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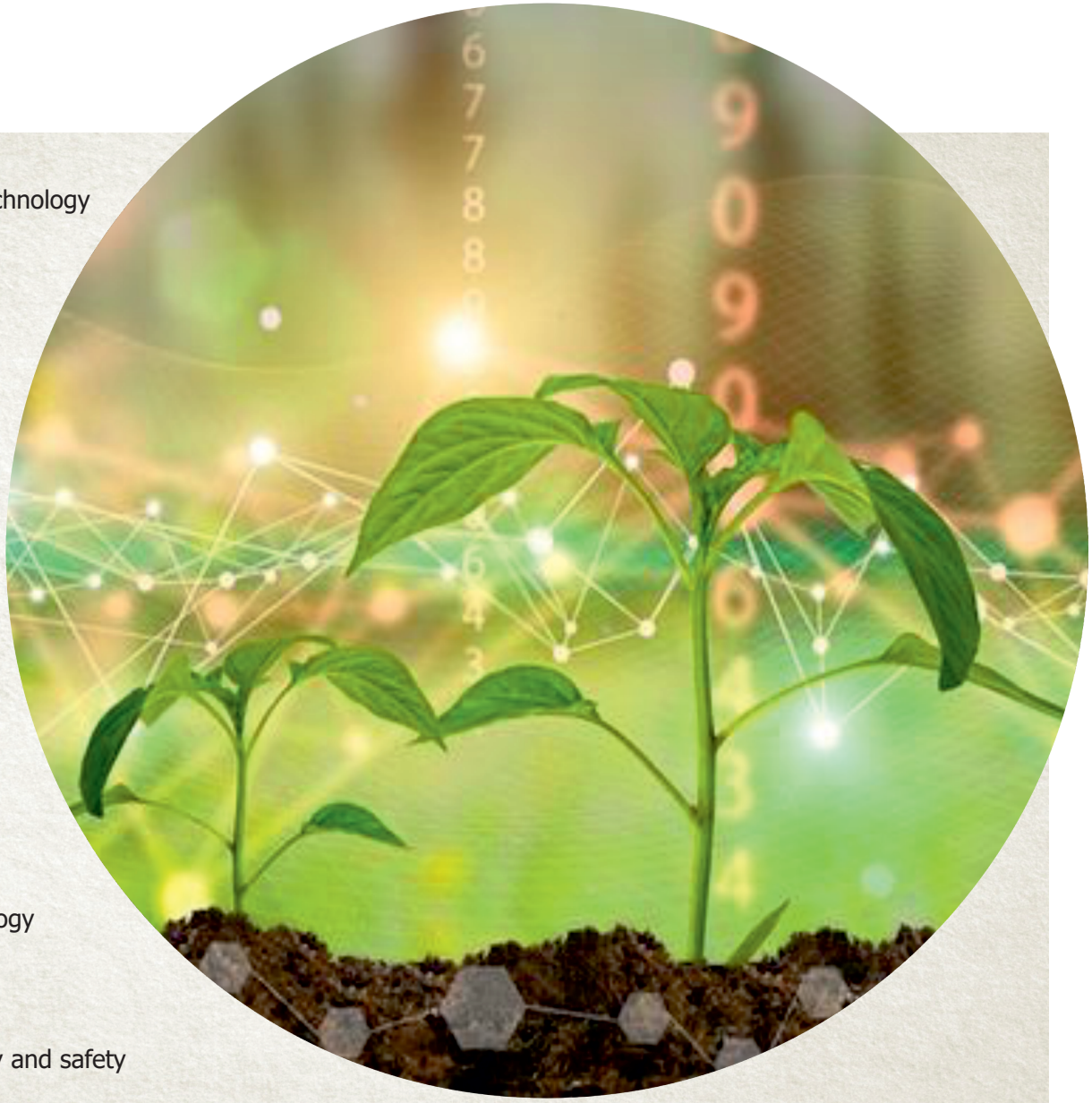

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Editorial
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Editor's column

The management of agricultural resources needs to be optimized and digitalized, and this is a major scientific task. To produce crops of a given quality and quantity, researchers are developing new farming techniques adapted to changing climate conditions and based on digital systems, agricultural landscape design, biotesting, and bioindicators for agroecosystems. Digital farming helps to reduce costs and increase yields and food production.

Large farmers are starting to use drones for a variety of purposes, e.g., to detect pests or diseases, as well as to spray crops with pesticides or fertilizers. With the help of sensors, they monitor the level of soil moisture and adjust it through irrigation or moisture control in greenhouses.

Digital systems, remote sensing and sensors are employed to assess the soil's health and condition by monitoring its pH and nutrient levels, as well as for its preservation and reclamation. With the help of sensors and data analytics, digital farming allows for accurate yield predictions, helping farmers to plan production and reduce losses. Virtual maps provide a digital representation of the fields, with accurate locations of different crops, field boundaries, and irrigation lines.

Agroindustrial machinery is equipped with sensors and software for autonomous operation. Automation improves accuracy and efficiency, let alone reduces the need for human intervention.

Agriculture has recently seen an increase in data exchange. Sharing digital information allows farmers to accurately measure their resources and manage them efficiently. Models have been created in which farmers benefit from data sharing, from purchases to loans. This exchange of digital information makes it easier for them to adopt new technologies.

Due to a reduction in arable land, farmers and agricultural enterprises have to find new ways to use the limited resources, such as vertical indoor farming or regenerative agriculture. Vertical farming allows for a year-round crop production in a limited space with 70% less water and fewer pesticides compared to standard farming methods.

Regenerative agriculture is also on the rise, aiming to improve the soil's health and biodiversity, as well as restore ecosystems. Agriculture accounts for about 25% of all CO_2 emissions. Combined with methane, these harmful greenhouse gases cause climate change. Regenerative agriculture offers effective tools for mitigating climate change, such as cover farming, composting, crop rotation, and rotational grazing. With this technology, plants extract CO_2 from the atmosphere through photosynthesis and store carbon in the soil.

Gennady P. Gamzikov, Doctor of Biological Sciences, Academician of the Russian Academy of Sciences, and an Honored Scientist of Russia, specializes in developing highly effective, non-traditional approaches in agrochemistry, soil science, farming, and ecology. He has opened a new page in the research of the nitrogen status of soils in the Asian part of Russia. Gennady P. Gamzikov's analog diagnostic system based on the autumn analysis of soils for nitrate nitrogen is highly instrumental in making recommendations for using fertilizers in Siberia, Trans-Urals, and Northern Kazakhstan.

The analytical database created by Academician Gennady P. Gamzikov can be used to describe, predict, and optimize the processes of circulation, balance, and transformation of soil nitrogen and phosphorus during short-term or long-term application of fertilizers, depending on farming systems.

Gennady P. Gamzikov is also a founder of the Siberian agrochemical school that occupies a prominent place in Russian and world agrochemistry, boasting numerous developments and publications on soils and plants.

In 2023, Academician Gennady P. Gamzikov celebrates his 85th birthday. We congratulate Gennady Pavlovich on his jubilee and wish him good health, inexhaustible vitality, optimism, and new creative achievements.

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

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Functional instant beverages

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Abstract:
Brown algae are a source of hydrothermal extracts that can serve as an effective raw material for instant beverages. This article offers new formulations of functional instant beverages made of concentrated fruit juices and algal extracts of *Saccharina japonica* and *Sargassum miyabei* Yendo. The research objective was to define their bioactive and antioxidant profiles.

The research featured *S. miyabei* Yendo and *S. japonica* brown algae from the Far East of Russia, their dry hydrothermal extracts, and instant drinks based on these extracts combined with concentrated juices of cranberry, sea buckthorn, and chokeberry. The list of methods included spectrophotometry, high-performance liquid chromatography, and gas chromatography.

The hydrothermal algal extracts of *S. miyabei* and *S. japonica* were rich in fucoidan, phenolic compounds, and iodine. The new instant beverages underwent a sensory evaluation. They contained iodine, phenolic compounds, vitamins (ascorbic acid), fucoidan, pectin, flavonoids, anthocyanins, catechins, carotenoids, and tocopherols. All the samples could be classified as functional, but the best antiradical properties belonged to the sample with black chokeberry juice and *S. miyabei*.

The new functional instant beverages had a high radical-binding activity, which reached 96.3%. One portion (200 mL) covered 27–30% of the recommended daily intake of iodine and 22–50% of vitamin C. The obtained results prove that instant beverages made of *S. japonica* and *S. miyabei* Yendo can be used as functional products.

Keywords: Brown algae, *Sargassum miyabei*, *Saccharina japonica*, instant beverages, iodine, fucoidan, cranberry, sea buckthorn, black chokeberry

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INTRODUCTION

As an integral part of human diet, beverages are responsible for water and electrolyte balance. They are consumed by all population strata. Beyond the scope of standard nutrition, they are part of therapeutic and preventive diets [1]. Non-alcoholic beverages include fruit, vegetable, and berry juices, mineral water, tonic extracts, decoctions, etc. Drinks can be easily modified to expand the product line with bioactive and antioxidant functional products.

Instant beverages possess a number of serious advantages, e.g., a convenient commodity form, a long shelf life, a multicomponent composition, etc. They can be used in almost any conditions, which makes them an important part of meals ready to eat for people who work in extreme conditions, e.g., the military, emergency responders, oilfield workers, etc. Instant beverages both replenish the water balance and provide valuable biologically active substances. Functional beverages can improve certain deficiencies, e.g., those of iodine, selenium, etc.

As a rule, instant beverages are based on land plants, e.g., fruits, vegetables, and berries that possess good sensory properties and are rich in vitamins, minerals, and antioxidants [2–4]. Seaweeds, or kelps, are known to contain a lot of biologically active substances with various antioxidant, antibacterial, anti-inflammatory, and anticarcinogenic properties [5–7]. However, kelps have a very limited application in beverage production. This research offers new formulations of functional beverages that combine biologically active substances of both marine and land origin.

Unfortunately, algal extracts have a very specific smell and taste. These sensory indicators have to be optimized for this raw material to find a wider application in beverage industry. Therefore, a functional

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beverage that would combine algal biologically active substances with an attractive sensory profile is an urgent task of the contemporary food science.

Saccharina japonica of the *Laminariales* family is brown algae that are widespread in the Sea of Japan. They develop in thickets at 0.5–12 m below the sea surface. *S. japonica* has a thallus, holdfast, stipe, and blade. This kelp contains numerous micro- and macroelements, e.g., iodine, calcium, zinc, magnesium, iron, and selenium, as well as vitamins and such polysaccharides as fucoidan, laminarins, alginic acids, etc. [8].

Sargassum miyabei of the *Sargassaceae (Decne) Kutz* family is another common kelp that grows in the Seas of Japan and Okhotsk in the Russian Far East. Its thallus is as long as 2.0–2.5 m and can weigh as much as 7 kg. *S. miyabei* is used in the food industry and pharmacy as a source of food and feed additives. This kelp is rich in vitamins A and B (B_1, B_6, and B_{12}), as well as in immunostimulatory and antitumor minerals and polysaccharides [9].

Hydrothermal *S. miyabei* extracts obtained by boiling and autoclaving demonstrated a significant content of phenolic substances and excellent antiradical properties [10].

Berry and fruit juices can be converted into functional beverages that possess an attractive sensory profile and contain biologically active substances. Sea buckthorn, cranberry, and chokeberry can serve as prospective raw material for such drinks. Concentrated juices are especially effective. They are obtained by vacuum evaporation or freezing to 44–62% solids and require no extra sugar. The resulting substance is thick and rich in color, which is usually similar to the color of the original juice. In addition, concentrated juices retain the original biologically active substances and vitamins.

In this research, the choice of fruit and berry juices depended on the content of biologically active substances. Sea buckthorn berries contain 3.5% sugars represented by glucose and fructose; organic acids represented by ascorbic, malic, and citric acids; vitamins B_1, B_2, E, and P; such trace elements as iron, boron, and manganese, as well as pectins, oils, and tannins [11]. Sea buckthorn (*Hippophae* L.) is a rare multivitamin plant in terms of water-soluble and fat-soluble substances, e.g., ascorbic acid, phenolic compounds, carotenoids (vitamin A), and tocopherols (vitamin E) [12]. Sea buckthorn berries are known for their anticarcinogenic and immunomodulatory properties [13, 14].

Cranberries (*Viburnum opulus* L.) are a source of vitamins C, K, and P, as well as various microelements [15]. They contain such biologically active substances as carotenoids and flavonoids that are well-known capillary strengtheners and choleretics. They also contain antitoxic pectins. The content of tannins in *V. opulus* is 2.01–4.15% [16]. Tannins possess P-vitamin properties, which makes them powerful antioxidants. The content of pectins in cranberries can reach 2.75% [17]. Their ability to bind with harmful substances make them excellent intestinal sorbents.

Black chokeberries (*Aronia melanocarpa* L.) are also rich in biologically active substances, which makes them a popular raw material for pharmaceuticals and food products. They contain carotenoids, bioflavonoids, pectins, organic acids, ascorbic acid, minerals, etc. Black chokeberries also contain such trace elements as boron, fluorine, iron, copper, manganese, and molybdenum. In addition, 100 g of fresh chokeberries contains 6–10 μg of iodine compounds. Also, chokeberries are a source of flavonoids that are known for their antioxidant properties and a beneficial effect on the cardiovascular system [18]. In fact, *Aronia* bioflavonoids proved to be an outstanding natural cardioprotector. A clinical study of 44 patients demonstrated that a chokeberry extract could reduce blood pressure and the level of low-density lipoproteins in the blood. The extract also appeared to be an effective antiaggregant and homocysteine inhibitor. According to some rat studies, chokeberry reduced the concentration of triglycerides and total cholesterol in the blood [19].

The present research objective was to develop granular instant beverages based on various combinations of algal extracts of *S. japonica* and *S. miyabei* and concentrated fruit juices. The resulting mixes were tested for biologically active substances and antioxidant properties.

STUDY OBJECTS AND METHODS

The research featured hydrothermal extracts of *Sargassum miyabei* and *Saccharina japonica*, as well as new instant beverages based on these extracts combined with concentrated sea buckthorn, cranberry, and black chokeberry juices.

The samples of *S. miyabei* and *S. japonica* were harvested in May 2020 in Nakhodka Bay of the Sea of Japan. They were placed in plastic bags, cooled with ice, and delivered to the laboratory, where they were washed with running tap water to remove salt, sand, and epiphytes. After that, the samples were soaked twice in distilled water. Each thallus sample was ground using a Waring mill and stored in a sealed container at –20°C. The hydrothermal extracts were obtained by boiling in water for 60 min. The ratio of raw material to water was 1:2. The suspension was filtered through three layers of gauze. The resulting filtrate underwent infrared drying until ≤ 8% residual moisture. The pulsed heating-cooling mode of the infrared drying was as follows: irradiation time – 7–11 s, cooling time – 14–22 s, specific energy flux density – 12–15 kW/m^2, limit temperature – 85–95°C, total processing time – 8 h. After that, the dry extract was ground.

The samples of 1,1-diphenyl-2-picrylhydrazyl (DPPH), BHT-2,6-ditretbutyl-4-methylphenol (ionol), and tannic acid were purchased from Sigma-Aldrich (USA). The Folin-Ciocalteu phenolic reagent was obtained from Fluka (Switzerland). Other reagents were of analytical grade.

To determine the ash content, we removed organic matter from the sample, burned it, and measured the

ash weight. We used the titrimetric method to define the iodine content according to the colored complex compound that iodine produced with sodium nitrite in an acidic medium. The Kjeldahl method helped determine the protein content. The content of alginic acid was determined by the titrimetric method. The excess sodium hydroxide after the alginic acid reaction in the test sample was titrated with sulfuric acid. The content of mannitol was determined using a UV-1800 scanning spectrophotometer (Shimadzu, Japan).

The amount of fucose in the kelp was determined spectrophotometrically by the color reaction between fucose, L-cysteine, and sulfuric acid. To determine the amount of fucoidan in the biomass, the fucose content was multiplied by two, based on the average fucoidan content in fucoidan, which was 50% [20].

The spectrophotometry method with the Folin-Ciokalteu reagent made it possible to determine the total content of phenolic compounds. A mix of phosphotungstic and phosphomolybdic acids was restored in an alkaline medium to determine the total content of phenols in medicinal plant raw materials and food products [21]. The experiment involved a UV-1800 scanning spectrophotometer (Shimadzu, Japan).

The quantitative content of carotenoids was determined on a UV-1800 scanning spectrophotometer (Shimadzu, Japan) in an acetone extract at a wavelength of 450 nm [22].

We used the titrimetric method to define the content of vitamin C [22].

The content of anthocyanins was determined spectrophotometrically with buffer solutions with pH of 1.0 and 4.5 [23]. The flavonoid profile was described according to the method introduced by Calado *et al*. [24]. The pectins were studied by the titrimetric method based on alkali titration of isolated pectin substances before and after hydrolysis. The quantitative content of catechins was determined spectrophotometrically at a wavelength of 504 nm [25]. The content of tocopherols was determined by high-efficiency liquid chromatography [26].

The sensory evaluation of the finished products included appearance, color, taste, and smell. The maximal score was eight points for appearance and color and nine points for taste and smell. The obtained score was ranked as follows: "excellent" – 22–25 points, "good" – 18–21 points, "fair" – 14–17 points, "not satisfactory" ≤ 14 points.

The nitrosamine test relied on the complex gas chromatography method with a fast chemiluminescent detector as described in [27]. The polychlorinated biphenyl test followed the gas-liquid chromatography procedure designed by Zabelina *et al*. [28].

We also tested the samples for such toxic elements as lead, cadmium, copper, and arsenic using an AA-7000 atomic absorption spectrophotometer (Shimadzu, Japan) according to the standard procedure [29]. The samples were mineralized with potassium permanganate and a mix of nitric and sulfuric acids. The detection limits for mercury, lead, cadmium, and arsenic were 0.01 mg/kg. We used the method of flameless atomic absorption to define the content of mercury in an Hg-1 mercury analyzer (Hiranuma, Japan). The qualitative and quantitative elemental composition was described by the method of atomic absorption spectrometry in an AA-7000 spectrophotometer (Shimadzu, Japan) with a graphite cuvette and a deuterium lamp background corrector. An average assay included three samples pre-dried at 80°C and mineralized with nitric acid. The elemental solutions were standard, state-certified, and registered by the Association of Official Analytical Chemists [29].

To define the content of Cesium-137 and Strontium-90, we measured their specific activity using a Progress-Gamma scintillator (Russia).

The radical test for 2,2-diphenyl-1-picrylhydrazyl (DPPH) included the following procedures. The antiradical properties of the finished products were evaluated using the stable free DPPH radical *in vitro* [30]. The optical density was measured at λ = 517 nm using a UV-1800 scanning spectrophotometer (Shimadzu, Japan) in 1-cm cuvettes at 25°C.

The radical-binding properties were described based on the radical-binding activity and the effective concentration which could scavenge 50% of DPPH free radicals (EC_{50}), mg/mL.

The radical-binding activity (RBA, %) was calculated according to the following formula:

$$RBA = [A_0 - A_1] / A_0 \times 100 \quad (1)$$

where A_0 was the optical density of the control solution; A_1 was the optical density of the test extract.

All the tests were conducted in triplicates. The experimental data were presented as M ± m. The obtained data were processed in Excel and Statistica 7.0. Statistical significance was tested by the Student's t-test at a 95% significance level.

RESULTS AND DISCUSSION

The method of hydrothermal extraction means that water-soluble substances move from the original raw materials to the extract. Figure 1 shows the chemical composition and content of biologically active substances in the algal extracts of *Sargassum miyabei* and *Saccharina japonica*. The hydrothermal extracts were salty, brown-green, and smelled of kelp. The extracts were highly soluble in water and formed a clear light green liquid with a slightly algal smell and salty taste.

