of *Jatropha* leaf increased, but the amount of cucumber remained constant.

The mixing of the pulp and sugar to make the jam followed standard procedures. The pulp and sugar were mixed and heated. The soluble solid formations were monitored during the process, until 55°C was attained [26]. The pectin served as a solution base. Pectin was added to hot water and heated until homogenous. It is at this stage that the fruit pulp, sugar, and citric acid were added into the pectin solution. Next, the jam was boiled until the layer of bubbles appeared, particularly at the sides of the vessel. After that, the hot jam was poured into clean dry wide-mouthed bottles and cooled to 35°C in a water bath until gelation started. At this point, the composite jam was ready for chemical, proximate, and sensory analyses.

Functional and sensory analyses of the jam. The functional analysis involved chemical and proximate aspects. The chemical measurements determined the contents of vitamins A (*β*-carotene) and C (ascorbic acid), mineral elements (calcium, zinc, magnesium, potassium, sodium, and iron), pH total titratable acidity (TTA), total sugar content (°Brix), and total soluble solids (TSS). The proximate measurements determined the moisture, protein, ash, fat, fiber, and carbohydrate contents. The sensory evaluation involved appearance, aroma, taste, spreadability, and overall liking.

Chemical measurements. Determination of vitamins A (*β*-carotene) and C (ascorbic acid). Vitamins A (*β*-carotene) and C (ascorbic acid) were determined using the AOAC method with slight modifications [27]. Approximately 10 g of each sample was weighed into a 250 mL flask, followed by 50 mL of acetone. The mix was left for 2 h with occasional shaking and then filtered. The filtrate was measured, and an equal volume of saturated NaCl was added to wash the filtrate (carotene extract). The resulting mixture was shaken and transferred into a separating funnel to remove the layer of the extracted carotene. The supernatant was washed again with an equal volume of 100% potassium trioxocarbonate (IV) (K_2CO_3), which was separated and washed with 10–20 mL of distilled water. After separating water carotene and extracting carotenoid, the absorbance was defined by a spectrophotometer at 326 nm wavelength using 50:50 acetones and low boiling petroleum ether solution as the blank.

Determination of minerals. The list of mineral elements included calcium (Ca), zinc (Zn), magnesium (Mg), potassium (K), sodium (Na), and iron (Fe). Their content was determined using the AOAC method with slight modifications [28]. Approximately 1 g of each sample was weighed into a 100 mL round bottom flask, and 5 mL of perchloric acid was added and heated over an electric heater in a fume chamber until the solution became colorless. The solutions were diluted with distilled water to 10 mL mark, and the diluted samples were set aside for further studies. The Ca, Zn, Mg, K, Na, and Fe contents were analyzed using an atomic absorption spectrophotometer (AAS).

Determination of pH. The pH was determined using the AOAC method with slight modifications [27]. It required the use of a pH meter, calibrated using standard buffer solutions. The electrode was rinsed with distilled water and then dipped into 5 g of the sample, which was dissolved in 50 mL of water.

Determination of total titratable acidity. The total titratable acid was determined using the AOAC method with slight modifications [27]. This involved ~ 10 g of the sample dissolved in 100 mL of distilled water. Thereafter, 10 mL of the supernatant was titrated with 0.1N NaOH and phenolphthalein as an indicator. The total titratable acidity (%) was defined based on citric acid according to the equation below:

$$citric\ acid = \text{volume of NaOH used} \times 0.1N \times \\ \times \text{mL equivalent of citric acid} \times 100 \quad (1)$$

Determination of total sugar content (°Brix). The AOAC method helped to determine the total sugar content [28]. The experiment involved a hand-held sugar refractometer. The prism of the refractometer was cleaned, and a drop of the sample was placed on the prism and closed. Total sugar content (°Brix) was read off the scale of the refractometer.