The algal extracts contained a lot of combustible matter, especially the sample with *S. japonica*. The protein content in both extracts was low, especially in the *S. miyabei* sample, because it was law in the initial raw material. As for biologically active substances, both samples contained fucoidan, laminaran, and alginic acid. The content of fucoidan was especially high and exceeded 5.1 g/100 g of solids in the *S. miyabei* sample;

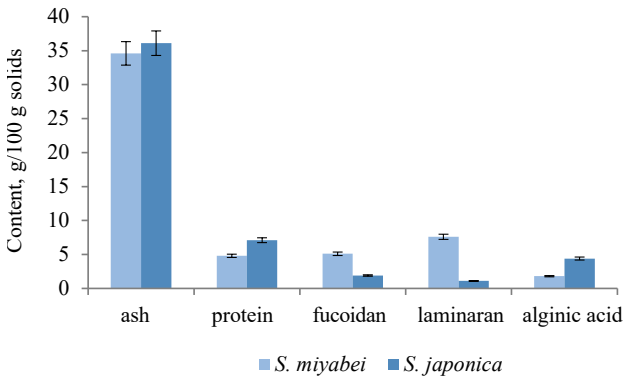

Figure 1 Chemical composition and bioactive profile of *Sargassum miyabei* and *Saccharina japonica* algal extracts

Table 1 Macro- and microelement profile of algal extracts of *Sargassum miyabei* and *Saccharina japonica* (solids)

Element	Content, g/100 g	
	Extract *Sargassum miyabei*	Extract *Saccharina japonica*
Na	0.049 ± 0.002	0.040 ± 0.002
K	0.172 ± 0.008	0.246 ± 0.0102
Ca	0.013 ± 0.0006	0.025 ± 0.0011
Mg	0.012 ± 0.0006	0.010 ± 0.004
Cr	0.00003 ± 0.000001	0.00004 ± 0.000002
Mn	0.00008 ± 0.000004	0.00005 ± 0.000002
Ni	0.000007 ± 0.0000003	0.000005 ± 0.0000002
Fe	0.005 ± 0.0002	0.004 ± 0.0002
Co	0.000001 ± 0.0000005	0.000002 ± 0.0000001
Zn	0.0000008 ± 0.00000008	0.000009 ± 0.0000003
Mo	0.000001 ± 0.0000005	0.000009 ± 0.0000004
Se	0.000005 ± 0.0000002	0.000003 ± 0.0000001

however, it was 2.7 times lower in the *S. japonica* sample. Fucoidan is a sulfated heteropolysaccharide with an extremely wide range of biological activities, e.g., antitumor, immunomodulatory, antibacterial, anti-inflammatory, etc. [31–33]. Fucoidan is a natural anticoagulant. Its effect is similar to that of heparin, but the mechanism is different [35]. In addition, fucoidan possesses an antiviral effect [36]. It is a powerful natural antioxidant that protects cells from damage by free radicals [37, 38]. Fucoidan has no cytotoxic effect. It affects both the primary tumor focus and its metastases, even distant ones [39].

The content of iodine was ≥ 0.0019 g/100 g of solids. The *S. japonica* sample contained 1.8 times more iodine than the *S. miyabei* sample. Iodine is an essential element that affects the metabolism of proteins, fats, and carbohydrates. It controls the balance of syntheses and catabolisms in the body. Iodine is important for the thyroid gland because it is responsible for such hormones as thyroxine and triiodothyronine [40]. In general, iodine facilitates the growth and differentiation of cells of all tissues, as well as transports sodium and hormones. Iodine deficiency triggers such conditions as endemic goiter, hypothyroidism, and arterial hypotension. It inhibits metabolism and cognitive development in children. Iodine deficiency also affects the reproductive system in women: it may cause miscarriages and stillbirths. Moreover, iodine balance is crucial for pre-natal and post-natal development [41, 42].

Phenols were another class of biologically active substances registered in the algal extracts. Phenolic compounds are often found in plants. If consumed regularly, phenols reduce the risk of cardiovascular diseases. Plant phenolic compounds owe their high biological activity to their antioxidant properties. In addition, phenols can affect the enzymic activity of xenobiotic metabolism [43].

The content of phenolic compounds in the *S. miyabei* and *S. japonica* extracts was 251 and 196 mg/g of solids in terms of tannic acid, respectively. The list of phenolic compounds included chlorogenic, coffee, 2,5-dihydroxybenzoic, coumaric, ferulic, salicylic, and syringic acids, as well as epigallocatechin gallate, epicatechin, and epicatechin gallate.

As they live in sea water, kelps possess a selective ability to accumulate macro- and microelements in much larger concentrations than in their environment. The mineral profile of kelps includes potassium, calcium, magnesium, iron, manganese, selenium, etc. (Table 1).

As for macro-elements, potassium predominated in both extracts, but its content in the *S. japonica* sample exceeded that in the *S. miyabei* sample by 43%. The *S. japonica* sample had a 92% greater calcium content than the *S. miyabei* extract. The content of magnesium and sodium was similar in both extracts. As for micro-elements, the extracts contained eight trace elements where iron and manganese predominated, especially in the *S. miyabei* sample. The content of nickel, cobalt, chromium, zinc, and selenium was similar in both extracts. However, the *S. japonica* extract had nine times more molybdenum.

The research included a traditional technology for non-alcoholic granulated instant drinks, which consisted of three stages: preparation of raw materials, mixing and drying, and dry granulation. The algal extract was mixed with powdered sugar in a mixer for 10–15 min at 100 rpm. After that, it was mixed with a certain amount of concentrated berry juice at 100 rpm for 10–15 min. The resulting mix was loaded into the dryer tank and dried in a fluidized bed at 35–40°C for 30–40 min until the residual moisture fell below 7%. After drying, the mass was granulated through a sieve with a mesh of 2.0 or 3.0 mm. The finished product was packaged in 10-g bags. After that, 10 g of substance was dissolved in 200 mL of drinking water at 25–40°C. The drink was restored by stirring for 1–2 min.

Table 2 illustrates the mix ratios of the instant fortified beverages. The experimental formulations were based on the best sensory evaluation, where the absence of specific algal smell and taste was the main requirement.

Table 2 Formulations of instant drinks based on a combination of algal extracts of *Sargassum miyabei* or *Saccharina japonica* and concentrated berry juices

Component	Content, per 100 kg of finished product			
	Powdered sugar, kg	*Sargassum miyabei* extract, kg	*Saccharina japonica* extract, kg	Concentrated berry juice, L
Concentrated black chokeberry juice	40	–	24	36
		26	–	34
Concentrated cranberry juice	44		23	31
		25		33
Concentrated sea buckthorn juice	42	–	21	37
		23	–	35

Table 3 Sensory profile of dissolved instant beverages based on a combination of algal extracts of *Sargassum miyabei* or *Saccharina japonica* and concentrated berry juices

	Sea buckthorn sample	Cranberry sample	Black chokeberry sample
Appearance	Transparent; no sediment	Transparent; no sediment	Opaque; no sediment
Color and taste	Orange-yellow, typical of sea buckthorn berries; sweet and sour	Burgundy-red, typical of cranberries; sour and tannic	Purple-red, typical of chokeberries; sweetish and tannic
Smell	Typical of sea buckthorn berries	Typical of cranberries	Typical of chokeberries

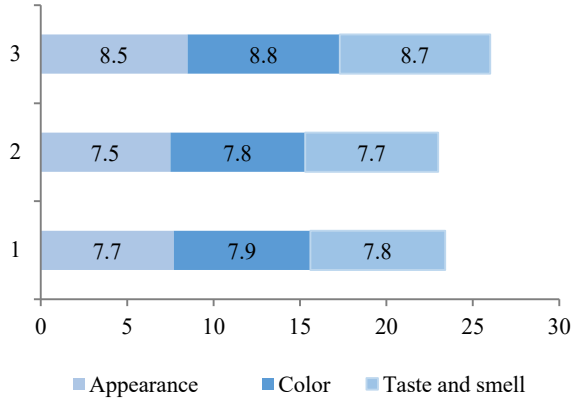

Figure 2 Sensory evaluation of dissolved instant beverages based on a combination of algal extracts of *Sargassum miyabei* or *Saccharina japonica* and concentrated berry juices:
1 – cranberry juice, 2 – chokeberry juice, 3 – sea buckthorn juice

The cranberry sample required the highest content of powdered sugar, which was at its lowest in the formulation with chokeberry juice. The content of algal extracts stayed below 24% to suppress the specific algal taste and smell. The optimal content of concentrated fruit juice was between 31 and 37%.

The dissolved concentrates were tested for such traditional sensory properties as appearance, color, taste, and smell (Table 3).

When dissolved in water, the new instant beverages were transparent or opaque; they maintained the taste and smell of the berries that were part of the formulation.

Figure 2 illustrates the sensory evaluation results of the developed functional beverages.

The buckthorn juice sample achieved the best score for appearance, taste, and smell, while the cranberry sample had the best color. The total sensory scores were as follows: cranberry sample – 23.4 points, chokeberry sample – 23 points, sea buckthorn sample – 26 points. All the samples were rated as excellent, but the sea buckthorn sample received the highest total score.

Sensory profile is important for prospective consumers. However, for functional foods the biological activity of the food system is more important, i.e., the content of biologically active substances, e.g., vitamins, carotenoids, flavonoids, mineral elements, etc., that produce a proven positive effect on human health. Table 4 demonstrates the content of biologically active substances in the new functional beverages.

The samples revealed a wide range of biologically active substances: iodine, phenolic compounds, ascorbic acid, fucoidan, pectin, flavonoids, anthocyanins, catechins, carotenoids, and tocopherols. The content of iodine was 42–89 μg per 200 mL and depended on the type of the algal extract in the formulation. *S. japonica* contributed more iodine than *S. miyabei*. The content of vitamin C was 20–45 mg per 200 mL and depended on the type of juice. It was maximal in the sea buckthorn sample and minimal in the chokeberry sample. The content of fucoidan was 40–80 mg per 200 mL and depended on the algal extract: *S. miyabei* provided two times more fucoidan than *S. japonica*. The content of pectin ranged from 25 to 63 mg per 200 mL. The maximal content belonged to the cranberry sample. The cranberry sample also demonstrated the biggest amount of flavonoids: up to 20 μg% per 200 mL. The chokeberry sample contained the greatest content of anthocyanins, which reached 2.4 mg% per 200 mL. The sea buckthorn and chokeberry samples proved to be rich in catechins: up to 21 mg% per 200 mL. Carotenoids were especially abundant in the sea buckthorn sample: 10–14 mg per 200 mL. The sea buckthorn sample also had the greatest content of tocopherols.

Table 4 Biologically active substances in dissolved instant beverages based on a combination of algal extracts of *Sargassum miyabei* or *Saccharina japonica* and concentrated berry juices

Indictor	Content per 200 mL					
	Sea buckthorn sample		Cranberry sample		Black chokeberry sample	
	Sargassum miyabei	*Saccharina japonica*	*Sargassum miyabei*	*Saccharina japonica*	*Sargassum miyabei*	*Saccharina japonica*
Iodine, µg	45.1 ± 2.2	89.1 ± 4.4	42.5 ± 2.1	83.3 ± 4.0	43.6 ± 2.1	85.6 ± 4.2
Vitamin C, mg	45.8 ± 1.5	35.0 ± 1.3	37.0 ± 1.6	39.0 ± 1.7	22.0 ± 1.0	20.7 ± 0.9
Fucoidan, mg	75.6 ± 3.2	47.3 ± 2.0	72.1 ± 3.0	46.5 ± 2.2	80.5 ± 3.8	40.3 ± 1.8
Pectin, mg	37.4 ± 1.6	45.7 ± 2.1	63.8 ± 2.9	59.4 ± 2.7	28.3 ± 1.3	25.0 ± 1.2
Flavonoids, mg%	29.3 ± 1.3	19.2 ± 0.9	20.2 ± 1.0	19.2 ± 0.8	20.1 ± 1.0	18.4 ± 0.9
Anthocyanins, mg%	1.6 ± 0.1	1.9 ± 0.1	1.3 ± 0	1.2 ± 0	2.4 ± 0.1	2.2 ± 0.1
Catechins, mg%	7.1 ± 0.3	18.5 ± 0.7	8.3 ± 0.4	8.0 ± 0.3	12.7 ± 0.6	11.7 ± 0.5
Carotenoids, mg	14.8 ± 0.5	10.6 ± 0.4	6.8 ± 0.3	6.0 ± 0.2	7.2 ± 0.3	6.5 ± 0.3
Tocopherols, mg	2.64 ± 0.11	2.31 ± 0.10	1.34 ± 0.06	1.61 ± 0.07	1.50 ± 0.07	1.93 ± 0.08

Mean values ± SD, n = 3

Table 5 Safety profile of dissolved instant beverages based on a combination of algal extracts of *Sargassum miyabei* or *Saccharina japonica* and concentrated berry juices

Indicator	Regulatory value (TR CU 021/2011)	Actual value					
Nitrosamines, mg/kg		Sea buckthorn sample		Cranberry sample		Black chokeberry sample	
		Sargassum miyabei	*Saccharina japonica*	*Sargassum miyabei*	*Saccharina japonica*	*Sargassum miyabei*	*Saccharina japonica*
	≤ 0.003	< 0.0002*	< 0.0001*	< 0.0002*	< 0.0001*	< 0.0001*	< 0.0002*
Polychlorinated biphenyls, mg/kg	≤ 2.0	< 0.001*	< 0.0001*	< 0.0002*	< 0.0001*	< 0.0002*	< 0.0001*
Toxic elements, mg/kg							
Lead	≤ 0.1	0.039	0.025	0.034	0.030	0.048	0.040
Arsenic	≤ 0.1	< 0.04*	< 0.04*	< 0.04*	< 0.04*	< 0.04*	< 0.04*
Cadmium	≤ 0.05	< 0.003*	< 0.003*	< 0.003*	< 0.003*	< 0.003*	< 0.003*
Mercury	≤ 0.03	< 0.004*	< 0.004*	< 0.004*	< 0.004*	< 0.004*	< 0.004*
Iron	≤ 5.0	0.7	1.0	0.9	0.85	1.18	0.59
Copper	≤ 0.4	< 0.03*	< 0.03*	< 0.03*	< 0.03*	< 0.03*	< 0.03*
Radionuclides: permissible levels, Bq/L							
Cesium-137: specific activity	≤ 40	5.01	8.05	6.47	4.82	7.54	8.45
Strontium-90: specific activity	≤ 80	3.26	7.14	5.96	3.93	8.08	10.1

* < detection limit

Since the new formulations were based on algal extracts, we tested the samples for food safety (Table 5).

The new instant beverages proved safe for food use as the content of nitrosamines, polychlorinated biphenyls, toxic elements, and radionuclides did not exceed the regulatory values.

To prove that the developed beverages can be classified as functional, we calculated the percentage of the recommended daily intake for iodine and vitamin C since their biological activity is a scientifically ascertained fact (Table 6). The data on the recommended daily intake came from the standards published by the Russian Federal Agency for Oversight of Natural Resource Usage (MP 2.3.1.0253-21).

One portion (200 mL) of the new functional beverages satisfied 19–49% of iodine recommended daily intake. It was maximal in the samples with *S. japonica*. One portion (200 mL) satisfied 22–38% of vitamin C recommended daily intake. The maximal result belonged to the cranberry sample. The sea buckthorn samples (200 mL) provided 15–18% of the physiological daily need in tocopherols. The chokeberry and sea buckthorn samples demonstrated good results in catechins. The results for anthocyanins appeared to be quite low: 12–15% of the recommended daily intake. The obvious functionality of the new beverages means that they can be used as correcting food systems for iodine and vitamin C deficiency. In addition, brown algae contain iodine in its organic form, which has a greater absorption capacity. The new instant beverages could serve as sources of tocopherols, catechins, and anthocyanins.

Table 6 Percentage of recommended daily intake: iodine and vitamin C in the functional instant beverages based on a combination of algal extracts of *Sargassum miyabei* or *Saccharina japonica* and concentrated berry juices

Biologically active substances	Physiological need for adults	Percentage of recommended daily intake per 200 mL, %					
		Sea buckthorn sample		Cranberry sample		Black chokeberry sample	
		Sargassum miyabei	*Saccharina japonica*	*Sargassum miyabei*	*Saccharina japonica*	*Sargassum miyabei*	*Saccharina japonica*
Iodine	150 µg/24 h	19	48	28	47	27	49
Vitamin C	100 mg/24 h	29	28	38	34	22	25
Tocopherols	15 mg toc. equivalent/24 h	18	15	9	11	10	13
Flavan-3-ols (catechins)	200 mg/24 h	14	19	14	14	16	16
Anthocyanins	50 mg/24 h	13	14	13	12	15	14

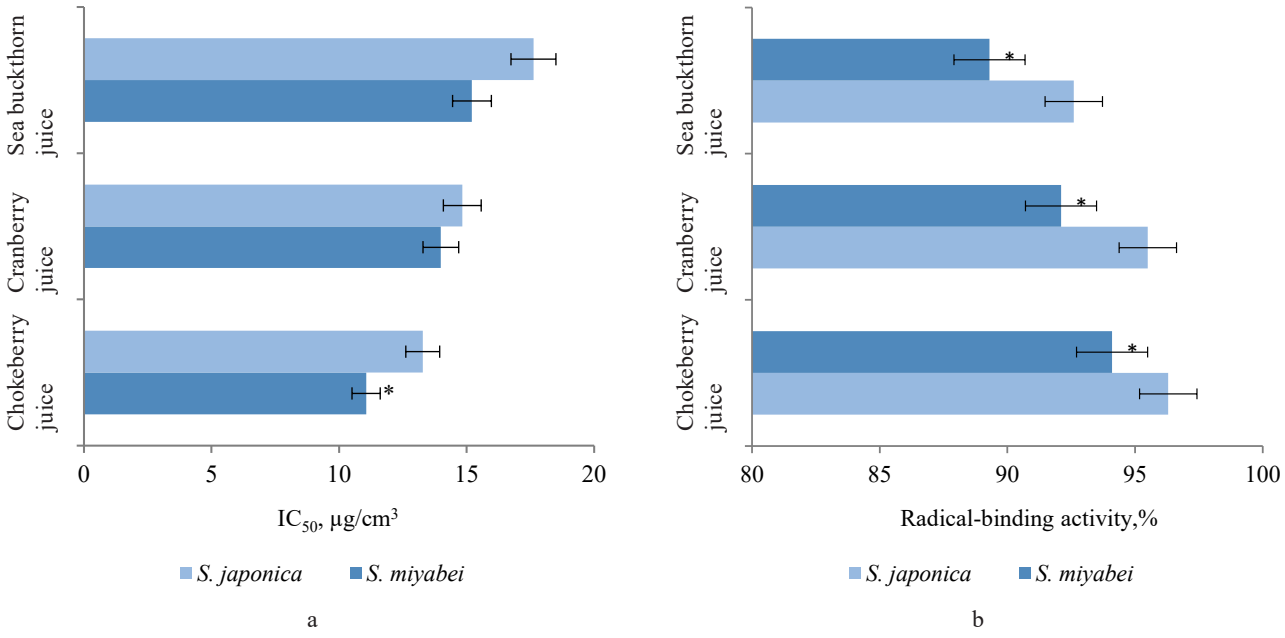

Figure 3 Antiradical properties of the functional instant beverages based on a combination of algal extracts of *Sargassum miyabei* or *Saccharina japonica* and concentrated berry juices: a – IC_{50} effective concentration, b – radical-binding activity. Mean values ± SD, n = 3

The antioxidant and anti-radical properties of food systems are an important manifestation of their biological activity. Such foods can neutralize free radicals in body cells, which helps prevent certain diseases. The antiradical activity of the new functional beverages comes from two sources: the biologically active substances in the algal extracts, which received scientific confirmation, and the biologically active substances in the concentrated berry juices, e.g., catechins, anthocyanins, and ascorbic acid [10]. The composition and bioactive profile in the new functional beverages demonstrated a potentially high antiradical activity. Figure 3 shows the antiradical properties of the new instant drinks.

The chokeberry sample had a higher radical-binding activity, which was at its lowest in the sea buckthorn sample. However, all the samples demonstrated a very high radical-binding activity, which amounted to 92.6–96.3%. IC_{50} is the concentration of an antioxidant-containing substance required to scavenge 50% of the initial DPPH radicals. The chokeberry sample also showed the maximal radical-binding properties, which were the lowest in the sea buckthorn sample. The cranberry sample demonstrated intermediate radical-binding activity and EC_{50}. The chokeberry sample had the highest content of anthocyanins, flavonoids, and catechins. The results suggested a high correlation between the content of biologically active substances and the antiradical properties. The *Sargassum miyabei* samples possessed higher antiradical properties than the samples based on *Saccharina japonica*, possibly due to more fucoidan.

CONCLUSION

The new functional instant beverages were based on a combination of algal extracts of *Sargassum miyabei* or *Saccharina japonica* and concentrated berry juices. All the samples had an excellent sensory profile, but the sea buckthorn sample obtained the highest sensory score.

The beverages contained a wide range of biologically active substances, e.g., iodine, phenolic compounds, vitamins (ascorbic acid), fucoidan, pectin, flavonoids, anthocyanins, catechins, carotenoids, tocopherols, etc. As a result, the new instant beverages can be classified as functional products. One portion of the novel beverage (200 mL) satisfied 27–30% of recommended daily intake of iodine and 22–50% of vitamin C. All the samples revealed a high radical-binding activity, which reached 96.3%. The sample based on chokeberry juice and *S. miyabei* extract showed the highest antiradical properties. Therefore, the new functional products have a positive impact on human health.

CONTRIBUTION

The authors were equally involved in the research and are equally responsible for any potential plagiarism.

CONFLICT OF INTEREST

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Effects of hydrolysis degree on the functional properties of hydrolysates from sour cherry kernel protein concentrate

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Abstract:
During the processing of sour cherries into different foodstuffs, a large amount of kernels is produced as waste material, which creates a significant disposal problem for the food industry. Sour cherry kernels containing 25.3–35.5% of protein can be used as a functional protein source in food production. Therefore, we aimed to study the effects of hydrolysis degree on the sour cherry kernel protein hydrolysates.

Proteins were extracted from the defatted flour by isoelectric precipitation. The resulting protein concentrate was hydrolyzed (5, 10, and 15% hydrolysis) using alcalase to yield hydrolysates. We determined their oil and water holding, emulsifying, gelation, and foaming properties, as well as apparent molecular weight distribution and proximate compositions.

No protein fractions greater than an apparent molecular weight of about 22 kDa were present in the hydrolysates. The hydrolysis of the protein concentrate mostly led to an increase in protein solubility. As the degree of hydrolysis increased from 5 to 15%, the water holding capacity of the hydrolysates decreased from 2.50 ± 0.03 to 2.03 ± 0.02 g water/g, indicating its deterioration.

The hydrolysates obtained at different degrees of hydrolysis had a better solubility than the intact protein concentrate. The oil holding capacity, the foaming stability, and the least gelation concentration of the protein concentrate could not be considerably improved by hydrolysis. In contrast, its emulsifying activity index and foaming capacity could be increased with a limited degree of hydrolysis (up to 10%).

Keywords: Sour cherry kernel protein, hydrolysis, Alcalase®, proximate composition, functional properties

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INTRODUCTION

Cherry belongs to the Prunus genus in the *Rosaceae* family. There are over several hundred species of cherry, but the two most common are sour cherry (*Prunus cerasus* L.) and sweet cherry (*Prunus avium* L.). Since sour cherry possesses a lower sugar/acid ratio than sweet cherry, it is commonly used to make fruit juice, puree, jam or marmalade, whereas sweet cherry is mostly eaten fresh [1]. In 2020, worldwide sour cherry production was approximately 1.48 million tons [2]. Roughly 85% of this output is converted into numerous foodstuffs, generating large volumes of seeds as waste material, which creates a significant disposal problem for the food industry [3]. Sour cherry kernels contain 17.0–41.9% of oil, 25.3–35.5% of protein, and 9.5–30.3% of dietary fiber [1, 4, 5].

Functional properties are physicochemical characteristics that govern the behavior of proteins in food systems during processing and storage. Emulsifying, oil and water holding, gelation, and foaming are some examples of functional characteristics. Proteins are included as functional components in foods to create definite textural and sensorial features, and/or enhance a nutritional value [6, 7]. The role of proteins in food systems can be understood through their functionality. Çelik *et al.* extracted proteins from sour cherry kernels and studied the functional attributes of the protein concentrate [5]. They found that the protein concentrate had lower emulsifying, foaming, and stability indices than sodium caseinate (Na-caseinate).

Therefore, research is needed to improve the functionality of sour cherry protein concentrate by

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applying various methods. Proteins are frequently modified by enzymatic, chemical, or physical processes that alter their structure and, therefore, physicochemical and functional attributes. Enzymatic hydrolysis is one of such processes that is most frequently used to enhance the functional attributes of proteins such as solubility, emulsifying, gelling, and foaming [8]. Various types of enzymes include Alcalase®, papain, trypsin, pepsin, and chymotrypsin. Partial proteolysis can improve functional characteristics by altering the conformation, molecular mass, and strength of the intra- and intermolecular bonds of the protein molecules while keeping their nutritional value.

Alcalase® is a serine-type protease from *Bacillus licheniformis* that has a broad specificity. The optimum pH and temperature for catalysis range from 7.0 to 9.0 and from 30 to 65°C, respectively. alcalase has been used to produce hydrolysates with superior nutritional or functional attributes compared to parent protein [9, 10].

To the best of our knowledge, no information is currently available on the functional attributes of hydrolysates prepared from sour cherry kernel proteins. Therefore, we aimed to (i) prepare protein hydrolysates with a different degree of hydrolysis (5, 10, and 15%) from sour cherry kernel protein concentrate using alcalase and (ii) determine the effects of the degree of hydrolysis on the selected functional properties, apparent molecular weight distribution, and proximate compositions of the resulting hydrolysates.

STUDY OBJECTS AND METHODS

Materials. The sour cherry kernels were purchased from a local enterprise in Turkey and kept in plastic pouches at 4°C until use. Na-caseinate containing 13.5–16.0% nitrogen was obtained from Sigma-Aldrich (St. Louis, MO, USA). Alcalase® (2.4L) from *Bacillus licheniformis* with endopeptidase activity, mainly subtilisin A (2.4 Anson units/g, with one Anson unit (AU) defined as the amount of enzyme that releases 1.0 mmol of L-tyrosine from urea-denatured hemoglobin per min at 25°C and pH 7.5) was obtained from Novozymes A/S (Bagsvaerd, Denmark). All the chemicals and reagents were of analytical grade and used without further purification.

Preparation of protein concentrate. Protein concentrate was produced via alkaline extraction and isoelectric precipitation as outlined by Çelik *et al.* with some modifications [5]. Briefly, sour cherry kernels were powdered (≤ 1 mm) with a coffee grinder. Then, the kernel powder was defatted 3 times (1 h each) with n-hexane and 2 times (1 h each) with petroleum ether in a 1:5 powder-to-solvent ratio at 25°C. The defatted powder was placed in a glass jar and kept at –18°C until use. The defatted powder was suspended in distilled water (5%, w/v) and the pH of the resulting slurry was set to 10.0 with 2 N NaOH for extraction. The slurry was agitated for 180 min at 25°C while the pH was maintained constant by re-adjusting every 30 min, if necessary. Next, the slurry was filtered through a Whatman Grade 1 filter paper and the filtrate pH was set to 4.5 with 2 N HCl, left for 15 min, and filtered through the same type of filter paper to collect aggregated proteins. The aggregated proteins were mixed with distilled water and the mixture pH was set to 7.0. Then, the mixture was dried in an air flow oven at 50°C for 12–18 h and kept at –18°C until use. The resulting protein-rich product was accepted as a protein concentrate since the protein content in dry matter (85%) was lower than 90%.

Enzymatic hydrolysis of protein concentrate. Protein concentrate was mixed with distilled water (1:25) and the pH of the mixture was adjusted to 8.0. The mixture was held for at least 30 min at 55°C. Then, the pH and temperature of the suspension were stabilized at 8.0 and 55°C, respectively. alcalase was added at various enzyme/protein ratios (3.6, 7.2, and 10.8 AU/100 g protein) to achieve three different degrees of hydrolysis (5, 10, and 15%) within 40–50 min. The degree of hydrolysis (DH, %) was calculated by the pH-stat method using the following Eq. (1) [11]:

$$DH = \frac{B \times N_b \times 100}{\alpha \times M_p \times h_{tot}} \quad (1)$$

where B is the amount of alkali, mL; N_b is the normality of alkali; M_p is the amount of protein, g; h_{tot} is the total peptide linkages in the protein (8.0 mEqv/g protein); α is the average ionization coefficient of the α-NH_2 groups (if the pH is 8.0 and the temperature is 55°C, then the pK is 7.1 and the α value is calculated as 0.888 with the Eq. (2)).

$$\alpha = \frac{10^{(pH-pK)}}{1+10^{(pH-pK)}} \quad (2)$$

When the desired degree of hydrolysis (5, 10 or 15%) was achieved, the hydrolysis process was terminated by heating the slurry in a water bath at 95°C for 10 min. Once the slurry was cooled to 50°C, the pH value was set to 7.0, and the slurry was dried using an air flow oven at 50°C for 12–18 h and kept at –18°C until use.

Proximate composition. Ash and total solid contents of the protein concentrate and its hydrolysates were determined by the gravimetric method [12, 13]. The lipid content was evaluated using an Ankom XT10 extractor (Macedon, NY, USA). The phenol-sulfuric acid method was used for the total carbohydrate and the micro-Kjeldahl method, for the nitrogen content (with 6.25 as the conversion factor) [14, 15].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. Apparent molecular weight distribution of the peptides/proteins from the protein concentrate and its hydrolysates were analyzed by the method described by Laemmli [16]. A sample containing 5 mg of protein was solubilized in 1 mL of sample buffer and then heated at 95°C for 5 min. After cooling, 10 µL of the sample was loaded onto the gel (1 mm thick, 4% stacking and 12% separating). A combination of standard proteins (6.5–200.0 kDa, catalogue number

S8445, Sigma-Aldrich, St. Louis, MO, USA) was loaded as a molecular weight marker. The gel was stained with Coomassie Brilliant Blue G-250 and de-stained with a 10% acetic acid solution. Currents of 25 mA were applied for the stacking gel and 35 mA, for the separating gel.

Color measurements. The CIELAB parameters (L^*, a^*, b^*) of protein concentrate and its hydrolysates were determined from 3 different locations by using a CR-300 Minolta colorimeter (Osaka, Japan). In this system, the a^* value ranges from green (–) to red (+), the b^* value ranges from blue (–) to yellow (+), and the L^* value is an estimation of lightness varying from 0 (black) to 100 (white). In addition, the total color change (ΔE^*) of hydrolysates with respect to the protein concentrate was calculated using the following Eq. (3):

$$\Delta E^* = [(L^* - L_0)^2 + (a^* - a_0)^2 + (b^* - b_0)^2]^{1/2} \quad (3)$$

where L_0 is the L^* value of protein concentrate; L^* is the L^* value of hydrolysate; a_0 is the a^* value of protein concentrate; a^* is the a^* value of hydrolysate; b_0 is the b^* value of protein concentrate; and b^* is the b^* value of hydrolysate.

Protein solubility. The solubility of the protein concentrate and its hydrolysates was tested as outlined by Güzel *et al.* and Du *et al.* with minor adjustments [7, 17]. Suspensions of 5% (w/v) protein concentrate or hydrolysates were prepared and their pH was set to 1.0–12.0 using 1 N NaOH or 1 N HCl. The suspensions were agitated at 25°C for 90 min. Meanwhile, their pH was checked at 30 and 60 min, and readjusted to the specified values, if necessary. Then, the suspensions were centrifuged at 4000×g for 30 min. Proteins in the supernatant were measured by the micro-Kjeldahl technique, and the protein solubility (%) was determined using the following Eq. (4) [15]:

$$\text{Protein solubility} = \frac{W_1 \times 100}{W_0} \quad (4)$$

where W_0 is the protein mass in the sample, g; and W_1 is the protein mass in the supernatant, g.

Water and oil holding capacity. The water and oil holding capacities of the protein concentrate and its hydrolysates were measured by the method of Çelik *et al.* and Wang *et al.* with some modifications [5, 18]. The protein concentrate or hydrolysates (1.0 g) were suspended in 8 mL of distilled water for the water holding capacity. The suspension (pH 7.0) was agitated for 30 s every 10 min and allowed standing for 70 min, and then centrifuged at 25°C for 15 min at 2000×g. The liquid phase was allowed draining for 10 min at a 45° angle. The increase in mass was noted as the water holding capacity (g water/g sample).

To determine the oil holding capacity, the protein concentrate or its hydrolysates (1.0 g) were suspended in 6 mL of sunflower oil. The suspension was agitated for 30 s every 5 min and held up for 30 min, and then centrifuged at 25°C for 25 min at 1600×g. The liquid phase was allowed draining for 10 min at a 45° angle. The increase in mass was noted as the oil holding capacity (g oil/g sample) and compared to the reference protein (Na-caseinate).

Emulsifying activity index and emulsifying stability index. The emulsifying activity index (EAI, m^2/g) and the emulsifying stability index (ESI, min) of the protein concentrate and its hydrolysates were examined as outlined by Pearce and Kinsella [19]. For emulsion formation, 6.6 mL of commercial sunflower oil was mixed with 20 mL of the protein concentrate or hydrolysate suspensions (pH 7.0, 0.1% protein, w/v) and homogenized (T18 Ultra Turrax, IKA, Staufen, Germany) at 20 000 rpm for 1 min. Fifty milliliters of the emulsion was added to 4.95 mL of 0.1% (w/v) sodium dodecyl sulphate (SDS) solution and instantly vortexed for 10 s, and the absorbance (A_0) of the mixture was read against 0.1% SDS solution at 500 nm. After 10 min, another 50 µL of the emulsion was added to 4.95 mL of 0.1% (w/v) SDS solution and promptly vortexed for 10 s, and finally the absorbance (A_{10}) was measured. The EAI and the ESI were calculated by using the Eqs. (5) and (6):

$$\text{EAI} = \frac{2 \times 2.303 \times A_0 \times N}{c \times \varphi \times 10000} \quad (5)$$

$$\text{ESI} = \frac{A_0 \times t}{A_0 - A_{10}} \quad (6)$$

where A_0 is the absorbance of the diluted emulsion after homogenization; c is the protein content of suspension (0.001 g/mL); N is the dilution factor (100); φ is the volume fraction of oil (6.6/26.6 = 0.248); A_{10} is the absorbance at 10 min; and t is the time interval (10 min).

Foaming capacity and stability. The foaming capacity (FC, %) and foaming stability (FS, %) of the protein concentrate and its hydrolysates were measured by the method outlined by Güzel *et al.* and Cui *et al.* with some modifications [7, 20]. The protein concentrate or its hydrolysates (0.25 g) were suspended in distilled water. The suspension's pH was set to 7.0, and the volume was made up to 20 mL (1.25%, w/v) with distilled water. The suspension was homogenized at 20 000 rpm for 2 min at 25°C to incorporate air, and then promptly poured into a 100-mL glass cylinder to record the total volume. The foaming capacity was calculated by using the following Eq. (7):

$$\text{FC} = \frac{(V_1 - V_2) \times 100}{V_2} \quad (7)$$

where V_1 is the total volume after homogenization; and V_2 is the total volume before homogenization (20 mL).

The foaming stability was determined using the Eq. (8) by recording changes in the foam volume after 0, 10, 30, 60, 90, and 120 min of storage at 25°C.

$$\text{FS} = \frac{V_t \times 100}{V_0} \quad (8)$$

where V_t is the foam volume at time t (10, 30, 60, 90, and 120 min); and V_0 is the foam volume at 0 min.

The least gelation concentration. The least gelation concentration of the protein concentrate and its hydrolysates was analyzed by the method of Güzel *et al.* and Rahman *et al.* with minor modifications [7, 21]. 5 mL of the protein concentrate or hydrolysates suspensions (pH 7.0, 2–14% w/v) were left in a boiling water bath for 1 h, followed by quick chilling to 4°C in an ice bath, and then kept for 2 h. Gel formation was assessed by turning the tubes with the suspensions upside down. The least gelation concentration was then recorded as a concentration at which the sample in the inverted tubes showed no signs of slipping or falling.

Statistical analysis. The results of three independent experiments were used to calculate means and standard deviations. Statistical evaluation of the data was performed using one-way analysis of variance, and the Duncan test was used to compare means in the IBM SPSS v. 25.0 statistical software at a significance level of 5% ($p < 0.05$). All the graphs were prepared with Microsoft Excel 2019.

RESULTS AND DISCUSSION

Proximate composition. The total solids, protein, lipid, total carbohydrate, and ash contents of the protein concentrate and its hydrolysates are presented in Table 1. The proximate composition of the protein concentrate was similar to that reported by Çelik *et al.*, except for total carbohydrates [5]. The total carbohydrate (9.70 ± 0.61%) content of the protein concentrate was significantly higher than the value (2.94 ± 0.36%) found by Çelik *et al.* [5].

The total solids contents of all the hydrolysates were statistically higher than that of the protein concentrate ($p < 0.05$). The protein content of the hydrolysates showed a significant ($p < 0.05$) decrease as the degree of hydrolysis increased. The possible reason for this decline was the increase in the ash content of the hydrolysates, resulting from the neutralization process with NaOH during the hydrolysis reaction. Similarly, Yust *et al.* found that the protein content of chickpea protein hydrolysates decreased as the degree of hydrolysis increased [22]. Interestingly, the lipid contents of the hydrolysates were significantly higher with the increase in the degree of hydrolysis ($p < 0.05$). This might be due to the fact that the enzymatic hydrolysis reaction changed the three-dimensional structure of proteins, leading to the release of non-extractable lipids trapped in the protein matrix. Similarly, Dias *et al.* reported that the amount of extractable lipids increased by the hydrolysis of almond proteins with neutral endoprotease from *Bacillus subtilis* [23]. The total carbohydrate contents of the protein concentrate and its hydrolysates varied from 9.7 to 10.5%, and no statistical difference was detected between the samples ($p > 0.05$). The ash content of the hydrolysates was significantly higher with the increase in the degree of hydrolysis ($p < 0.05$). As mentioned earlier, this increase was due to the addition of NaOH to neutralize the carboxyl groups released during the hydrolysis reaction. Similar findings were obtained for the hydrolysis of chickpea proteins with alcalase [22].

Apparent molecular weight distribution. The sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis was carried out to determine the apparent molecular weight (AMW) distribution of the peptide/protein fractions in the protein concentrate and its hydrolysates. We found that alcalase was an efficient enzyme for the hydrolysis of sour cherry kernel proteins. As can be seen from Fig. 1, the AMW of proteins in the protein concentrate ranged from 6.5 to 66.0 kDa under denaturing and reducing conditions.

The hydrolysis of the protein concentrate at different levels (5, 10, and 15%) resulted in the protein bands of almost the same number and the AMW, but different concentrations. As can be seen from Fig. 1, no protein fractions greater than an AMW of about 22 kDa are present in the hydrolysates. In other words, the protein fractions greater than an AMW of about 22 kDa were completely broken down into smaller peptides. As the degree of hydrolysis increased from 5 to 15%, the color of the protein fraction with an AMW of 22 kDa became fainter, but the fractions smaller than 6.5 kDa became darker.

Color changes. The color parameters (CIELAB L^*, a^*, b^* and ΔE^*) of all the samples are presented in Table 2. The highest L^* value (63.90 ± 0.28) was observed in the 5% hydrolysis degree sample, although it was not significantly different from the L^* value of the protein concentrate ($p > 0.05$). The L^* values of the hydrolysates showed a significant decline as the degree of hydrolysis increased

Table 1 Proximate composition of sour cherry kernel protein concentrate and its hydrolysates with different hydrolysis degree

Parameters, %	Protein concentrate	Hydrolysates		
		5%	10%	15%
Total solids	93.30 ± 0.28[a]	94.90 ± 0.11[b]	93.80 ± 0.09[c]	94.00 ± 0.06[c]
Protein	79.30 ± 0.43[a]	73.90 ± 0.03[b]	72.20 ± 0.65[c]	70.10 ± 0.09[d]
Lipid	1.70 ± 0.01[a]	2.50 ± 0.01[b]	3.10 ± 0.01[c]	3.30 ± 0.02[d]
Total carbohydrates	9.70 ± 0.61[a]	10.10 ± 0.59[a]	10.50 ± 0.55[a]	10.00 ± 0.28[a]
Ash	4.40 ± 0.01[a]	7.80 ± 0.01[b]	8.90 ± 0.01[c]	10.00 ± 0.01[d]

All the data are expressed as mean ± standard deviations and are the means of three replicates (n)

[a, b, c, d] Means followed by different letters within the same line represent significant differences ($p < 0.05$)

Table 2 Color properties of sour cherry kernel protein concentrate and its hydrolysates

Samples	*L**	*a**	*b**	Δ*E**
Protein concentrate	63.50 ± 1.40^{ab}	3.70 ± 0.20^{a}	21.90 ± 0.14^{a}	0^{a}
5% hydrolysate	63.90 ± 0.28^{b}	2.90 ± 0.05^{b}	20.00 ± 0.20^{b}	2.13 ± 0.14^{b}
10% hydrolysate	62.60 ± 0.33^{a}	3.10 ± 0.05^{bc}	19.30 ± 0.16^{c}	2.80 ± 0.24^{c}
15% hydrolysate	59.70 ± 0.35^{c}	3.20 ± 0.16^{c}	20.20 ± 0.28^{b}	4.13 ± 0.33^{d}

All the data are expressed as mean ± standard deviations and are the means of three replicates (n)

$^{a, b, c, d}$ Means followed by different letters within the same column represent significant differences ($p < 0.05$)

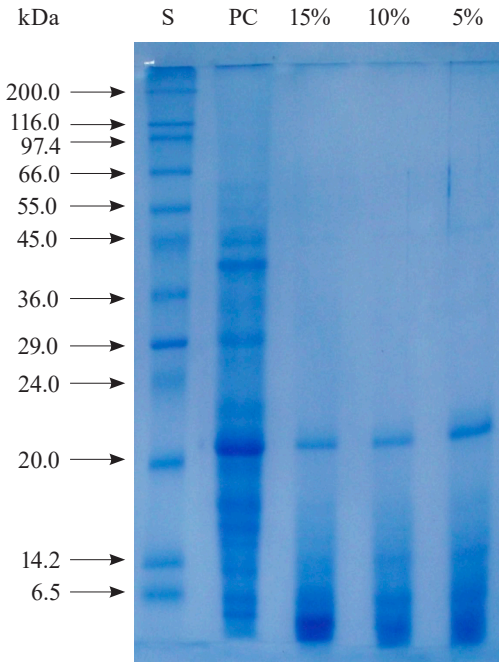

Figure 1 Sodium dodecyl sulfate polyacrylamide gel electrophoretogram of sour cherry kernel protein concentrate (PC) and its hydrolysates. S – molecular weight standard; 5, 10, and 15% – samples with 5, 10, and 15% degree of hydrolysis, respectively

from 5 to 15% ($p < 0.05$). This might be due to a combined effect of the hydrolysis reaction (by increasing the number of reactive amino groups participating in the Maillard type browning reactions) and the heat treatment applied to inactivate alcalase (by accelerating the Maillard type browning reactions). The protein concentrate had a redder (higher *a**) and yellower (higher *b**) color compared to the hydrolysates ($p < 0.05$). We found that the *a** values of the hydrolysates showed a gradual increase, while the *b** values decreased or increased irregularly. The 15% degree of hydrolysis sample had the highest redness and yellowness compared to the other hydrolysates, implying that the Maillard type browning reaction was higher in this sample.