Determination of total soluble solids. The AOAC method with slight modifications made it possible to determine the total soluble solids in the sample [28]. Dry empty dishes were weighed, and 5 g of the samples was put onto them. The dishes were then placed on a boiling water bath and left there until the water evaporated from the samples. The samples were then placed in an oven at 102°C for ~ 2 h. Readings were taken after cooling. The equation below was used to calculate total solids (%):

$$total\ solid = \frac{Weight\ of\ residue \times 100}{Weight\ of\ the\ sample} \quad (2)$$

Proximate measurements. Determination of moisture. The moisture of the sample was determined using the AOAC method with slight modifications [28]. Approximately 2 g of the samples were weighed in Petri dishes, then transferred into an oven, uncovered, and heated at 130–150°C for 3 h. After heating, the samples were removed and placed in a desiccator, where they were allowed to cool for 15 min before weighing. The procedure was repeated until constant mass. The loss in weight was reported as the percentage moisture content, using the equation below:

$$Moisture\ Content = \frac{Weight\ loss \times 100\%}{Weight\ of\ sample} \quad (3)$$

Determination of crude protein. The crude protein in the samples was determined using the AOAC method with slight modifications [28]. Approximately 0.8 g of each sample was digested in the Kjeldahl digestion system under a fume chamber. The digestion was allowed to cool and then distilled into boric acid containing bromocresor green indicators after it had been appropriately diluted first with water and then with solutions of sodium thiosulphate and sodium hydroxide. After that, the samples were titrated with 0.1N hydrochloric acid (HCl) solutions. Blank titrations were similarly carried out and the percentage protein content was calculated using the equation below:

$$Crude\ protein = \text{Nitrogen} \times 6.25$$
$$(1\ \text{mL of 0.1N HCl} = 0.0014\text{gN}) \quad (4)$$

Determination of ash. The ash of the sample was determined using the AOAC method with slight modifications [28]. Approximately 5 g of the sample was weighed into previously weighed ash dishes, placed in muffle furnace, and ignited at 550 ± 10°C for 5 h. After cooling, it was weighed to constant mass. The resulting ash (%) was calculated as below:

$$Ash\ content = \frac{W_2 - W_3}{W_2 - W_1} \times 100 \quad (5)$$

were W_1 is the weight of empty crucible; W_2 is the weight of crucible + weight of sample before ashing; and W_3 is the weight of crucible + weight of sample after ashing.

Determination of crude fat. The crude fat of the samples was determined using the AOAC method with slight modifications [28]. The procedure involved ~ 2 g of the prepared sample weighed into Soxhlet thimbles and fixed into the extraction flask of a given weight. Extraction with diethyl ether lasted for 5 h. At the completion, the diethyl ether was removed by evaporation on an electrical bath. The remaining fat in the flask dried in the oven at 60°C for 30 min, then it was cooled for 15 min and weighed. The fat content (%) was calculated as below:

$$Fat\ content = \frac{Weight\ of\ fat}{Weight\ of sample} \times 100 \quad (6)$$

Determination of crude fiber. The crude fiber of the samples was determined using the AOAC method with slight modifications [28]. The procedure involved weighing ~ 1 g of the sample and adding 100 mL of trichloroacetic acid as digesting reagent. The solution was brought to boil and reflux for approximately 40 min at 50–60°C. The flask was removed from the heater and cooled a little, followed by filtering the solution through Whatman filter paper. The residue was washed in hot water and methylated spirit. The filtrate was transferred to the muffle furnace, ignited at 550°C for 30 min, cooled, and weighed. The percentage of crude fiber content was calculated as follows:

$$Crude\ fiber = \text{the loss in weight after incineration} \times 100 \quad (7)$$

Determination of carbohydrate. The carbohydrate percent of the samples was determined according to the AOAC method and calculated using the equation below [27]:

$$Carbohydrate = 100 - (\%\ \text{Moisture} + \%\ \text{Fat} + \%\ \text{Protein} + \%\ \text{Crude fiber} + \%\ \text{Ash}) \quad (8)$$