The total color difference (Δ*E**) helps identify inconsistencies between the colors of the samples and to control the color more effectively. We observed that the total color difference between the hydrolysates increased significantly with the increase in the hydrolysis degree from 5 to 15% ($p < 0.05$). The color values of the protein concentrate in our study were comparable to the color values reported by Çelik *et al.* (*L** = 55.43 ± 0.96, *a** = 5.67 ± 0.21, and *b** = 23.71 ± 0.50) for the sour cherry kernel protein concentrate [5].

Protein solubility at different pH values. Protein solubility is an essential criterion for the food industry because it influences other functional attributes such as viscosity, gelling, and foaming. It is also a beneficial marker of denaturation and interactions between proteins. Protein solubility is affected by factors such as temperature, concentration, ionic strength, pH, and the presence of other molecules [7]. As can be seen from Fig. 2, the solubility of the protein concentrate was obviously pH dependent. The maximum (86.90 ± 0.30%) and minimum (18.80 ± 0.45%) solubility values of the protein concentrate were observed at pH 12.0 and 4.0, respectively. They were consistent with the values reported by Çelik *et al.* for the sour cherry kernel protein concentrate [5]. In general, the hydrolysis of protein concentrate with alcalase increased protein solubility in broad pH ranges, especially at acidic pH values. Similar to our results, the solubility of the resulting hydrolysates increased depending on the hydrolysis degree in the studies on rice endosperm protein concentrate, oat bran protein concentrate, chickpea protein isolate, and peanut protein isolate [8, 22, 24, 25].

While the 5% hydrolysis degree sample showed higher solubility than the protein concentrate at all pH values ($p < 0.05$), there was no shift in pH values at which minimum and maximum solubility was observed. The 10% hydrolysis and 15% samples had higher solubility at all pH values ($p < 0.05$) except pH 12.0 than the protein concentrate. In addition, the 10% sample showed higher solubility at pH 4.0–11.0 than the 15% hydrolysis degree sample. These results were inconsistent with the theoretical knowledge that the higher the hydrolysis degree, the higher the solubility. They might be due to the heat treatment applied to the hydrolysates at 95°C to destroy alcalase activity. Depending on the peptides and proteins present in the environment, insoluble complexes can be formed as a result of peptide-peptide, protein-protein, and/or peptide-protein hydrophobic interactions promoted by high temperatures [26].

Effects of enzymatic hydrolysis on water holding capacity. The water holding capacity (WHC) reflects a protein's ability to physically hold water against gravity. It is a functional attribute critical for high viscosity foods such as bakery products, sauces, soups,

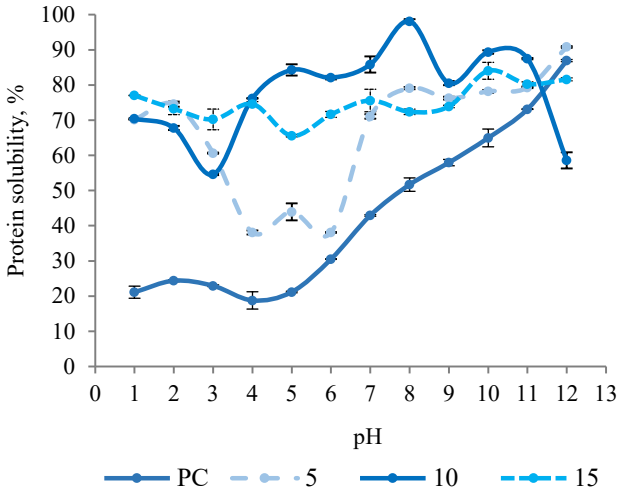

Figure 2 Solubility of sour cherry kernel protein concentrate (PC) and its hydrolysates at different pH. 5, 10, and 15% – samples with 5, 10, and 15% degree of hydrolysis, respectively

and gravies. The water holding capability of a protein molecule is a function of its size and shape, as well as the hydrophobic and hydrophilic interactions it participates in [6, 7].

The WHC of the protein concentrate (2.84 ± 0.01 g water/g sample) in our study was higher than that of the sour cherry kernel protein concentrate (2.42 ± 0.09 g water/g) reported by Çelik *et al.* [5]. We found that the WHC values of the 5, 10, and 15% samples were 2.50 ± 0.03, 2.18 ± 0.03, and 2.03 ± 0.02 g water/g, respectively. As the degree of hydrolysis increased from 5 to 15%, the WHC of the hydrolysates decreased from 2.50 ± 0.03 to 2.03 ± 0.02 g water/g. This indicated that the WHC of the protein concentrate could not be improved by hydrolysis. This result was not unexpected since hydrolysis causes proteins to dissolve in water instead of holding it. The 5% hydrolysis degree sample showed the highest WHC among the hydrolysates ($p < 0.05$). The 15% sample had the lowest WHC and differed significantly from the other samples ($p < 0.05$). Similar to our results, Nisov *et al.* found that the WHC of rice endosperm protein hydrolysates decreased with an increase in the hydrolysis degree [24]. Guo *et al.* also reported lower WHC in *Elaeagnus mollis* protein hydrolysates with an increase in the hydrolysis degree [27]. Contrary to our results, however, Vioque *et al.* reported higher WHC with an increase in the hydrolysis degree [28].

Effects of enzymatic hydrolysis on oil holding capacity. The interaction between lipids and proteins controls the sensorial properties of many foods. These interactions can be driven by ionic strength, pH, temperature, and other variables in the system. High oil holding capacity is critical for certain food systems, such as cake batters, sausages, salad dressings, and mayonnaise [7].

In our study, the oil holding capacity (OHC) of the protein concentrate was 1.76 ± 0.03 g oil/g sample. It was significantly lower than the OHC of Na-caseinate (1.93 ± 0.04 g oil/g) used as a reference protein ($p < 0.05$). This value was higher than the OHC of the protein concentrate (1.73 ± 0.17 g oil/g) reported by Çelik *et al.* [5]. The OHCs of 5, 10, and 15% hydrolysis degree samples were 1.71 ± 0.04, 1.64 ± 0.02, and 1.72 ± 0.01 g oil/g, respectively, with no differences among the OHC values of the hydrolysates ($p > 0.05$). While the 10% sample showed a significantly lower OHC than the protein concentrate ($p < 0.05$), the 5 and 15% samples had an OHC similar to the protein concentrate ($p > 0.05$). This indicated that the OHC of the hydrolysates did not depend solely on the hydrolysis degree. On the one hand, this inconsistent result was possibly due to the fact that the hydrolysis process increased the OHC by revealing nonpolar groups, while on the other hand, it decreased the OHC by reducing the oil-trapping surfaces [25, 28]. These results showed that the OHC of the protein concentrate could not be improved by hydrolysis. Similar to our results, the hydrolysis of oat bran protein concentrate with trypsin enzyme produced hydrolysates with a lower OHC [25]. On the contrary, Vioque *et al.* reported that the hydrolysis of rapeseed protein isolate with alcalase produces hydrolysates with a higher OHC [28].

Effects of enzymatic hydrolysis on emulsifying activity and stability indices. Surface hydrophobicity and concentration are the most significant features affecting the emulsifying capability of a protein [7, 29]. The emulsifying activity index (EAI) is an indicator of how quickly a protein can adsorb to the water/lipid interface during emulsion formation. In our study, the EAI values of the protein concentrate, Na-caseinate (a reference protein), as well as the 5, 10, and 15% hydrolysis degree samples at pH 7.0 were 22.7 ± 0.3, 176.4 ± 2.6, 24.2 ± 0.4, 26.8 ± 0.9, and 29.6 ± 1.2 m^2/g, respectively. The EAI values of the protein concentrate, as well as the hydrolysates were significantly lower than that of Na-caseinate ($p < 0.05$). This suggested that the proteins in the protein concentrate and its hydrolysates probably could not adsorb to the water/lipid interface as quickly as Na-caseinate. The EAI values of the hydrolysates increased depending on the degree of hydrolysis. We found that the 15% sample had the highest EAI value among the samples. In addition, the 10 and 15% samples had significantly higher EAI values than the protein concentrate ($p < 0.05$). This increase in the EAI might be due to increased solubility and the exposure of hydrophobic groups by hydrolysis. These results showed that the EAI of the protein concentrate could be improved by enzymatic hydrolysis, albeit to a certain limit. Similar to our findings, Guan *et al.* reported that the hydrolysates had higher EAI values than the intact oat bran protein concentrate [25]. On the contrary, Zhao *et al.* found that the hydrolysates showed lower EAI values when they hydrolyzed peanut protein isolate with alcalase [8].

The emulsifying stability index (ESI) reflects a protein's capacity to form a stable emulsion over a period of time [29]. The emulsifying attributes of protein isolates and concentrates are mostly parallel to their water solubility profile [7]. In our study, the ESI values of Na-caseinate, protein concentrate, as well as the 5, 10, and 15% samples were 1,187.5 ± 17.7, 116.4 ± 6.5, 63.7 ± 3.2, 62.9 ± 1.7, and 177.2 ± 7.1 min, respectively. It was obvious that the protein concentrate and its hydrolysates had considerably lower ESI values than Na-caseinate ($p < 0.05$). Therefore, we assumed that the proteins in the protein concentrate and its hydrolysates had a limited capability to diminish the interfacial tension and to form a protective film around the oil droplets. The ESI values of the hydrolysates decreased significantly compared to the protein concentrate at up to 10% hydrolysis ($p < 0.05$). However, when the hydrolysis degree reached 15%, the ESI value grew significantly compared to the protein concentrate ($p < 0.05$). This result showed that the ESI of the protein concentrate could be improved by about 1.5 times with 15% hydrolysis. Similarly, higher ESI values were produced by the hydrolysis of oat bran protein concentrate with trypsin and the hydrolysis of peanut protein isolate with alcalase [25, 30].

Effects of enzymatic hydrolysis on foaming capacity and stability. Foam formation is determined by the type of protein, its concentration, preparation method, solubility, composition, pH, the presence of salts, as well as hydrophobic interactions. Important factors for the foaming capacity include the diffusion rate of protein molecules towards the air-water interface, rapid conformational changes, and the rearrangement of protein molecules at the interface (molecular flexibility) [6, 7].

In our study, the foaming capacity of the protein concentrate (21.3 ± 1.8%) at pH 7.0 was considerably lower than that of Na-caseinate (46.3 ± 8.8%) used as a reference protein ($p < 0.05$) (Table 3). This result showed that the proteins in the protein concentrate were possibly less flexible than those in Na-caseinate. In addition, the foaming capacity of the protein concentrate was lower than that of sour cherry kernel protein concentrate (21.3 ± 1.8 vs 35.0 ± 3.5%) reported by Çelik *et al.* [5]. Although not statistically significant ($p > 0.05$), the protein concentrate exhibited a lower foaming capacity than the 5% (25.0 ± 0.1%) and 10% (23.8 ± 1.8%) samples, but higher than the 15% sample (17.5 ± 0.1%). These results showed that the foaming capacity of the protein concentrate could be improved with a limited degree of hydrolysis (up to 10%). Similarly, an increase in the foaming capacity resulted from the hydrolysis of the rapeseed protein isolate with alcalase, the oat bran protein concentrate with trypsin, and the chickpea protein isolate with Alcalase [22, 25, 28]. However, contrary to our findings, the hydrolysis of the peanut protein isolate with alcalase led to a decrease in the foaming capacity [30].

The foaming stability is a critical feature as the effectiveness of a foam-forming agent depends on its ability to retain gas bubbles for as long as possible. The formation of an adhesive multilayer protein film around each gas bubble makes the foam resistant to liquid drainage and coalescence [6]. Time-dependent (0–120 min) variations in the foaming stability of the protein concentrate, Na-caseinate, and the hydrolysates are shown in Table 3. Although not statistically significant ($p > 0.05$), the protein concentrate had a higher foaming stability than Na-caseinate after storage at 25°C for 10 and 30 min. Furthermore, the protein concentrate had a significantly higher foaming stability than Na-caseinate after 60, 90, and 120 min of storage ($p < 0.05$). All the hydrolysates (5, 10, and 15% hydrolysis degree) showed lower foaming stability values than both the protein concentrate and Na-caseinate over 120 min. The foaming stability of the 10 and 15% samples even decreased to zero after 90 min. These results showed that the foaming stability of the protein concentrate could not be improved by enzymatic hydrolysis. Similar to our findings, Guan *et al.* and Jamdar *et al.* also found a decrease in the foaming stability as a result of hydrolysis [25, 30].

Effects of enzymatic hydrolysis on the least gelation concentration. The gelling features of proteins are especially crucial in emulsion meat products such as sausage and salami. The gelling capacity is determined by the protein type and concentration, by pH, ionic strength, temperature, and the quantity of

Table 3 Foaming capacities and stabilities of sour cherry kernel protein concentrate, sodium caseinate, and hydrolysates with different degree of hydrolysis

Properties, %	Sodium caseinate	Protein concentrate	Hydrolysates		
			5%	10%	15%
Foaming capacity	46.3 ± 8.8^{a}	21.3 ± 1.8^{c}	25.0 ± 0.1^{c}	23.8 ± 1.8^{c}	17.5 ± 0.1^{c}
Foaming stability at 10 min	84.7 ± 1.2^{a}	85.4 ± 5.0^{a}	69.7 ± 4.3^{ab}	43.2 ± 9.6^{c}	41.7 ± 11.8^{c}
Foaming stability at 30 min	73.7 ± 3.2^{a}	85.4 ± 5.0^{a}	30.7 ± 8.0^{e}	14.1 ± 5.9^{e}	29.9 ± 10.8^{e}
Foaming stability at 60 min	60.6 ± 4.9^{a}	75.3 ± 3.6^{c}	17.4 ± 1.1^{bd}	9.6 ± 0.6^{e}	11.8 ± 1.0^{de}
Foaming stability at 90 min	45.8 ± 2.6^{a}	65.2 ± 2.1^{b}	12.9 ± 5.4^{e}	0^{d}	0^{d}
Foaming stability at 120 min	41.4 ± 2.0^{a}	59.6 ± 5.7^{c}	0	0^{d}	0^{d}

All the data are expressed as mean ± standard deviations and are the means of three replicates (n)

$^{a, b, c, d}$ Means followed by different letters within the same line represent significant differences ($p < 0.05$)

sulfhydryl and hydrophobic groups. Gels are stabilized by hydrogen bonds, ionic interactions, and disulfide linkages [6, 7].

The least gelation concentration (LGC) reflects the gel forming capacity of a protein: the lesser the LGC, the better the gelling capacity. In our study, the LGCs of the protein concentrate, as well as the 5, 10, and 15% samples at pH 7.0 were 10, 11, 11, and 11%, respectively. The LGC of the protein concentrate was higher than the value (10 vs. 8%) found by Çelik *et al.* [5]. The LGCs of the hydrolyzed samples were higher than that of the protein concentrate. This showed that the LGC of the protein concentrate could not be improved by hydrolysis. A possible reason might be that the reduction in peptide size caused by hydrolysis resulted in aggregation rather than gelation due to excessive peptide-peptide interactions. Similarly, Severin and Xia reported the LGCs of the samples as 8, 6, 12, 14, and > 20% when the whey protein concentrate was hydrolyzed with alcalase at 0, 5, 10, 15, and 20%, respectively [26].

CONCLUSION

We prepared protein hydrolysates from the protein concentrate with varying degrees of hydrolysis (5, 10, and 15%) using alcalase and determined the effects of the hydrolysis degree on their functional properties, apparent molecular weight distribution, and chemical compositions. According to our results, the solubility of the hydrolysates improved compared to the intact protein concentrate. However, the oil holding capacity, the foaming stability, and the least gelation concentration of the protein concentrate could not be considerably improved by hydrolysis. In contrast, the emulsifying activity index and the foaming capacity of the protein concentrate could be increased with a limited degree of hydrolysis (up to 10%). Further studies are needed to investigate the effect of different proteolytic enzymes, as well as various drying and separation methods on the functional properties of sour cherry kernel protein concentrate.

CONTRIBUTION

M. Yildirim contributed to the study conception and design. A. Cingöz performed material preparation, data collection, and analysis. A. Cingöz wrote the first draft of the manuscript and M. Yildirim commented on its previous versions. Both authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Effect of cocoa husk Criollo tea on hypercholesterolemia in animal model

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Abstract:
Organic waste is a problem the cocoa industry has to handle. The industry produces a lot of cocoa bean husk, also called *Criollo cocoa husk*. Cocoa bean husk is an underutilized cocoa waste that contains bioactive components in the form of phenols and flavonoids. Processed cocoa bean husk can be brewed as a functional beverage.
The research objective was to test cocoa husk tea for sensory properties, bioactive components, and impact on blood cholesterol. This study used a randomized experimental design with six repetitions. Sensory data were processed using the Friedman and Wilcoxon signed-rank tests ($\alpha = 0.05$) to determine the difference in sensory properties between each formulation of cocoa husk tea.
The sensory evaluation involved 30 untrained panelists who gave the highest score to the formulation with 62.5% cocoa bean husk, 25% lemongrass, and 12.5% aromatic ginger, which could also reduce 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals (IC_{50} = 264.8675). The animal test showed that the cocoa husk formulation produced no significant difference ($p > 0.05$) in pre- and post-treatment, but was able to keep cholesterol within normal limits.
Cocoa bean husk showed health benefits by its antioxidant properties and ability to control blood cholesterol.

Keywords: Criollo cocoa husk, sensory characteristic, bioactive value, blood cholesterol, food waste

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INTRODUCTION

Organic waste produced by the food or beverage industry covers a wide range of substances that cannot be consumed for nutrition purposes [1]. Dried cocoa beans and fermented cocoa beans are the main raw materials for all types of cocoa products, while cocoa bean husk is a by-product of the cocoa industry. The three most popular types of cocoa are *Criollo*, *Forastero*, and *Trinitario*, which together make up 95% of the total cocoa produced in the world. Criollo cocoa husk is a by-product with highly valuable bioactive components. However, it is discarded without recycling [2]. The weight of cocoa husk ranges from 10–17% of the total weight of cocoa beans [3]. A proper utilization of this by-product could prevent many environmental problems that arise from it being dumped as waste.

Cocoa husk has a good biofunctional potential for human health. It contains a lot of polyphenols that possess numerous biofunctional properties and health benefits. Flavonoids are the main polyphenol class in cocoa and their by-products [4]. According to scientific evidence, polyphenols are good for human health due to their antioxidant properties: they act as free radical scavengers and reduce oxidative stress. The bioactive substances contained in cocoa husk have antibacterial, antiviral, anticarcinogenic, antidiabetic,

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Table 1 Cocoa bean husk beverage formulations

Ingredients	Formulation									
	Control		1		2		3		4	
	g	%	g	%	g	%	g	%	g	%
Cocoa bean husk	8.0	100.0	5.0	62.5	5.0	62.5	5.0	62.5	5.0	62.5
Lemongrass	–	–	2.0	25.0	1.0	12.5	2.0	25.0	1.0	12.5
Ginger	–	–	1.0	12.5	1.0	12.5	–	–	–	–
Turmeric	–	–	–	–	1.0	12.5	–	–	1.0	12.5
Aromatic ginger	–	–	–	–	–	–	1.0	12.5	1.0	12.5
Mineral water	200.0	96.2	200.0	96.2	200.0	96.2	200.0	96.2	200.0	96.2

and neuroprotective properties, not to mention their beneficial effect on the cardiovascular system [5]. The total polyphenol content in one cocoa pod is slightly higher than in cocoa husk, but the total flavonoid content in cocoa husk is almost twice higher than in cocoa pods [6].

Cocoa polyphenols can affect the lipid profile and enhance the antiatherogenic effect. Saad tested cocoa polyphenols *in vitro* and in cell culture to demonstrate the inhibition of low-density lipoprotein oxidation and reduction of low-density lipoprotein oxidative susceptibility [7]. Experimental rats after a month on a cocoa powder diet improved their lipid profile and demonstrated a low cardiovascular risk. Polyphenols have a protective effect against atherosclerosis: they alter the hepatic cholesterol homeostasis by reducing cholesterol absorption. Polyphenols were also reported to lower blood pressure and the activity of enzymes in the renin-angiotensin-aldosterone system, which are involved in the renin-angiotensin-aldosterone system [8].

A functional cocoa-husk beverage is a solution to the cocoa waste problem, not to mention its potential for human health. Cocoa bean husk can be processed into a functional beverage with a good antiradical and cardioprotective potential because it contains antioxidants in the form of polyphenols (flavonoids). Cocoa bean husk is processed into functional beverage starting with sorting and sterilization, followed by packaging into brewed bags. The present research objective was to find out the sensory profile of cocoa husk functional beverages and their effect on blood cholesterol.