Sensory evaluation. The sensory evaluation was based on a 9-point hedonic scale according to the method described by Iwe, with slight modifications [29]. Ten panelists (N = 10) compared the sensorial variations between the different jams based on particular attributes. All the panelists reported zero allergy to the jam ingredients and participated in all sensory tests. Participation was voluntary, with verbal consent obtained prior to the evaluation. Participants were served with ~ 10 g of each jam on white disposable plates with a slice of bread from the same loaf. The plates were coded with three-digit random numbers and distributed randomly among the panelists. The appearance, aroma, taste, and spreadability of the samples were evaluated according to a nine-point hedonic scale, where 1 represented "dislike extremely" and 9 represented "like extremely". The overall liking

Table 2 Vitamin and mineral content of jam from cucumber, pineapple, and *Jatropha* leaf in different proportions

Samples P:C:J	Vitamin C, mg/kg	*β*-carotene, mg/kg	Ca, mg/100g	Zn, mg/100g	Mg, mg/100g	K, mg/100g	Na, mg/100g	Fe, mg/100g
100:0:0 (control)	$691.35^{a} \pm 0.49$	$1.44^{a} \pm 0.02$	$0.12^{e} \pm 0.00$	$1.87^{e} \pm 0.03$	$0.03^{d} \pm 0.00$	$0.11^{e} \pm 0.00$	$1.78^{b} \pm 0.00$	$0.10^{e} \pm 0.01$
85:10:05	$680.65^{b} \pm 0.21$	$1.25^{b} \pm 0.04$	$0.34^{d} \pm 0.00$	$2.00^{d} \pm 0.00$	$0.06^{c} \pm 0.00$	$0.34^{d} \pm 0.00$	$1.79^{b} \pm 0.00$	$0.20^{d} \pm 0.00$
80:10:10	$569.35^{c} \pm 0.49$	$1.12^{c} \pm 0.02$	$0.40^{c} \pm 0.00$	$2.20^{c} \pm 0.00$	$0.07^{b} \pm 0.00$	$0.45^{c} \pm 0.00$	$1.81^{b} \pm 0.02$	$0.33^{c} \pm 0.01$
75:10:15	$477.45^{d} \pm 0.21$	$0.81^{d} \pm 0.01$	$0.55^{b} \pm 0.00$	$2.71^{b} \pm 0.02$	$0.09^{a} \pm 0.00$	$0.65^{b} \pm 0.00$	$1.87^{b} \pm 0.02$	$0.45^{b} \pm 0.01$
70:10:20	$436.95^{e} \pm 0.07$	$0.73^{e} \pm 0.01$	$0.61^{a} \pm 0.02$	$2.87^{a} \pm 0.00$	$0.10^{a} \pm 0.00$	$0.71^{a} \pm 0.01$	$2.59^{a} \pm 0.14$	$0.67^{a} \pm 0.03$
LSD	0.887	0.056	0.019	0.037	0.00	0.010	0.163	0.041

Values are mean ± standard deviations (SD) of two determinations

Values with same superscript within a column are not significantly different ($P > 0.05$)

P:C:J = pineapple:cucumber:*Jatropha* leaf

LSD = least significant difference

was considered as the mean of other attributes. The panelists had clean potable water to rinse/clean their mouths between each taste session to ensure the integrity of the experiment. The participants completed the score sheets after tasting.

Statistical analysis. The obtained data underwent a one-way analysis of variance (ANOVA). The results were expressed as the mean values ± standard deviation (SD) of duplicate measurements. The Fisher's least significant difference (LSD) test helped to resolve the differences between mean values. The level of statistical significance was set at $P < 0.05$ (95% confidence interval). IBM SPSS version 20.0 software (version 2011) was used to run the data analysis.

RESULT AND DISCUSSION

Variations in chemical components. Tables 2 and 3 show the vitamin and mineral composition of pineapple jams with cucumber and *Jatropha* leaf, as well as their total titratable acidity, pH, Brix, and total soluble solids. Among the experimental samples, significant differences ($P < 0.05$) were found in the chemical component composition compared to the control. The pH and TTA of the sample containing pineapple, cucumber, and *Jatropha* leaf in the ratio of 85:10:5 were similar ($P > 0.05$) to those of the sample 80:10:10. Compared to the experimental samples, however, the control sample (100:0:0) obtained noticeably higher values of vitamin C, *β*-carotene, TTA, and Brix, but lower values of Ca, Zn, Mg, K, Na, Fe, pH, and TSS.