STUDY OBJECTS AND METHODS

Research design. We used a complete randomized experimental design with six repetitions. We developed several tea formulations based on criollo cocoa husk. The research included a qualitative analysis of bioactive and sensory components based on such parameters as color, smell, taste, and concentration, as well as a pre-clinical animal test. The panelists gave an informed consent before the sensory evaluation. The evaluation procedure had no adverse effect on the panelists. The research was approved by the Health Research Ethics Committee, Department of Dental Medicine, Universitas Airlangga (Number: 379/HRECC.FODM/ VIII/2020).

Cocoa husk tea development. We designed four cocoa husk tea formulations (Table 1). Formulations 1 and 2 contained such spices as lemongrass, ginger, and turmeric. Formulations 3 and 4 included lemongrass, turmeric, and aromatic ginger. The spices were intended to improve the taste. Ginger (*Zingiber officinale* L.) was chosen because it contains active compounds with anti-inflammatory and antioxidant properties [9, 10]. Lemongrass (*Cymbopogon citratus* L.) is known to prevent several diseases because it has antibacterial, antifungal, antioxidant, antiseptic, anti-inflammatory, analgesic, and antipyretic properties [11, 12]. Turmeric (*Curcuma longa* L.) is useful as an anti-inflammatory, anti-oxidant, anti-microbial, cancer-prevention, and anti-tumor agent. It can reduce fat and cholesterol in the blood and acts as a blood purifier [13]. It also lowers blood pressure and improves rheumatism [14]. Likewise, aromatic ginger (*Kaempferia galanga* L.) also contains antioxidants, like other spices [15]. The production process required an oven, a baking sheet, a tray, a measuring spoon, a digital scale, and a grinding machine, as well as tea brewing equipment in the form of hollow tea bags.

CHC phytochemical screening. Dry cocoa beans (*Theobroma cacao* L.) served as the main raw material. The list of reagents included Dragendorff's reagent, Mayer's reagent, and Stiasny's reagent. Dragendorff's reagent was prepared by mixing 0.8 g of bismuth nitrate and 20 mL of HNO_3 (p) with 27.2 grams of Kl dissolved in 50 mL of water. The solutions were allowed to stand for 24 h, filtered, and brought up to 100 mL with Aqua Dest. Mayer's reagent was made by mixing 1.36 g of $HgCl_2$ and 60 mL of Aqua Dest with 5 g of Kl dissolved in 10 mL of water. The two solutions were mixed and brought up to 100 mL with Aqua Dest. Stiasny's reagent was prepared by mixing two parts of 30% formaldehyde with one part of concentrated hydrochloric acid. The method used in this research was a laboratory experimental method. The tests covered flavonoids, tannins, quinones, saponins, steroids/triterpenoids, and alkaloids.

The flavonoid examination referred to Farnsworth: the sample was heated with hot water and mixed with magnesium powder, hydrochloric acid, and amyl

alcohol solution [16]. Yellow, orange, and red staining indicated the presence of flavonoid compounds. This test as described by Stefova *et al.* and Nugroho used the standard High-Performance Liquid Chromatography (HPLC) method [17, 18].

The method for examining tannins was approved by WHO and the Association of Official Agricultural Chemists [19]. The tannin testing followed the procedure described by Farnsworth: the heated sample solution was mixed with three different reagents, namely iron (III) chloride, gelatin, and Stiasny's reagent [16]. The positive test for tannins was indicated by a change in color for each reagent: blue-black, white, and pink, respectively.

The quinone test was based on the method developed by Farnsworth [16]. The sample solution was processed through the initial stages of heating. The boiling solution was then mixed with sodium hydroxide. Red staining indicated a positive quinone test. The same initial preparation process was also valid for the saponin tests: after adding a reagent of hydrochloric acid, the vile was shaken vertically for 10 s.

The test for steroids/triterpenoids involved adding 20 mL of ether to 1 g of sample followed by grinding and filtering. The filtrate was put into a vaporizer cup and allowed evaporating. Then few drops of Liebermann-Burchard reagent were added. Green-blue or red-purple staining indicated the presence of steroids/triterpenoids. For the alkaloid test, a solution of 10 mL HCl was mixed with 2 g of sample, crushed in a mortar, and then filtered. After that, 5 mL of 25% ammonia was added to the filtrate and extracted with 20 mL of chloroform. The chloroform layer was removed, and part of it was dropped on filter paper, where it reacted with Dragendorff's reagent. Orange staining indicated a positive alkaloid test.

Cocoa husk antioxidant test. The antioxidant activity test relied on the procedure described by Filbert with several modifications [20]. It involved a sample solution with a concentration of 1000 μg/mL and a 0.4 mM DPPH solution. The sample stock solution was diluted to various concentrations with a total volume of 1.6 mL. The solution was then put into a test tube as a test solution, followed by producing a blank solution. At the next stage, the test solution was mixed with 0.4 mL of DPPH in the test tube and underwent an incubation process for 30 min in the dark. After that, the blank and the test solution were measured for absorbance value using UV-Vis spectrophotometry at a maximal wavelength of 516 nm. Each sample was tested for absorbance value of the inhibition percentage (%) and IC_{50} value [21].

Sensory evaluation. The sensory evaluation revealed the consumer appeal and feasibility [22]. It included such parameters as color, smell, taste, and concentration of cocoa bean husk beverages. Five trained panelists and 30 untrained panelists filled in a questionnaire. The panelists were asked to assess the samples based on their level of preference on a 7-point scale: 1 = disliked very much, 2 = disliked, 3 = disliked a little, 4 = neutral, 5 = liked a little, 6 = liked, and 7 = liked very much. The samples were tea from the cocoa husk with mineral water and spices. The panelists were given mineral water and advised to drink it before moving to the next tea sample.

Blood cholesterol test in experimental animals. The experimental animals used in this study were male Wistar strains rats (*Rattus norvegicus* L.) aged 2–3 months with a weight of 160–240 g. Before entering the treatment stage, all experimental animals were adapted for 7 days. Each rat was placed in a different cage according to the treatment group. The rats were treated and controlled in a fixed environment to make them able to adapt to the new conditions. The conditions presupposed room temperature and sufficient light. Food and drinks were given *ad libitum*. Induction of hypercholesterolemia was performed by testing cholesterol tolerance by giving extra egg yolk.

The experimental animals were grouped into three phases: the adaptation experiment phase, the induction phase, and the intervention phase. During the adaptation stage, all the rats were treated normally using pellet feed for 7 days. During the induction stage, they were divided into five groups: positive control, negative control, Treatment 1, Treatment 2, and Treatment 3. During the induction stage, all the groups were induced using egg yolk, except for the negative control group (–), where the pellet induction lasted two weeks.

The intervention stage involved three types of treatment using simvastatin at a dose of 1 mg/kg BW, as well as 200 and 400 mg cocoa bean husk extract. Figure 1 summarizes the details.

Statistical analysis. The sensory data were first tested for normality and homogeneity by the Saphiro-Wilk test. If the data were distributed normally, they were processed by the analysis of variance (ANOVA) to determine the difference between parameters. The parameters that were found to be different underwent Fisher's test ($p \leq 0.05$). If the assumption of normality was not met, the data were subjected to the Friedman test and the Wilcoxon test ($\alpha = 0.05$).

The statistical data analysis of the cholesterol test was conducted by using the normality test according to the Saphiro-Wilk test. If the data were distributed normally ($p > 0.05$), it was followed by the paired sample t-test analysis. If the data were not distributed normally, then the Wilcoxon test was used. The test was conducted to determine the differences in cholesterol levels in the pre- and post-test through the post hoc test. If the significance value obtained was < 0.05, it meant that there was a difference between cholesterol levels in the pre-test and post-test in all treatments. The statistical analysis was tested using the IBM Statistics SPSS 22 software.

RESULTS AND DISCUSSION

Cocoa husk tea development. The cocoa bean husk tea included several types of spices (Table 1). The spices were intended to improve the taste. Ginger (*Zingiber*

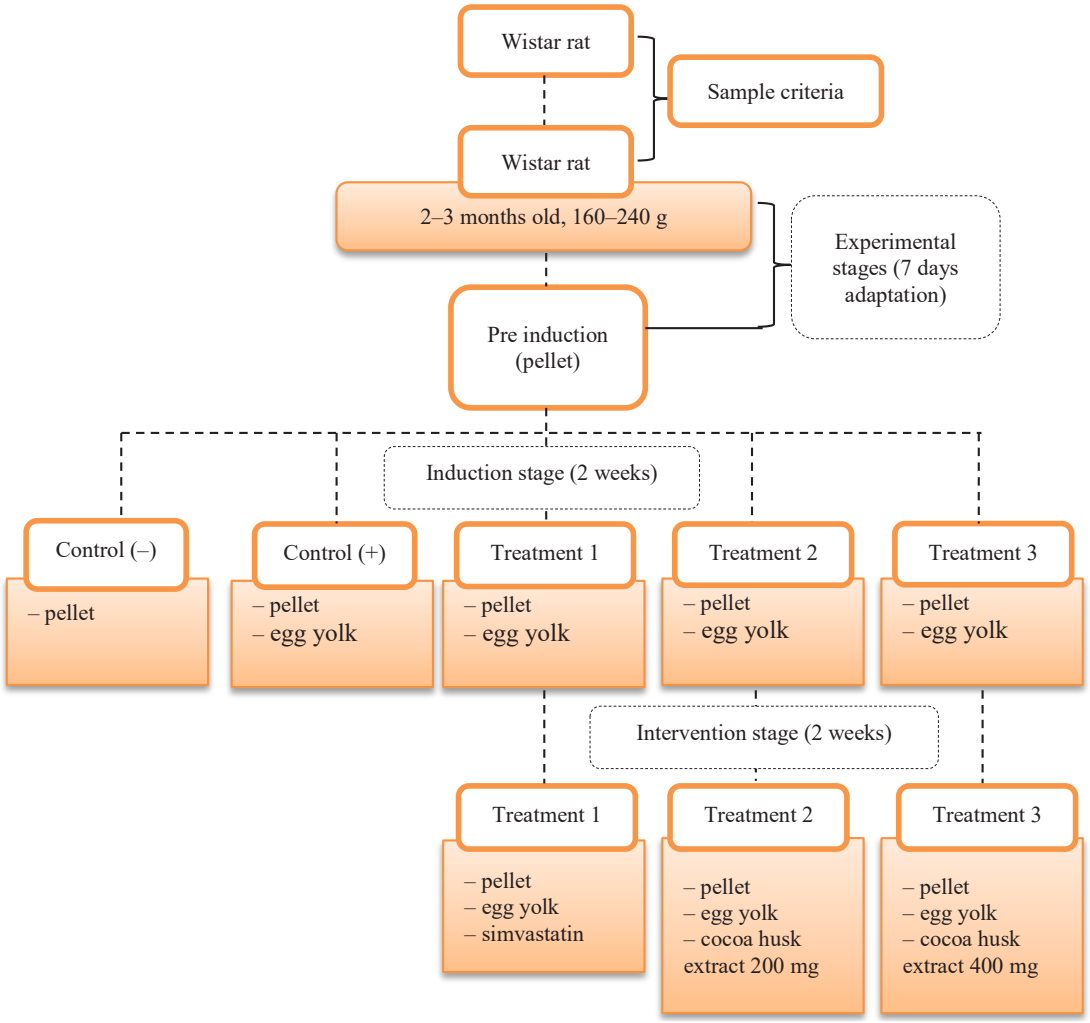

Figure 1 Cholesterol tests in experimental animals

Table 2 Phytochemical screening of cocoa bean husk

Compounds	Result
Alkaloids	–
Flavonoids	+
Saponins	–
Quinones	–
Hydrolyzable tannins	–
Condensed tannins	+
Steroids/triterpenoids	+

+: secondary metabolite detected

–: secondary metabolite undetected

officinale L.) was chosen for its bioactive compounds that have anti-inflammatory and antioxidant properties [9, 10]. Lemongrass (*Cymbopogon citratus* L.) is known to prevent several diseases because it possesses antibacterial, antifungal, antioxidant, antiseptic, anti-inflammatory, analgesic, and antipyretic properties [11, 12]. Turmeric (*Curcuma longa* L.) is an anticoagulant and anti-oxidant. Aromatic ginger (*Kaempferia galanga* L.) also contains antioxidants [15].

Phytochemical screening results. The phytochemical screening employed laboratory experimental methods. It included tests for flavonoids, tannins, quinones, saponins, steroids/triterpenoids, and alkaloids (Table 2).

The results of the phytochemical screening showed that cocoa bean husk contained flavonoids, condensed tannins, and steroids. The fact that it also contained flavonoid compounds, condensed tannins, and steroids/triterpenoids was in line with Yumas, who subjected cocoa bean husk to phytochemical screening and reported flavonoids, tannins, and triterpenoids [23]. The abovementioned substances belong to secondary metabolites. The screening detected no alkaloid compounds, saponins, quinones, or hydrolyzable tannins.

Flavonoids are secondary metabolite compounds that are commonly found in plant tissues. Flavonoids belong to phenolic compounds with a chemical structure of C6-C3-C6 [24]. They are powerful antioxidants as they are able to release hydrogen atoms [25].

Tannins are another type of secondary metabolites found in plants. Tannins are antioxidants: the larger their content, the greater their antioxidant activity. Tannins owe their antioxidant activity to the fact that they contain polyphenolic compounds that are able to capture free radicals [26].

Steroids are natural compounds with a carbon skeleton. They belong to the type of secondary metabolite compounds. Steroids are found in nature and derived from triterpene. Steroids of plant origin are derived from cycloartenol triterpenes. Early in their formation, acetic acid converts into cycloartenol through mevalonic acid and squalene [27]. Steroids are also classified as secondary metabolites and are known to possess antioxidant and antibacterial properties [28].

Antioxidant test results on cocoa bean husk simplicial. Simplicia is a natural material that has not undergone any changes or processing, e.g., it has not been dried [29]. We tested the antioxidant activity of cocoa bean husk simplicia to measure the effect of phytochemical substances in the initial raw material, i.e., before processing.

The simplicia calibration showed that the percentage of inhibition increased together with concentration, which was raised gradually. The resulting value of $R^2 = 0.9956$ demonstrated that these two variables had a strong effect.

The maximal wavelength was 516 nm. The control absorbance values were 0.973, 0.905, and 0.934 with a mean value of 0.937. The values of IC_{50} and IAA for cocoa bean husk simplicia were very weak – 1302.414 and 0.034551, respectively. Thus, the antioxidant activity was weak: IC_{50} < 50 ppm = very strong antioxidant; 50–100 ppm = strong; 101–150 ppm = moderate; and 150–200 ppm = weak [30].

Antioxidant activity of cocoa bean husk extract. The next antioxidant test was carried out on the cocoa bean husk extract to determine the effect of processing on the antioxidant activity of cocoa bean husk. The antioxidant test preceded both the sensory test and the experimental animal test.

The results of the extract calibration showed that the percentage of inhibition increased together with concentration. The obtained value of $R^2 = 0.9949$ demonstrated that these two variables had a strong effect.

The maximal wavelength was 516 nm. The control absorbance values were 0.977, 0.951, and 0.954 with a mean value of 0.961. The IC_{50} and IAA values of cocoa bean husk extract were found weak – 264.8675 and 0.169896, respectively. Thus, the antioxidant activity of cocoa bean extract was still weak. Compared with the results for simplicia, the extract showed even lower IC_{50} values.

The antioxidant activity test was based on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. Cocoa bean husk simplicia demonstrated antioxidant properties. However, when compared to the antioxidant standards, the antioxidant activity of cocoa bean husk fell into the weak category, as indicated by its IC_{50} value. This antioxidant ability was in line with the phytochemical screening results for flavonoid content (Table 2). The weak antioxidant power might have been due to the low content of flavonoid compounds (not yet quantified) or the type of flavonoid compound that had a chemical structure with a weak electron transfer ability (structural elucidation was not conducted). Therefore, the antioxidant effect could be provided only by regular and continuous consumption of cocoa bean husk infusion.

Table 3 Average sensory test score of cocoa bean husk beverage

	Formulation				
Indicators	Control	1	2	3	4
Color	3.0	5.0	6.5	6.0	6.0
Smell	6.0	4.5	5.0	6.0	5.0
Taste	2.0	5.0	3.8	5.0	4.0
Concentration	1.5	5.5	4.5	5.5	5.5
Total	12.5	20.0	19.8	22.5	20.5
Average	3.1	5.0	4.9	5.6	5.1

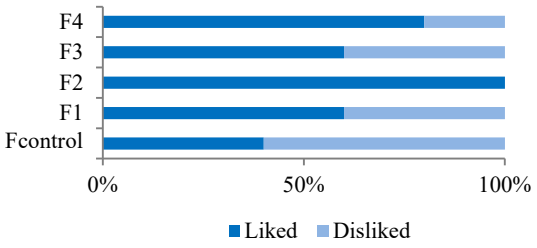

Figure 2 Color assessment of cocoa bean husk beverage, %

Sensory profile. Five formulations of cocoa bean husk beverages (Table 1) where bean husk was substituted with various spices were tested for consumer appeal. Table 3 shows the results of the sensory test for each parameter. Among the five samples, Formulation 3 (62.5% cocoa bean husk with 25% lemongrass and 12.5% aromatic ginger) attained the highest preference value based on the mean value of all parameters, i.e., color, smell, taste, and concentration.

Color is an important aspect that can affect food and beverage preferences [31]. The sensory assessment of color showed that the range of color acceptance scores was 3.0–6.5, which means that it ranged from "disliked a little" to "liked". The highest color score was obtained by Formulation 2 (62.5% cocoa bean husk, 12.5% lemongrass, 12.5% ginger, 12.5% turmeric, 12.5% aromatic ginger), while the lowest preference score was obtained by the control formulation with 100% cocoa bean husk.

The panelists preferred a lighter shade to the dark one: the lighter samples got a higher score than the dark-colored ones. The more cocoa bean husk was added, the darker the color became [22]. When a part of cocoa husk was substituted with spices, it produced a yellowish color or affected the brightness. Extra spices made the drink slightly yellow or bright in color. Turmeric was responsible for the yellow color as it is known to contain three pigments: curcumin, dimethoxy-curcumin, and bis dimethoxy-curcumin [32]. Ginger powder alone produces a yellowish-white color when dissolved in water [33]. Pure lemongrass powder dissolved in water produces a yellowish color [34].

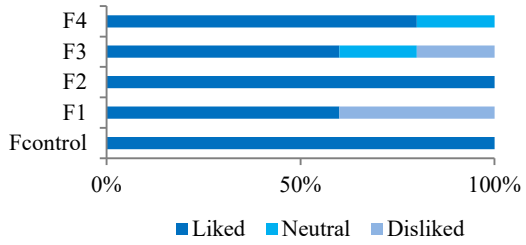

Figure 3 Smell assessment of cocoa bean husk beverage, %

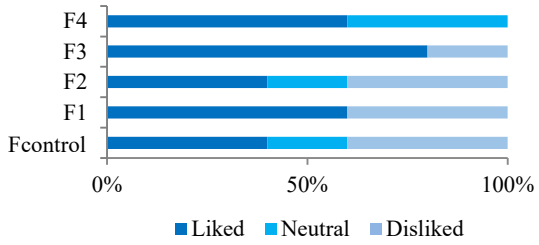

Figure 4 Taste assessment of cocoa bean husk beverage, %

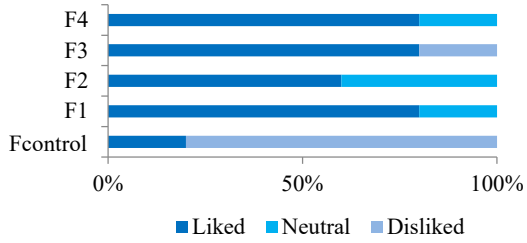

Figure 5 Concentration assessment of cocoa bean husk beverage, %

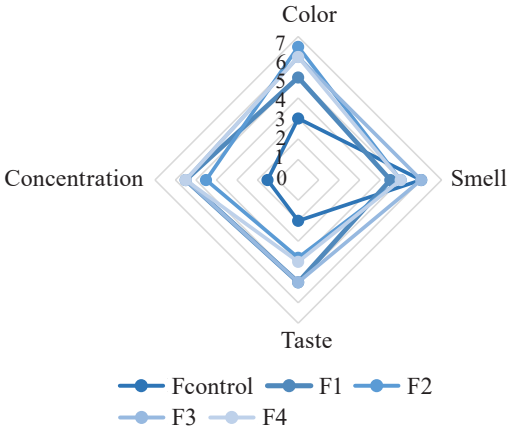

Figure 6 Hedonic test results

The percentage of panelists who claimed to like the color was as follows: Formulation 0 – 40%, Formulation 1 – 60%, Formulation 2 – 100%, Formulation 3 – 60%, and Formulation 4 – 80%. Formulation 2 had the best score for color: all 30 panelists appreciated it (Fig. 2).

Figure 3 demonstrates smell evaluation of the cocoa bean husk beverages. The highest score for smell went to the control sample and Formulation 2 (62.5% cocoa bean husk, 12,5% lemongrass, 12.5% ginger, 12,5% turmeric). The lowest smell score belonged to Formulation 1 (62.5% cocoa bean husk, 25% lemongrass, 12.5% ginger). The panelists preferred the beverage with a distinctive cocoa flavor. The characteristic cocoa smell appears as a result of the reaction between cocoa smell precursors (free amino acids and peptides) and sugar that enter the Maillard reaction to produce such smell components as alcohol, ether, furan, thiazole, pyrone, acid, ester, aldehyde, amine, amine, oxazole, pyrazine, and pyrrole. One of the compounds responsible for the characteristic smell of cocoa is 2,3-butanediol [35].

The highest taste score belonged to Formulation 3 (62.5% cocoa bean husk, 25% lemongrass, 12.5% aromatic ginger), while the lowest score belonged to the control (Fig. 4). The panelists preferred the samples with a lower proportion of cocoa bean husk. Polyphenols that give cocoa bean husk its antioxidant properties produce a slightly bitter and sour taste [23].

The score for the concentration ranged from 1.5 to 5.5, which means that it ranged from “disliked very much” to “liked a little” (Fig. 5). The highest concentration score belonged to Formulation 1 (62.5% cocoa bean husk, 25% lemongrass, 12.5% ginger), Formulation 3 (62.5% cocoa bean husk, 25% lemongrass, 12.5% aromatic ginger), and Formulation 4 (62.5% cocoa bean husk, 12.5% lemongrass, 12.5% turmeric, 12.5% aromatic ginger). The control sample received the lowest concentration score.

The acceptability value of cocoa bean husk beverages was based on the sensory test which included color, smell, taste, and concentration (Fig. 6).

Formulation 3 (62.5% cocoa bean husk, 25% lemongrass, 12.5% aromatic ginger) received the highest preference value based on all the sensory indicators, i.e., color, smell, taste, and concentration.

Table 7 Paired sample t-test: pre- and post-intervention cholesterol levels

Treatment groups	Cholesterol levels, mg/dL		A *p*-value of paired t-test results	Conclusion
	Pre-test	Post-test		
Negative control	29.80 ± 4.38	38.00 ± 8.64	0.902	No difference (normal cholesterol level)
Positive control	33.80 ± 5.54	86.50 ± 8.87	0.002	Difference detected (high cholesterol level)
Treatment 1	34.80 ± 6.18	58.00 ± 4.55	0.606	No difference (high cholesterol level)
Treatment 2	44.00 ± 3.39	42.00 ± 14.16	0.841	No difference (normal cholesterol level)
Treatment 3	33.00 ± 2.86	33.80 ± 8.32	0.942	No difference (normal cholesterol level)

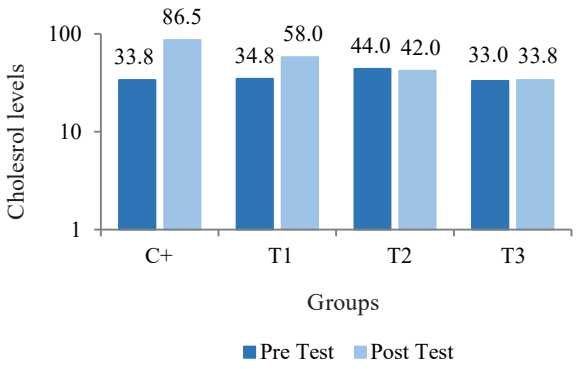

Figure 7 Effect of cocoa bean husk extract on cholesterol in rats

Blood cholesterol value in experimental animals. The data collected for blood cholesterol tests were the initial data before the intervention and the final data collected two weeks after the intervention period. The pre-test and post-test data were compared to reveal differences in the results of the intervention activities (Table 4).

Normal blood cholesterol level in rats is 10–54 mg/dL [36]. The paired sample t-test showed that the negative control treatment with pellets increased cholesterol from 29.8 mg/dL in pre-intervention to 38 mg/dL in post-intervention (Table 7). The negative control demonstrated no significant difference in cholesterol levels (p-value = 0.902 (> 0.05)). Thus, the negative control treatment had no significant effect on cholesterol.

The positive control group received duck egg yolk. As a result, the pre-cholesterol level of 33.8 mg/Dl (normal cholesterol) increased to 86.5 mg/dL (high cholesterol). The difference between pre-test and post-test was significant with a p-value of 0.002 (< 0.05). Hence, the positive control treatment had a significant effect on the changes in cholesterol (Fig. 7).

Treatment 1 included pellets, duck egg yolk, and simvastatin. As a result, the pre-cholesterol level of 34.8 mg/dL reached 58 mg/dL. No significant difference in cholesterol levels was detected (p-value = 0.841 (> 0.05)). Thus, Treatment 1 had no significant effect on cholesterol.

Treatment 2 involved pellets, duck egg yolks, and 200 mg/kg BW of cocoa bean husk extract. From the precholesterol level of 44 mg/dL, it fell down to 42 mg/dL. Therefore, we detected no significant difference in cholesterol levels between pre-test and post-test (p-value = 0.841). Treatment 2 produced no significant effect on cholesterol but was able to maintain it within normal limits.

Treatment 3 involved pellets, duck egg yolks, and 400 mg/kg BW of cocoa bean husk extract. The pre-cholesterol level of 33.3 mg/dL remained almost the same: 33.8 mg/dL. The difference between pre-test and post-test was not significant (p-value = 0.942). Thus, Treatment 3 had no significant effect on cholesterol but was able to maintain it within normal limits.

Treatment 2 and Treatment 3, which involved cocoa bean husk extract, maintained cholesterol levels in the normal range but provided no significant changes. Treatment 1, which involved simvastatin, demonstrated a significant difference in blood cholesterol levels. The positive control also showed a significant difference, which was inversely proportional to the negative control. According to Ahmad & Amy, cocoa injections in rats reduced their low-density lipoprotein (LDL) levels. Darand *et al.* also mentioned the effect of cocoa consumption which could significantly reduce blood cholesterol levels in humans [37, 38]. Sweety also stated that cocoa products had a significant anti-cholesterol impact [39].

The mechanism of reducing LDL cholesterol and total cholesterol in experimental animals could be explained by the high flavonoid content in cocoa bean husk. Flavonoids with their antioxidant and anti-inflammation properties increased the function of high-density lipoprotein (HDL) cholesterol, which decreased the total cholesterol levels. HDL cholesterol removed reverse cholesterol transport in the blood [40]. For instance, Martinez *et al.* also reported that an increase in the consumption of cocoa flavonoids improved the level of HDL cholesterol [41]. Flavonoid-rich foods could improve the endothelial function in cells [42]. Out of the five types of intervention trials, Treatments 2 and 3, which involved cocoa bean extract, managed to keep cholesterol levels in rats within the normal limits, although they provided no significant change.

CONCLUSION

Cocoa bean husk extract, a by-product of the cocoa industry, showed health benefits through its antioxidant activity. It was able to reduce DPPH free radicals (IC_{50} = 264.8675) better than cocoa bean husk simplicia (IC_{50} = 1302.414). The blood cholesterol animal tests registered no significant difference, but the extract was able to maintain cholesterol within normal limits. The lighter-colored samples with little cocoa husk received the best score for color and concentration, while the samples with a higher proportion of cocoa bean husk received a higher score for the characteristic cocoa smell. As for the taste, the panelists preferred the sample with less cocoa bean husk. The highest mean score for sensory properties belonged to the sample with 62.5% cocoa bean husk, 25% lemongrass, and 12.5% aromatic ginger.

CONTRIBUTION

Conceptualization: A.C. Adi and H. Rachmawati; methodology: H. Rachmawati and F. Farapti; software: W. Salisa; validation: A.I. Tawakal; formal analysis: W. Salisa and M.F. Rasyidi; investigation: W. Salisa and A.I. Tawakal; resources: F. Farapti; data curation: W. Salisa; original draft: M. Rasyidi; review and editing: H. Rachmawati, F. Farapti, M.F. Rasyidi; visualization: W. Salisa; supervision: A.C. Adi and H. Rachmawati;

project administration: A.C. Adi and H. Rachmawati. All the authors have read and agreed to the published version of the manuscript.

CONFLICTS OF INTEREST

The authors declared no conflict of interests regarding the publication of this article.

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Effects of triticale flour on the quality of honey cookies

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Abstract:
Flour confectionery products are highly popular in Russia, especially honey cookies (pryanik). In order to increase their nutritional value, wheat flour can be replaced by triticale flour rich in essential amino acids and minerals. This study aimed to determine the effects of triticale flour on the quality of honey cookies.

The control cookie sample was made from premium wheat flour, while the test samples were made from mixtures of wheat and triticale flours in various ratios (10–90%), as well as from 100% triticale flour of grade T-80. Standard methods were applied to determine the cookies' sensory and physicochemical characteristics, as well as their nutritional value and contents of minerals, vitamins, and amino acids.

All the samples with triticale flour showed good sensory characteristics. Adding up to 30% of triticale flour did not change the taste and aroma of honey cookies, whereas larger amounts of triticale flour made them sweeter and more aromatic. The test samples from 100% of triticale flour had higher contents of essential amino acids (arginine, valine, histidine, isoleucine, leucine, lysine, methionine, and threonine) than the control sample from premium wheat flour. The contents of micro- and macroelements also increased with larger amounts of triticale flour in the formulation. While adding triticale flour increased the protein content in the test honey cookies, it had no significant effect on their fat and carbohydrate contents, or the calorie content. The tests showed that 60:40% was the optimal ratio of wheat and triticale flours.

Replacing wheat flour with 40% of triticale flour provided the finished product with good sensory properties and high contents of proteins, vitamins, essential amino acids, as well as micro- and macroelements. Thus, triticale flour proved to be a good replacement for wheat flour in the production of honey cookies.

Keywords: Honey cookie, premium wheat flour, triticale flour, formulation, quality indicators, nutritional value

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INTRODUCTION

Triticale is an artificial type of cereals obtained by combining the genomes of wheat (*Tritium* genus) and rye (*Secale* genus). The protein content in triticale grain slightly exceeds the protein content in rye and wheat. Triticale grain can form wheat-type bound gluten, although its content is lower than in wheat. Moreover, triticale gluten is of lower quality compared to wheat due to rye-type proteins. On the other hand, triticale grain contains more water-soluble proteins than wheat and rye. Its biological value is higher than that of wheat due to a larger amount of free essential amino acids (lysine, valine, leucine, etc.). Additionally, triticale grain contains a variety of minerals, including large amounts of phosphorus and potassium, as well as magnesium, calcium, manganese, iron, copper, and others [1–13].

Rye traits inherited by triticale contribute to an increased activity of its amylolytic enzymes, in particular amylase, and a lower starch gelatinization temperature. As a result, triticale accumulates a significant amount of dextrins and maltose formed during the enzymatic hydrolysis of starch [14–21].

In recent years, Russia has seen a greater demand for flour confectionery products made from non-traditional raw materials of plant origin. Honey cookies (Rus. "pryaniks") are among the most popular types of flour confectionery due to their taste and aroma. They have a high energy value, but rather low levels of nutrients (essential amino acids, vitamins, macro- and microelements). Therefore, formulators seek to improve their nutritional value.

Historically, Russian honey cookies date back to the XI century, when they were called "honey bread". They

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were made from a mixture of whole-grain rye flour with various berry juices and honey, which accounted for almost half of all the ingredients [22–24]. Over time, their formulation changed due to improved technology and new ingredients. As a result, they have become one of the most popular types of confectionary products in Russia [16, 25–32].

The current demand for honey cookies in Russia is on the rise. From 2015 to 2020, their sales increased by 14.3%. The popularity of honey cookies is mainly due to their affordability and a long shelf life compared to other confectionery products. Yet, their appeal is also down to their pronounced honey aroma, subtle notes of spices, and a sweet taste.

Despite a high energy value and an appealing taste, honey cookies have an unbalanced composition of essential amino acids and a low content of vitamins and trace elements. However, their nutritional value can be improved by enriching them with biologically valuable components [17, 33–36].

Today, flour confectionery products are fortified with non-traditional raw materials that are rich in valuable nutrients. They include chickpeas, peas, soybeans, chia, buckwheat, amaranth, triticale, rice, and many other components [37–40].

In this study, we used triticale flour, which contains all essential amino acids, vitamins, and microelements that humans need. Its baking characteristics are similar to those of wheat and rye, making triticale flour an excellent replacement for premium wheat flour in confectionery production [41–43].

Thus, we aimed to study the effects of triticale flour on the quality of honey cookies.

STUDY OBJECTS AND METHODS

Samples of honey cookies (Rus. “pryaniks”) were made from premium wheat flour (control), mixtures of wheat and triticale flours in various ratios (10–90%), and 100% triticale flour of T-80 grade. The effects of triticale flour on the quality of honey cookies were determined according to the following standards: State Standard 26574-2017 (for premium wheat flour), State Standard 34142-2017 (for triticale flour of grade T-80), State Standard 33222-2015 (for white sugar), State Standard 33917-2016 (for starch syrup), State Standard 32188-2013 (for margarine), State Standard 19709-2019 (for enzyme-interesterified fat), State Standard 32802-2014 (for baking powder), and State Standard R 51232-98 (for drinking water). The quality indicators of wheat and triticale flour samples are presented in Table 1.

The main raw materials were premium wheat flour and T-80 grade triticale flour (78% yield of baking flour from milling triticale grains) [12]. Sugar, in cookie production, is most often used in the form of syrups (sugar, invert, sugar-honey, or sugar-treacle). Treacle, honey, and invert syrup are used to increase the products’ hygroscopicity, thus prolonging their shelf life and preventing them from quick hardening. Other ingredients include margarine (82% fat), butter, and confectionery fats with a melting point of 34–37°C. Sodium bicarbonate, or baking soda (0.15% by weight of flour) and ammonium carbonate (0.4% by weight of flour) are used as baking powders. Most formulations of honey cookies also contain a mixture of cinnamon, cloves, allspice, black pepper, cardamom, and ginger to add flavor.

We used a traditional formulation of honey cookies, with such ingredients as premium wheat flour, white sugar, treacle, margarine, baking powder, and water. The dough was kneaded in a Kitchen Aid batch kneader. The ingredients were loaded in the following order: sugar-treacle syrup was mixed with margarine or enzyme-interesterified fat for 2 min until an emulsion formed, then the flour mixture with baking powder was introduced and the dough was kneaded for 5 min (22°C and 24% moisture for the final dough).

The dough was cut with a special cutter and baked in an electric oven at 200–220°C for 10 min. After baking, the finished products were cooled at room temperature. In the test samples (11 pieces), wheat flour was replaced with triticale flour (10 to 100%). The control sample was made from premium wheat flour.

We aimed to determine whether our samples’ sensory and physicochemical characteristics complied with State Standard 15810–2014 and to identify the optimal amount of triticale flour to replace wheat flour.

RESULTS AND DISCUSSION

Our samples were based on the traditional formulation of honey cookies (Rus. “pryaniks”), with varying ratios of wheat and triticale flours. First, a sugar-treacle syrup was prepared from water, sugar, and treacle.

Table 1 Quality indicators of wheat and triticale flour samples

Indicator	Premium wheat four	Triticale flour of grade T-80
Moisture, %	14.2	10.3
Crude gluten, %	28.0	20.0
Gluten quality, units (gluten strain meter)	83	90
Autolytic activity, s	266	110
Acidity, degrees	3.1	3.0
Ash, %	0.55	0.80
Flour whiteness, units (RZ-BPL whiteness meter)	54.0	36.0
Falling number, s	200	160

Table 2 Sensory characteristics of honey cookies made from wheat flour, triticale flour, and their mixtures

<table>
<tr><th rowspan="2">Ratio of wheat and triticale flours</th><th colspan="6">Cookie quality indicators</th></tr>
<tr><th>Taste and aroma</th><th>Texture</th><th>Color</th><th>Cross-section</th><th>Surface</th><th>Shape</th></tr>
<tr><td>100% wheat (control)</td><td rowspan="4">Sweet taste and aroma corresponding to flavoring additives</td><td rowspan="6">Products with a soft, bonded texture that do not crumble when broken</td><td rowspan="4">Creamy surface, uniform creamy-white crumb</td><td rowspan="6">Well-baked products, with a uniform well-developed porosity, without voids, hardening or traces of undermixing</td><td>Smooth upper surface, without cracks or swellings, with noticeable tears on the sides</td><td rowspan="6">Regular shape, without slackness or dents, with a convex upper surface</td></tr>
<tr><td>90% wheat + 10% triticale</td><td>Smooth upper surface, without cracks or swellings, with tears on the sides</td></tr>
<tr><td>80% wheat + 20% triticale</td><td rowspan="2">Smooth upper surface, without cracks or swellings, with slight tears on the sides</td></tr>
<tr><td>70% wheat + 30% triticale</td></tr>
<tr><td>60% wheat + 40% triticale</td><td rowspan="3">Highly sweet taste and pleasant aroma corresponding to flavoring additives</td><td rowspan="3">Dark creamy surface, uniform creamy-white crumb</td></tr>
<tr><td>50% wheat + 50% triticale</td></tr>
<tr><td>40% wheat + 60% triticale</td></tr>
<tr><td>30% wheat + 70% triticale</td><td rowspan="4">Pronounced sweet taste and pleasant aroma corresponding to flavoring additives</td><td rowspan="4">Light brown surface, uniform creamy-white crumb</td><td rowspan="4">Smooth upper surface, without cracks, swellings, or tears</td></tr>
<tr><td>20% wheat + 80% triticale</td></tr>
<tr><td>10% wheat + 90% triticale</td></tr>
<tr><td>100% triticale</td></tr>
</table>

It was heated with constant stirring to 60°C until the sugar completely dissolved and then cooled to 30–40°C. To make the dough, the syrup was first mixed with fat and dry yeast, and then with flour and baking powder. The dough was kneaded for 5 min to reach 20–22°C and 23.5–25.5% moisture. After kneading, 40-g samples were cut and baked at 200°C for 15 min. After baking, the cookies were cooled for sensory and physicochemical analysis [9].

In addition to sensory characteristics (Table 2), we determined the samples' moisture, alkalinity, water absorption, and density in accordance with State Standard 15810-2014.

As can be seen from Table 2, the samples with 30% of triticale flour had the same taste and aroma as the control, those with 40–60% of triticale flour had a highly sweet taste and a pleasant aroma, while the cookies with 70–100% of triticale flour acquired a pronounced sweet taste and a pleasant aroma.

With 10–100% of triticale flour, the samples had an invariably soft texture and did not crumble when broken.

As for the color, the cookies with up to 30% of triticale flour had a creamy surface and a uniform creamy-white crumb, those with 40–60% of triticale flour had a dark creamy surface and the same crumb color, while those with 70–100% of triticale flour had a light creamy surface and the same crumb color.

When broken, the samples with 10–100% of triticale flour looked well-baked, had a uniform well-developed porosity, and no voids or traces of undermixing.

The control cookie had a smooth upper surface, with no cracks or swellings, but with noticeable tears on the sides. The surface of the test samples was the same, smooth with no cracks or swellings. However, the samples with 10–20% of triticale flour had some tears on the sides, those with 30–50% of triticale flour had slight tears on the sides, and the ones with 60–100% of triticale flour had no tears.

All the test samples had a regular shape, without tears or dents, and a convex upper surface.

Table 3 presents the physicochemical quality indicators of honey cookies made from wheat flour, triticale flour, and their mixtures.

As can be seen from Table 3, water absorption, density, and alkalinity of the samples tended to decrease as the amount of triticale flour increased. These changes in water absorption and density made the cookies more compact in texture and less crumbly. The decrease in alkalinity was due to triticale flour's lower alkalinity compared to premium wheat flour.

According to our data, the samples with increased amounts of triticale flour had a smoother surface with no side tears. The reason for that is that triticale flour contains more water- and salt-soluble proteins and less residual proteins compared to wheat flour [4]. These differences in protein components make the

Table 3 Physicochemical quality indicators of honey cookies.