Considering the pH among the experimental samples, the marginal decreases in acidity probably happened due to the addition of blended *Jatropha* leaf pulp, in spite of the fact that the cucumber amount was constant in samples 85:10:05 and 70:10:20. Such pH variations might agree with the data reported by Rahman about comparative studies of pineapple, papaya, and ash gourd jam preserves and candies [30]. Despite being significantly lower ($P < 0.05$) than in the control, the Brix of the blends of the current study might compete well with those of carrot-cucumber jam sweetened with honey [3].

Adding to the above-mentioned significant differences, some trends in the parameters under study were observed among the experimental samples herein. For instance, as amounts of *Jatropha* leaf increased and those of pineapple decreased, significant increases ($P < 0.05$) were found in Ca, Zn, Mg, K, Na, Fe, pH, and TSS.

On the other hand, vitamin C, *β*-carotene, TTA, and Brix demonstrated significant ($P < 0.05$) decreases. Vitamin C contents in the experimental samples could compete favorably with those in the grape and apricot jams reported by Mohd-Naeem *et al*. [31]. Indeed,

Table 3 TTA, pH, Brix, and TSS of jam from cucumber, pineapple and, *Jatropha* leaf in different proportions

Samples P:C:J	TTA, %	pH	Brix, °brix	TSS, %
100:0:0 (control)	$0.17^{a} \pm 0.98$	$3.16^{c} \pm 0.09$	$79.05^{a} \pm 0.07$	$84.38^{c} \pm 0.02$
85:10:05	$0.13^{b} \pm 0.28$	$3.18^{c} \pm 0.01$	$73.15^{b} \pm 0.07$	$84.79^{c} \pm 0.02$
80:10:10	$0.12^{b} \pm 0.35$	$3.23^{c} \pm 0.03$	$69.00^{c} \pm 0.00$	$87.31^{bc} \pm 3.59$
75:10:15	$0.09^{c} \pm 0.35$	$3.41^{b} \pm 0.01$	$66.20^{d} \pm 0.14$	$89.11^{b} \pm 0.02$
70:10:20	$0.09^{c} \pm 0.21$	$3.85^{a} \pm 0.07$	$64.10^{e} \pm 0.14$	$96.17^{a} \pm 0.04$
LSD	0.013	0.14	0.26	4.12

Values are mean ± standard deviations (SD) of two determinations

Values with same superscript within a column are not significantly different ($P > 0.05$)

TTA = total titratable acidity; TSS = total soluble solids

P:C:J = pineapple:cucumber:*Jatropha* leaf

LSD = least significant difference

Table 4 Proximate components in pineapple jam with cucumber and *Jatropha* leaf in different proportions

Sample	Proximate components, %					
P:C:J	Moisture	Protein	Ash	Fat	Fiber	Carbohydrate
100:0:0	$3.82^{e} \pm 0.03$	$0.061^{e} \pm 0.001$	$0.0140^{c} \pm 0.0010$	$0.023^{d} \pm 0.004$	$0.123^{e} \pm 0.001$	$96.14^{a} \pm 0.00$
85:10:05	$10.17^{d} \pm 0.02$	$0.134^{d} \pm 0.016$	$0.0390^{b} \pm 0.0001$	$0.145^{a} \pm 0.004$	$0.217^{d} \pm 0.009$	$89.29^{b} \pm 0.01$
80:10:10	$15.20^{b} \pm 0.02$	$0.405^{c} \pm 0.008$	$0.0410^{b} \pm 0.0001$	$0.136^{ab} \pm 0.001$	$0.305^{c} \pm 0.008$	$83.90^{d} \pm 0.01$
75:10:15	$15.61^{a} \pm 0.02$	$0.506^{b} \pm 0.008$	$0.0800^{ab} \pm 0.0020$	$0.134^{bc} \pm 0.001$	$0.417^{b} \pm 0.007$	$83.25^{c} \pm 0.01$
70:10:20	$10.88^{c} \pm 0.02$	$0.707^{a} \pm 0.007$	$0.1100^{a} \pm 0.0010$	$0.124^{c} \pm 0.006$	$0.592^{a} \pm 0.007$	$87.58^{c} \pm 0.00$
LSD	0.08	0.02	0.00	0.01	0.02	0.26