Ratio of wheat and triticale flours	Quality indicators			
	Moisture, %	Water absorption, %	Alkalinity, degrees	Density, g/cm³
100% wheat (control)	14.2	215.3	1.9	0.64
90% wheat + 10% triticale	14.3	212.7	1.9	0.65
80% wheat + 20% triticale	14.5	211.4	1.9	0.65
70% wheat + 30% triticale	14.7	209.6	1.8	0.66
60% wheat + 40% triticale	14.6	208.8	1.8	0.67
50% wheat + 50% triticale	14.8	207.2	1.8	0.67
40% wheat + 60% triticale	14.7	206.9	1.7	0.68
30% wheat + 70% triticale	14.8	206.1	1.7	0.67
20% wheat + 80% triticale	14.7	205.4	1.7	0.68
10% wheat + 90% triticale	14.9	205.1	1.6	0.69
100% triticale	14.4	203.3	1.6	0.69

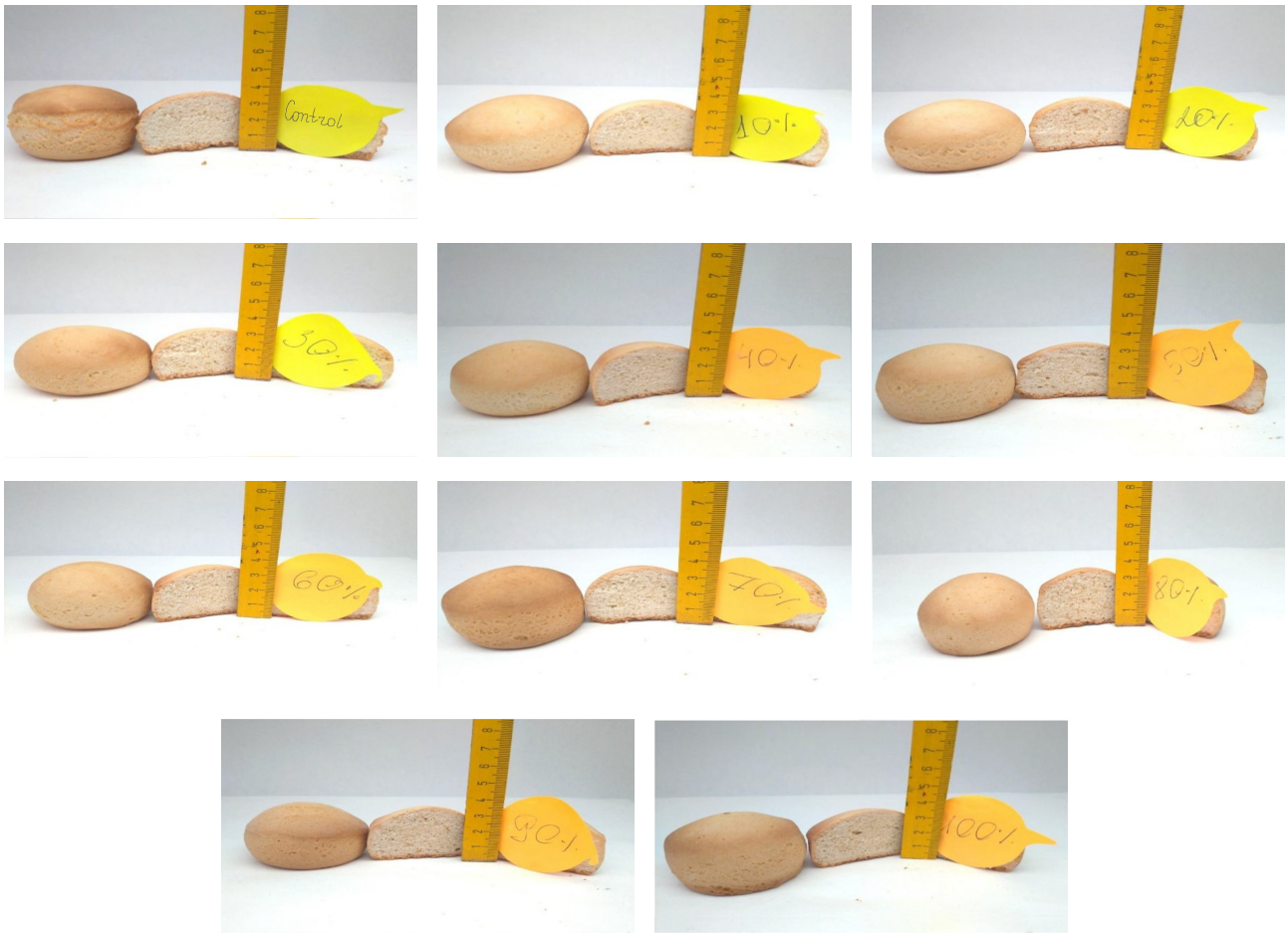

Figure 1 Honey cookies from wheat flour (control), mixtures of wheat and triticale flours (10–90%), and from 100% triticale flour

test dough pieces more extensible and less elastic, which prevents their deformation during cookie formation.

Also, the samples containing larger amounts of triticale flour had lower density compared to those with predominantly wheat flour. The changes in color and higher sweetness are associated with an increase in dextrins and sugars resulting from the enzymatic hydrolysis of starch, which gives the product a pleasant aftertaste.

Figure 1 show the samples of honey cookies made from premium wheat flour (control) and mixtures of wheat and triticale flours in different ratios.

Table 4 presents the nutritional value of the honey cookies made from wheat flour, triticale flour, and their mixtures.

According to Table 4, the protein content in the test sample from triticale flour increased by 22.8% compared to the control wheat flour sample. However,

Table 4 Nutritional value of honey cookies

Ratio of wheat and triticale flours	Components (in 100 g)			
	Proteins, g	Fats, g	Carbohydrates, g	Calorie content, kcal
100% wheat (control)	7.55	10.3	72.3	451.3
90% wheat + 10% triticale	7.87	10.6	73.2	457.2
80% wheat + 20% triticale	7.94	10.8	73.5	461.4
70% wheat + 30% triticale	7.99	11.1	73.7	465.8
60% wheat + 40% triticale	8.06	10.9	74.1	470.1
50% wheat + 50% triticale	9.02	11.2	74.4	474.7
40% wheat + 60% triticale	9.08	11.0	74.9	478.5
30% wheat + 70% triticale	9.12	11.1	75.3	483.2
20% wheat + 80% triticale	9.14	11.2	75.8	487.9
10% wheat + 90% triticale	9.18	11.2	76.1	492.3
100% triticale	9.27	11.1	76.9	497.6

Table 5 Vitamin contents in honey cookies

Ratio of wheat and triticale flours	Vitamins, mg/100 g						
	E	B_1	B_2	B_3	B_4	B_5	B_6
100% wheat (control)	1.3	0.2	0.1	1.2	11.4	0.2	0
90% wheat + 10% triticale	1.3	0.2	0.1	1.4	10.4	0.4	0
80% wheat + 20% triticale	1.4	0.3	0.1	1.6	9.3	0.6	0.1
70% wheat + 30% triticale	1.5	0.2	0.1	1.7	8.3	0.9	0.1
60% wheat + 40% triticale	1.6	0.3	0.1	1.8	7.2	1.0	0.2
50% wheat + 50% triticale	1.7	0.3	0.1	2.0	6.2	1.2	0.2
40% wheat + 60% triticale	1.7	0.3	0.1	2.2	5.2	1.4	0.2
30% wheat + 70% triticale	1.8	0.4	0.1	2.4	4.1	1.6	0.3
20% wheat + 80% triticale	1.8	0.3	0.1	2.5	3.1	1.7	0.3
10% wheat + 90% triticale	2.0	0.3	0.1	2.7	2.0	2.0	0.4
100% triticale	2.1	0.4	0.1	2.9	1.0	2.2	0.4

Table 6 Contents of macro- and microelements in honey cookies

Ratio of wheat and triticale flours	Macro- and microelements, mg/100 g								
	Ca	Fe	Mg	P	K	Na	Zn	Cu	Mn
100% wheat (control)	79.4	1.41	25.4	129.4	151.4	110.6	1.0	0.3	0.8
90% wheat + 10% triticale	80.9	1.51	38.2	150.4	183.1	110.5	1.2	0.4	1.1
80% wheat + 20% triticale	82.4	1.61	50.9	172.1	214.8	110.4	1.3	0.4	1.5
70% wheat + 30% triticale	83.9	1.81	63.8	193.5	246.5	110.3	1.5	0.4	1.9
60% wheat + 40% triticale	85.4	1.91	76.6	214.5	278.2	110.2	1.7	0.4	2.5
50% wheat + 50% triticale	86.9	2.01	89.4	236.3	309.9	110.1	1.8	0.5	2.5
40% wheat + 60% triticale	88.4	2.21	102.2	257.7	341.6	110.3	2.0	0.5	2.5
30% wheat + 70% triticale	89.9	2.31	115.0	279.7	373.3	110.4	2.2	0.6	3.1
20% wheat + 80% triticale	91.4	2.51	127.8	300.5	405.0	110.5	2.3	0.6	3.5
10% wheat + 90% triticale	92.9	2.50	140.6	321.9	436.7	110.6	2.5	0.5	3.9
100% triticale	94.4	2.71	153.4	343.3	486.4	110.6	2.7	0.6	4.2

both the control and the test samples showed no significant changes in the contents of fat and carbohydrates, as well as the calorie content.

Table 5 shows the vitamin composition of the honey cookies made from wheat flour, triticale flour, and their mixtures.

As can be seen from Table 5, the contents of vitamins E, B_1, B_3, B_5 and B_6 slightly increases with larger amounts of triticale flour in the test samples. Vitamin B_2 remained unchanged, while vitamin B_4 decreased with higher contents of triticale flour in the wheat-triticale flour ratios.

Table 6 shows the contents of macro- and microelements in the honey cookies made from wheat flour, triticale flour, and their mixtures.

According to Table 6, the samples from triticale flour had their contents of Ca, Fe, Mg, P, K, Zn, Cu, and Mn increased by 18.9, 92.2, 503.9, 165.3, 212.3, 170.0,

Table 7 Amino acid composition of honey cookies

Ratio of wheat and triticale flours	Essential amino acids, mg/100 g							
	Arginine	Valine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Threonine
100% wheat (control)	0.367	0.390	0.197	0.327	0.627	0.260	0.150	0.264
90% wheat + 10% triticale	0.443	0.434	0.238	0.369	0.731	0.242	0.186	0.294
80% wheat + 20% triticale	0.470	0.455	0.247	0.383	0.752	0.256	0.187	0.307
70% wheat + 30% triticale	0.495	0.474	0.255	0.395	0.773	0.271	0.190	0.320
60% wheat + 40% triticale	0.521	0.495	0.264	0.408	0.794	0.285	0.192	0.333
50% wheat + 50% triticale	0.548	0.514	0.272	0.420	0.815	0.298	0.194	0.345
40% wheat + 60% triticale	0.574	0.535	0.280	0.433	0.836	0.312	0.197	0.357
30% wheat + 70% triticale	0.600	0.554	0.289	0.446	0.857	0.326	0.199	0.370
20% wheat + 80% triticale	0.625	0.575	0.297	0.458	0.878	0.341	0.202	0.383
10% wheat + 90% triticale	0.647	0.592	0.303	0.469	0.891	0.358	0.204	0.394
100% triticale	0.678	0.615	0.314	0.484	0.920	0.369	0.206	0.409

100.0, and 425.0% compared to the control sample from wheat flour. The Na content remained virtually unchanged for all the samples.

Table 7 shows the amino acid composition of the honey cookies made from wheat flour, triticale flour, and their mixtures.

As can be seen from Table 7, the cookie samples from triticale flour had their contents of arginine, valine, histidine, isoleucine, leucine, lysine, methionine, and threonine increased by 84.7, 57.7, 59.4, 48.0, 46.7, 41.9, 37.3, and 54.9% compared to the control sample from wheat flour.

Thus, our tests proved that triticale flour of grade T-80 can be used in the production of honey cookies to enrich them with valuable substances, including vitamins, macro- and microelements, and essential amino acids. In addition, triticale flour has a positive effect on the quality of finished products, improving their sensory and physicochemical properties.

CONCLUSION

According to our results, triticale flour of grade T-80 can be used to replace premium wheat flour in the production of honey cookies (Rus. “pryaniks”). All the samples made from mixtures of wheat and triticale flours in various ratios, as well as from 100% triticale flour, had an excellent appearance.

Using up to 40% of triticale flour instead of wheat flour gives honey cookies good sensory characteristics and higher nutritional and biological values due to increased amounts of vitamins, macro- and microelements, protein, and essential amino acids.

CONFLICT OF INTEREST

The author declares that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Using casein and gluten protein fractions to obtain functional ingredients

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Abstract:
Today, the food industry widely uses both animal and plant proteins. Animal proteins have a balanced amino acid composition, while plant proteins have more pronounced functional properties. However, both types of proteins can act as allergens, which limits their practical application. Therefore, we aimed to select optimal conditions for obtaining hypoallergenic mixtures based on casein hydrolysates and gluten proteins, which have good functional properties and a balanced amino acid composition.
We used wheat flour (Makfa, Russia) with 12.6% of crude protein and 69.4% of starch, as well as rennet casein (Atletic Food, Russia) with 90% of protein. The methods included the Lowry method, the Anson method, Laemmli electrophoresis, ion-exchange chromatography, and the enzyme-linked immunosorbent assay.
Protex 6L was an optimal enzyme preparation for the hydrolysis of gliadin, while chymotrypsin was optimal for the hydrolysis of glutenin and casein. The optimal amount for all the enzymes was 40 units/g of substrate. We analyzed the effect of casein, glutenin, and gliadin enzymolysis time on the functional properties of the hydrolysates and found that the latter had relatively low water- and fat-holding capacities. The highest foaming capacity was observed in gliadin hydrolysates, while the highest emulsifying capacity was registered in casein and glutenin hydrolysates. Further, protein enzymolysis significantly decreased allergenicity, so the hydrolysates can be used to obtain functional additives for hypoallergenic products. Finally, the mixtures of casein hydrolysate and gliadin or glutenin hydrolysates had a balanced amino acid composition and a high amino acid score. Also, they retained high emulsifying and foaming capacities.
The study proved the need for mixtures based on wheat protein and casein hydrolysates, which have good functional properties and hypoallergenicity.

Keywords: Gliadin, glutenin, casein, allergenicity, functional foods, enzymatic hydrolysis, amino acid score

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INTRODUCTION

Russia prioritizes the quality of food and seeks to improve it by developing healthy foods, including functional products [1]. The modern food industry widely uses proteins of both animal and plant origin. This is meant to make up the deficiency of protein caused by a rapid population growth, the shortage of cultivated areas, and unfavorable environmental conditions. The quality of food could be effectively improved by 20–30% in highly nutritional products, including healthy foods rich in protein, essential amino acids, vitamins, as well as micro- and macronutrients [2, 3].

Today, the main sources of plant protein are soybeans, wheat, nuts, oilseeds, and legumes. Soy protein is the most common meat substitute, with a low cost and high crop yield. It accounts for 20 to 40% of the human diet [4].

However, the role of cereal proteins, primarily wheat, is currently on the rise. The nutritional value of plant proteins is primarily determined by their fractional and amino acid composition. Cereal protein fractions are classified on the basis of solubility. Globulins and albumins are extracted by treating flour with a 5% sodium chloride solution and water. Prolamins and glutelins are extracted by treating flour with 60% alcohol and a 0.1% sodium hydroxide solution [5]. Cereal proteins also contain scleroproteins that perform a structural function [6, 7].

Wheat protein, gluten, is one of the most common in Russia. Due to its low cost, gluten is used in a variety

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of food products, including functional foods. In Europe and the USA, flour is often enriched with gluten to give bakery products a marketable appearance and make them less friable. Functional additives based on gluten and its components are also used as emulsifiers and foaming agents.

One of the disadvantages of gluten is that some people have an intolerance, or allergy, to it called "celiac disease". This allergy is usually caused by an IgE-mediated reaction to ω-5 gliadin, one of gluten components (gliadin and/or glutenin in wheat) [8–10]. It is the main allergen that causes anaphylactic reaction to gluten. Also allergenic are proteins related to serine proteases (α-amylase/trypsin inhibitor – Tri a 15-AAI monomer, Tri a 39-serine protease inhibitor), agglutenins, peroxidase, nonspecific proteins (lipid carriers), and other components of gliadin [7, 11, 12].

In order to make gluten less allergic, it can be modified by enzymatic or acid hydrolysis, with its most allergenic fraction (gliadin) removed from it.

Enzymatic hydrolysis of wheat gluten is complicated by the fact that it consists of two fractions – gliadin and glutenin. These fractions are most effectively hydrolyzed by different enzymes, so multi-enzymatic compositions or alcalase need to be used. The use of alcalase requires a two-stage process with a product removal, since protein cleavage is inhibited by the reaction products. This produces a mixture of incompletely hydrolyzed but water-soluble proteins, peptides, and amino acids, as well as enhances the surfactant properties of the hydrolysate. The final result of enzymolysis significantly depends on the reaction time, which affects the composition and functional properties of hydrolysates [13]. Enzymatic hydrolysis of wheat protein is also used to reduce its allergenicity caused by wheat prolamins. Finally, gluten can be hydrolyzed by microbial proteases that destroy peptide bonds in the region of proline residues, abundant in gliadin [14].

Casein is of great practical importance among proteins of animal origin. It is the main protein of milk, cheese, cottage cheese, and other dairy products. Casein contains physiologically available calcium and phosphorus, which determine its nutritional value [15, 16].

Due to its structure, casein is easily broken down by proteolytic enzymes during digestion, even without prior denaturation [17, 18].

Abundant in nutrients and functional components, milk proteins are widely used in many food formulations (dairy desserts, nutritional drinks, ice cream, yogurt, meat products, confectionery, and baked goods). Milk proteins perform various key functions, including emulsification, thickening, gelling, and foaming. A wide range of products based on milk proteins includes caseins and caseinates, whey protein concentrates, isolates and hydrolysates, as well as milk protein concentrates [19–21].

Allergenicity is the main obstacle to using casein in food products [22, 23]. Casein fraction is represented by four types: αS1-casein, αS2-casein, β-casein, and κ-casein, with αS1-casein being the main allergen [24]. To date, there are no effective methods of treatment or drugs for the allergy to milk components that do not cause noticeable side effects. Previously, dairy products were simply excluded from the diet of people with the allergy. However, this caused a deficiency of important nutrients contained in dairy products [24].

Modern food scientists have developed a number of milk processing methods to reduce casein allergenicity. The main ones are heat treatment, enzymatic hydrolysis, and glycation [25]. Enzymatic hydrolysis seems quite promising, since casein fractions are resistant to heat. Enzyme preparations can be specially selected to ensure a desirable product (including products with good sensory properties), as well as to optimize the technology for processing milk-protein raw materials [26, 27]. The degree of casein hydrolysis determines the taste and aromatic qualities, as well as the functional properties of hydrolysates, such as emulsifying, gelling, and foaming abilities, as well as hygroscopicity. The higher the degree, the larger the amount of free amino acids in the hydrolysate, which improves its biological value [27, 28]. Also, the degree of hydrolysis can affect the solubility of the hydrolysate: the deeper the hydrolysis, the better the solubility. Denatured and dried proteins dissolve better even with incomplete hydrolysis. However, deep hydrolysis of milk proteins can worsen their sensory properties, especially the smell and the taste.

According to previous studies, chymotrypsin and thermolysin, as well as a temperature of 50°C, are optimal for the enzymatic hydrolysis of milk proteins. These conditions preserve the amino acid composition of the final hydrolysate, improve its nutritional value, and greatly reduce or completely eliminate its antigenic activity [29, 30]. Milk proteins are also hydrolyzed by proteolytic systems of lactic acid bacteria [30].

Milk casein is fundamentally different from wheat flour proteins in the fractional composition and the content of individual amino acids. Wheat proteins are high in valine and phenylalanine but low in tryptophan, lysine, and methionine, while casein is rich in leucine, valine, lysine, methionine, and tryptophan [31]. Thus, products with a balanced amino acid composition can be obtained by mixing casein and wheat proteins.

We aimed to select the conditions for obtaining hypoallergenic mixtures based on casein and gluten hydrolysates which have functional properties and a balanced amino acid composition.

STUDY OBJECTS AND METHODS

Study objects. We studied the hydrolysates of casein, gliadin, and glutenin, as well as their combinations: casein + gliadin at proportions of 0.5:1, 1:1, and 2:1 and casein + glutenin at proportions of 0.5:1, 1:1 and 2:1.

Materials. We used wheat flour of the highest grade (Makfa, Russia) with 12.6% of crude protein and 69.4% of starch, as well as rennet casein (Atletic Food, Russia) with 90% of protein. Enzymatic protein hydrolysates were obtained by using enzyme preparations with specific proteolytic activity measured by the Anson method (State Standard 20264.2-88). They included chymotrypsin (Samson-Med; 1900 u/g protein), Protex 6L (Genencor; 2100 u/g protein), pancreatin (Biosintez; 177 u/g protein), trypsin (Diaem; 1800 u/g protein), and beef pepsin (Moscow Rennet Plant; 7500 u/g protein).

Enzymatic hydrolysis of proteins. Proteins (10 g/L of a solution) were hydrolyzed for 2 h, with the enzyme preparation activity of 40 u/g of substrate, under optimal temperature and pH conditions. A modified Lowry method was used to measure the concentration of hydrolysis products in the supernatant [32]. The degree of protein hydrolysis was determined as a ratio of the low-molecular-weight protein fraction in the hydrolysate to the initial protein concentration.

Enzyme preparations. Three enzyme preparations were used, namely: Protex 6L, pancreatin, and chymotrypsin. Hydrolysis was carried out at a substrate concentration of 10 g/L, proteolytic activity of 20–60 u/g substrate, at 40°C and pH 7.6–8.2 for 2 h. The hydrolysates were analyzed for the content of the low-molecular-weight protein fraction by the modified Lowry method and the degree of protein hydrolysis was determined as described above [32].

Gliadin extraction. The protein fraction of wheat gliadin was extracted from wheat gluten with a 40% ethanol solution (1:9) at 40°C for 2 h. Then, gliadin was precipitated with acetone at an ethanol extract:acetone ratio of 1:5. Its resulting fraction contained 72% of protein.