Values are mean ± standard deviations (SD) of two determinations

Values with same superscript within a column are not significantly different ($P > 0.05$)

P:C:J = pineappl:cucumber:*Jatropha* leaf

LSD = least significant difference

the presence of blended cucumber and *Jatropha* leaf pulp brought about fluctuations among the parameters under study. Pineapple, cucumber, and *Jatropha* leaf certainly helped to fortify the composite jam of this study with minerals and vitamins, which makes it a useful source of health-promoting compounds. When Zn becomes deficient, certain individuals may demonstrate carbohydrate intolerance [32]. Increase in Ca intake could help to manage and reduce the diabetic and osteoporosis situations in senior citizens [33].

Variations in proximate components. Table 4 shows the proximate components in cucumber, pineapple, and *Jatropha* leaf composite jam. The experiment demonstrated significant differences ($P < 0.05$) in moisture, protein, ash, fat, fiber, and carbohydrate among the experimental samples, compared to the control. Specifically, the proximate components of moisture, protein, ash, fat, and fiber in the control (P:C:J = 100:0:0) were significantly lower ($P < 0.05$) than in the experimental samples. Probably, it happened due to the absence of cucumber and *Jatropha* leaf in the control. Only the amount of carbohydrates in the control sample was significantly higher ($P < 0.05$) than in the experimental samples.

However, the sample P:C:J = 85:10:5 demonstrated drastic increases and decreases in proximate components, which clearly shows the impact of cucumber pulp and *Jatropha* leaf. In particular, with increasing *Jatropha* leaf and decreasing pineapple amounts, some substances increased significantly ($P < 0.05$) among the experimental jams. Thus, protein, fat, and fiber increased from 0.134 to 0.707%, from 0.039 to 0.110%, and from 0.217 to 0.592%, respectively. However, this increase did not happen in the experimental sample P:C:J = 70:10:20, where moisture and fat contents decreased significantly ($P < 0.05$).

Given its carbon and nitrogen provision for the gluconeogenesis and energy synthesis, protein remains among the key macro-nutrients gaining increasing attention, particularly in terms of ecology and economy [34]. Additionally, ash content considers the total minerals in food: it is the inorganic material left after organic matter has been oxidized [35]. In the current study, the addition of cucumber and *Jatropha* leaf increased the content of crude protein, thus elevating the curcumin in the experimental samples. Curcumin is a protein moiety with anti-inflammatory and antioxidant properties [36, 37]. The chemical constituent of curcumin could improve the episodic memory in cadmium induced (memory) impairment via acetycholiesterase and adenosine deaminase activities [38, 39].

Table 5 Sensory attributes of pineapple jam with cucumber and *Jatropha* leaf in different proportions

Sample	Sensory properties				
P:C:J	Appearance	Taste	Aroma	Spreadability	Overall liking
100:0:0	8.20^{a}	7.93^{a}	7.67^{a}	3.87^{b}	7.27^{a}
85:10:05	7.07^{b}	7.93^{a}	7.67^{a}	7.27^{a}	7.73^{a}
80:10:10	6.47^{bc}	7.93^{a}	7.27^{a}	7.67^{a}	7.60^{a}
75:10:15	5.73^{cd}	7.33^{a}	6.93^{ab}	7.80^{a}	7.53^{a}
70:10:20	5.07^{d}	7.07^{a}	6.47^{b}	7.60^{a}	6.87^{a}
LSD	1.05	n.s.	0.70	1.05	n.s.

Values are mean sensory scores obtained from ten panelists

Values with same superscript within a column are not significantly different ($P > 0.05$)

P:C:J = pineapple:cucumber:*Jatropha* leaf

LSD = least significant difference

n.s = not significant

Vegetables play essential role in human nutrition and can provide some carbohydrates, proteins, and energy [35]. The crude fiber of jam could help to secure the intestinal mucous, thereby excluding any malignant growth-causing elements [40]. In the current work, the moisture peaked in the experimental sample P:C:J = 75:10:15. The increase in ash also indicates that this sample is a promising source of minerals [13]. Carbohydrate content in the experimental samples decreased significantly ($P < 0.05$), specifically in the samples 85:10:5 (89.29%), 80:10:10 (83.90%), and 75:10:15 (83.25%). Such decreases in carbohydrate suggest the composite jam can be highly promising for diabetes management. However, the sample 70:10:20 demonstrated the increased carbohydrate content (87.58%).

Sensory attributes. Sensory evaluation includes such aspects as appearance, aroma, taste, etc., which cumulatively helps to reveal the overall liking. Typically, aroma plays a key role in the overall liking [25]. Table 5 shows the sensory attributes of cucumber, *Jatropha* leaf, and pineapple composite jam. The experimental samples appeared significantly different ($P < 0.05$) in appearance, aroma, and spreadability but not ($P > 0.05$) in taste and overall liking, compared to the control.

For appearance, the control sample (P:C:J = 100:0:0) scored much higher than the experimental samples. With decreasing pineapple and increasing *Jatropha* leaf proportions, the appearance obtained somewhat decreasing trend: 85:10:5 > 80:10:10 > 75:10:15 > 70:10:20. For aroma, the score of the control (100:0:0) sample did not differ significantly ($P > 0.05$) from that of 85:10:5, 80:10:10, and 75:10:15. For spreadability, the control scored significantly lower than the experimental samples 85:10:5, 80:10:10, and 75:10:15.

Compared to the control, the variations in appearance, aroma, and spreadability can be attributed to cucumber and *Jatropha* leaf blends. However, such variations could also arise from the (slight) differences in thermal treatment during the jam making process. Moreover, either decreasing the pineapple or increasing *Jatropha* leaf amounts did not significantly affect ($P > 0.05$) the spreadability. It is highly unlikely that the respective peaks and drops of *Jatropha* leaf and pineapple amounts had an impact on the taste of the experimental samples. Additionally, the overall liking did not show significant changes ($P > 0.05$) among the experimental samples. Potentially, the sample P:C:J = 85:10:5 appeared more preferable based on consumer acceptability, given the higher scores for appearance and taste, compared to the other samples.

CONCLUSION

This study featured the effect of different proportions of pineapple, cucumber, and *Jatropha* leaf on the functional and sensory properties of the composite jam.

The vitamin and mineral composition, as well as total titratable acidity (TTA), pH, Brix, and total soluble solids (TSS) showed significant differences ($P < 0.05$), with the minor exceptions of pH and TTA. The proximate components (ash, fat, fiber, moisture, and protein) of the control (P:C:J = 100:0:0) appeared significantly ($P < 0.05$) lower compared to the experimental samples. The appearance, aroma, and spreadability demonstrated significant differences ($P < 0.05$), but the taste and overall liking remained similar ($P > 0.05$). The composite jam proved functionally nutritious and demonstrated a good sensory appeal.

Jatropha leaf appears to be on the rise in scientific literature. Despite this, it still remains understudied. Further research should be aimed to promote it as a functional food ingredient and ensure the consumer safety of the finished product. In the future, pineapple jam with cucumber and *Jatropha* leaf should be tested for quality and shelf-life under various storage conditions.

CONTRIBUTION

A.F. Ogori, J. Amove, and P. Evi-Parker conceived, designed, and performed the analysis, collected the data, and wrote the manuscript. A.F. Ogori, J. Amove, G. Sardo, C.O.R. Okpala, G. Bono, and M. Korzeniowska contributed to the data analysis. G. Sardo, C.O.R. Okpala, G. Bono, and M. Korzeniowska proofread the manuscript.

CONFLICTS OF INTEREST

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

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