Glutenin preparation. After the extraction of gliadin, the insoluble residue was treated with a 2% sodium hydroxide solution for 2 h, followed by precipitation from an aqueous solution at pH 5–6. The resulting glutenin fraction contained 68% of protein.

The effect of enzymatic hydrolysis time on the functional properties of hydrolysates. Hydrolysates were prepared from gliadin, glutenin, and casein by enzymolysis varying in time. For this, 1 g of each hydrolysate was mixed with water (1:25) and then an enzyme preparation was added until the proteolytic activity in the solution reached 40 u/g of protein. Hydrolysis was carried out at the temperature and pH optimal for the selected enzyme preparation for 15, 30, 60, 90, and 120 min. Heating protein hydrolysates may produce an unpleasant odor due to side chemical reactions, for example, the Maillard and Strecker reactions [34]. Therefore, the enzymes were inactivated by cooling to –10°C. The hydrolysate samples were dried at 50°C, after which their functional properties were studied.

Protein content. The content of protein in the hydrolysate samples was measured by the modified Lowry method, with a separate determination of high- and low-molecular-weight protein fractions (HMF and NMF, respectively) by a preliminary precipitation of HMF with 50% trichloroacetic acid.

Total proteolytic activity. The total proteolytic activity was measured by a modified Anson method (State Standard 20264.2-88). A unit of proteolytic activity was understood as the enzyme's ability to convert sodium caseinate into a form unprecipitable by trichloroacetic acid in an amount corresponding to 1 μmoL of tyrosine in 1 min at 30°C.

The allergenicity of gliadin and glutenin hydrolysates was quantified by enzyme immunoassay according to ALINORM Standard 08/31/26 for food products (Methodological Guidelines 4.1.2880-11 4.1). We took into account the specific interaction between the allergenic protein in the test sample and the antibodies to it contained in the test solution (Siemens, Germany). For the immunoassay, we placed 100 μL of a 1% solution of the test sample into a well of the plate and added 100 μL of a conjugate test solution containing antibodies to gliadin. The optical density was measured at 450 nm and then recalculated for gliadin (one unit of optical density corresponded to 40 μg/mL of gliadin). Allergenicity was considered low if the gluten content was under 20 mg/kg and moderate if it amounted to 20–100 mg/kg of the end product.

Amino acid composition. The amino acid composition of the hydrolysate samples and their mixtures was determined by ion exchange chromatography on an ARACUS amino acid analyzer (MembraPure GmbH, Bodenheim, Germany) equipped with a C18 column and a refractive index detector. High-performance liquid chromatography was supplemented with mass spectrometry, with electrospray ionization for separation of amino acids followed by ninhydrin reaction and photometric detection [34].

Electrophoresis by the Laemmli method. The molecular weight of casein hydrolysate components was determined by electrophoresis using the Laemmli method in polyacrylamide gel with 12.5% SDS. For comparison, we used a marker that included 11 standards of certain molecular weights, namely 250, 150, 100, 70, 50, 40, 30, 20, 15, 10, and 5 kDa (Fermentas, Lithuania). Electrophoresis was carried out in a VE-10 chamber (Helicon, Crenshaw, Alabama, USA) at room temperature without additional cooling at 60 V for the first 30 min and then at 120 V until the samples reached the lower edge of the gel. The resulting electropherograms were stained with Coomassie Brilliant Blue G 250, followed by washing with acetic acid [35].

Fat-holding capacity. 0.5 g of the test sample was placed in glass centrifuge tubes and 0.125 to 0.625 mL of vegetable oil was added with an interval of 0.125 mL. The contents of the tubes were stirred for 10 min, then the samples were kept under stirring for 15 min, cooled to room temperature, and centrifuged at 1500 rpm for 15 min. The fat-holding capacity was determined as the

maximum amount of added oil at which no separation of the oil phase was observed during the test, expressed in terms of 1 g of preparation [36].

Water-holding capacity. 0.5 g of the test sample was placed in glass centrifuge tubes and 1.5 to 2.5 mL of water was added with an interval of 0.25 mL. The experiment continued as described above (for fat-holding capacity). The water-holding capacity was determined as the maximum amount of added water, at which no separation of the aqueous phase was observed during the test, expressed in terms of 1 g of preparation [36].

Emulsifying capacity. 1 g of the test sample was placed in glass centrifuge tubes and mixed with 5 mL of water and 5 mL of oil. The contents of the test tubes were stirred for 10 min, followed by the procedures described above. The emulsifying capacity was determined as a percentage ratio between the aqueous and oil phases separated from the emulsion [36].

Foaming capacity. 0.25 g of the test sample was placed in 50 mL conical flasks and mixed with 25 mL of water. The resulting solution was shaken with a shaker for 30 s. Then it was poured into a measuring cylinder to measure the height of the foam column [37].

Statistical analysis was carried out using the Statistics 2020 programs.

RESULTS AND DISCUSSION

According to literature, hydrolysates of casein, gliadin, and glutenin are not allergenic, unlike the original proteins. In addition, they have much better functional properties (foaming, fat- and water-holding, emulsifying) than the original proteins [38].

The functional properties of protein hydrolysates are affected by the enzymolysis time and the choice of an enzyme preparation. We evaluated the efficiency of the most common protease enzyme preparations, namely chymotrypsin (1917 u/g), pancreatin (1501 u/g), and Protex 6L (2156 u/g).

The enzymatic hydrolysis of the gliadin and glutenin samples obtained by the above methods, as well as an industrial sample of casein, was carried out at the temperature and pH conditions optimal for each enzyme preparation with the activity of 40 u/g of substrate (Table 1).

According to Table 1, Protex 6L was selected for the hydrolysis of gliadin, while chymotrypsin was selected for casein and glutenin. Further, the best activities of these enzyme preparations were selected to ensure the maximum degree of protein hydrolysis (Fig. 1).

Table 1 Selection of an enzyme preparation for casein, gliadin, and glutenin hydrolysis

Enzyme preparation	Degree of hydrolysis, %		
	Gliadin	Glutenin	Casein
Chymotrypsin	25.4	34.1	48.1
Pancreatin	51.1	6.51	36.1
Protex 6L	60.5	13.0	40.1

As can be seen, an increase in the activity of proteases above 40 u/g of substrate did not lead to a noticeable increase in the degree of hydrolysis. Therefore, this activity value was taken as recommended.

At the next stage, we determined the functional properties of the hydrolysates (Fig. 2). As we know, the functional properties (water-holding, fat-holding, emulsifying, and foaming ones) depend on the degree of protein hydrolysis, which, in its turn, depends on the hydrolysis time.

According to Fig. 2, the water-holding capacity decreased in the course of hydrolysis in all the cases. Its slight increase during the first 15 min of glutenin hydrolysis might be explained by a larger specific surface area of the hydrolyzed substrate. This is due to the fact that during hydrolysis, proteins lose their ability to maintain a water-retaining structure. Longer hydrolysis increases the degree of protein degradation. The resulting peptides have a high solubility, which reduces their water-holding capacity. The lower water-holding capacity is also likely to decrease the hygroscopicity of hydrolysates and their gelling ability.

The fat-holding capacity showed a similar trend (Fig. 2). This capacity depends on fat retention in the native structure of the protein, which is destroyed during enzymatic hydrolysis. Protein hydrolysis results in low-molecular-weight peptides with a lower hydrophobicity than that of the original protein. This decreases the fat-holding capacity.

During short hydrolysis, the emulsifying and foaming capacities increased for glutenin and casein, respectively (Fig. 2). This might due to the fact that short protein hydrolysis produces peptides with surfactant properties that promote foaming and emulsification. However, longer hydrolysis leads to the destruction of these peptides and to a decrease in emulsifying and foaming capacities. Plant protein hydrolysates, which have significant surfactant properties, can become a more cost-effective substitute for animal protein hydrolysates in the products that

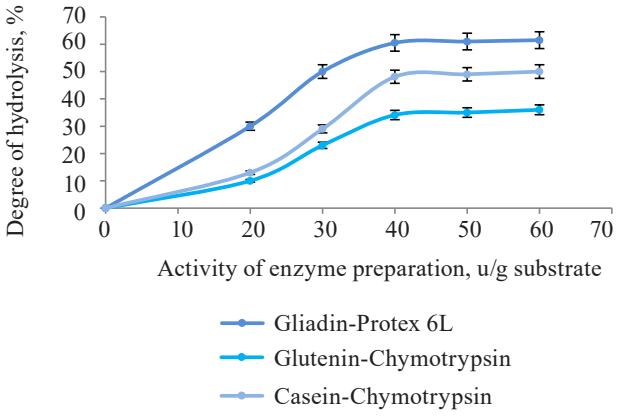

Figure 1 Effect of enzyme preparation activity on the degree of casein, gliadin, and glutenin hydrolysis

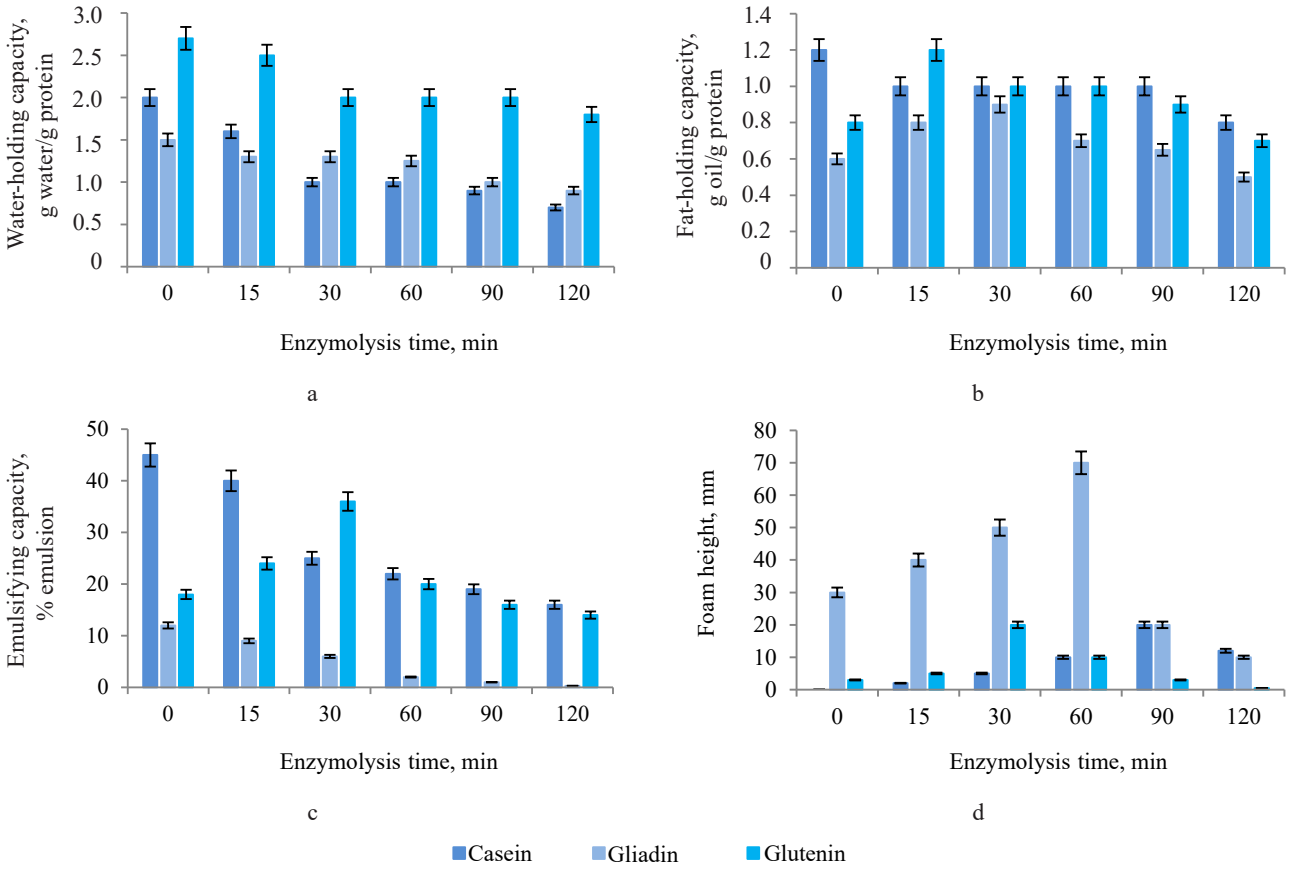

Figure 2 Effect of casein, gliadin, and glutenin enzymolysis time on the functional properties of hydrolysates: (a) water-holding capacity; (b) fat-holding capacity; (c) emulsifying capacity; and (d) foaming capacity

Table 2 Allergenicity of gliadin and glutenin hydrolysates

Protein sample	Enzymolysis time, min	Allergenicity determination		
		Degree of hydrolysis, %	Allergen concentration in hydrolysate, μg/g	Allergenicity level
Gliadin	0	7.0 ± 1.0	181.0 ± 9.0	High
	15	24.0 ± 1.0	127.0 ± 7.0	High
	30	37.0 ± 2.0	76.0 ± 5.0	Moderate
	60	46.0 ± 2.0	16.4 ± 3.0	Moderate
	90	54.0 ± 3.0	15.0 ± 1.0	Low
	120	60.0 ± 3.0	12.0 ± 1.0	Low
Glutenin	0	5.6 ± 0.3	115.0 ± 6.0	High
	15	14.0 ± 1.0	63.0 ± 4.0	Moderate
	30	18.0 ± 1.0	18.0 ± 2.0	Low
	60	25.0 ± 1.0	15.0 ± 1.0	Low
	90	30.0 ± 2.0	10.0 ± 1.0	Low
	120	34.0 ± 2.0	7.0 ± 1.0	Low

need good emulsifying and foaming properties, such as yoghurts and shakes.

At the next stage, we determined the allergenicity of wheat protein and casein enzyme lysates (Table 2).

As can be seen in Table 2, the original proteins had a high level of allergenicity, which decreased after 90 min of enzymolysis for gliadin and 60 min of enzymolysis for glutenin.

According to literature, the allergenicity of casein hydrolysates significantly decreases if they have a minimal content of proteins with molecular weights over 25 kDa and a predominance of peptides with molecular weights of 10–15 kDa. The main allergens of milk are casein (25–98 kDa), *β*-lactoglobulin (18.4 kDa), and *α*-lactalbumin (15 kDa). Therefore, low allergenicity can be provided by the predominance of peptide fractions with molecular weights below 15 kDa [39, 40]. In our study, we determined the molecular weights of hydrolysate components by Laemmli electrophoresis in polyacrylamide gel (Table 3) [35].

Table 3 Molecular weight distribution of protein fractions in casein hydrolysates depending on enzymolysis time

Enzymolysis time, min	Degree of protein hydrolysis, %	Molecular weight range, kDa			
		25–30	20–25	15–20	10–15
0	8.1 ± 1.2	47.0 ± 4.0	28.0 ± 3.0	22.0 ± 2.0	3.0 ± 0.3
15	17.0 ± 1.0	34.0 ± 3.0	37.0 ± 3.0	22.0 ± 2.0	7.0 ± 1.2
30	24.0 ± 1.0	25.0 ± 3.0	20.0 ± 2.0	40.0 ± 3.0	15.0 ± 1.0
60	37.0 ± 2.0	14.0 ± 1.0	15.0 ± 1.0	45.0 ± 4.0	26.0 ± 2.0
90	45.0 ± 2.0	8.0 ± 1.0	10.0 ± 1.0	41.0 ± 3.0	41.0 ± 3.0
120	48.0 ± 2.0	2.0 ± 0.3	3.0 ± 0.5	42.0 ± 4.0	53.0 ± 5.0

Table 4 Amino acid composition of casein, gliadin, and glutenin hydrolysates and their mixtures

Amino acid	Hydrolysate								
	Casein, 90 min	Gliadin, 60 min	Glutenin, 30 min	Casein, 90 min +					
				Gliadin 60 min, casein:gliadin ratio			Glutenin 30 min, casein:glutenin ratio		
				0.5:1	1:1	2:1	0.5:1	1:1	1:2
Gly	2.7 ± 0.1	1.9 ± 0.1	3.4 ± 0.2	2.2 ± 0.2	2.3 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	2.9 ± 0.1	3.2 ± 0.2
Ala	3.0 ± 0.2	2.5 ± 0.1	2.5 ± 0.1	2.7 ± 0.1	2.8 ± 0.1	2.8 ± 0.1	2.8 ± 0.1	2.8 ± 0.1	2.7 ± 0.1
Val	7.2 ± 0.4	5.2 ± 0.3	4.5 ± 0.2	5.9 ± 0.3	6.2 ± 0.3	5.9 ± 0.3	6.5 ± 0.3	6.3 ± 0.3	5.4 ± 0.3
Ile	6.1 ± 0.3	5.1 ± 0.3	3.7 ± 0.2	5.4 ± 0.3	5.6 ± 0.3	4.9 ± 0.2	5.8 ± 0.3	5.3 ± 0.3	4.5 ± 0.2
Leu	9.2 ± 0.5	8.4 ± 0.4	7.3 ± 0.4	8.7 ± 0.4	8.8 ± 0.4	8.3 ± 0.4	8.9 ± 0,4	8.6 ± 0.4	7.9 ± 0.4
Pro	11.0 ± 1.0	18.0 ± 1.0	17.0 ± 1.0	16.0 ± 1.0	15.0 ± 1.0	14.0 ± 1.0	14.0 ± 1.0	13.0 ± 1.0	15.0 ± 1.0
Ser	6.3 ± 0.3	4.1 ± 0.2	3.3 ± 0.2	4.8 ± 0.2	5.2 ± 0.3	4.8 ± 0.2	5.6 ± 0.3	5.3 ± 0.3	4.3 ± 0.2
Thr	4.9 ± 0.2	2.2 ± 0.1	2.7 ± 0.1	3.1 ± 0.2	3.6 ± 0.2	3.8 ± 0.2	4.0 ± 0.2	4.2 ± 0.2	3.4 ± 0.2
Cys	0.30 ± 0.02	5.1 ± 0.3	5.7 ± 0.3	3.5 ± 0.2	2.7 ± 0.1	3.0 ± 0.2	1.9 ± 0.1	2.1 ± 0.1	3.9 ± 0.2
Met	2.8 ± 0.1	1.6 ± 0.1	1.8 ± 0.1	2.0 ± 0.1	2.2 ± 0.1	2.3 ± 0.1	2.4 ± 0.1	2.5 ± 0.1	2.1 ± 0.1
Asp	7.1 ± 0.4	2.8 ± 0.1	2.7 ± 0.1	4.2 ± 0.2	5.0 ± 0.3	4.9 ± 0.2	5.7 ± 0.3	5.7 ± 0.3	4.2 ± 0.2
Glu	22.4 ± 1.1	51.0 ± 3.0	49.0 ± 2.0	41.0 ± 2.0	37.0 ± 2.0	36.0 ± 2.0	32.0 ± 2.0	32.0 ± 2.0	41.0 ± 2.0
Lys	8.2 ± 0.4	0.73 ± 0.04	1.2 ± 0.1	3.2 ± 0.2	4.5 ± 0.2	4.7 ± 0.2	5.7 ± 0.3	5.9 ± 0.3	3.5 ± 0.2
Arg	4.1 ± 0.2	2.9 ± 0.2	2.9 ± 0.1	3.3 ± 0.2	3.5 ± 0.2	3.5 ± 0.2	3.7 ± 0.2	3.7 ± 0.2	3.3 ± 0.2
His	3.1 ± 0.2	1.8 ± 0.1	1.5 ± 0.1	2.2 ± 0.1	2.5 ± 0.1	2.3 ± 0.1	2.7 ± 0.1	2.6 ± 0.1	2.0 ± 0.1
Phe	5.0 ± 0.3	6.6 ± 0.3	6.0 ± 0.3	6.1 ± 0.3	5.8 ± 0.3	5.5 ± 0.3	5.5 ± 0.3	5.3 ± 0.3	5.7 ± 0.3
Trp	1.2 ± 0.1	0.10 ± 0.01	1.1 ± 0.1	0.54 ± 0.03	0.73 ± 0.04	1.1 ± 0.1	0.82 ± 0.04	1.1 ± 0.1	1.1 ± 0.1
Tyr	6.3 ± 0.3	3.0 ± 0.2	3.5 ± 0.2	4.1 ± 0.2	4.7 ± 0.2	4.9 ± 0.2	5.2 ± 0.3	5.4 ± 0.3	4.4 ± 0.2

Table 5 Functional properties of mixed hydrolysates of wheat proteins and casein

Mixtures of protein hydrolysates	Water-holding, g H_2O/g protein	Fat-holding, g oil/g protein	Emulsifying, % emulsion	Foaming, mm
Casein 90 min + Gliadin 60 min (0.5:1)	1.67 ± 0.08	1.16 ± 0.06	8.0 ± 1.0	35.0 ± 2.0
Casein 90 min + Gliadin 60 min (1:1)	1.25 ± 0.06	0.75 ± 0.04	14.0 ± 1.0	42.0 ± 3.0
Casein 90 min + Gliadin 60 min (2:1)	0.95 ± 0.03	0.68 ± 0.03	20.0 ± 1.0	56.0 ± 3.0
Casein 90 min + Glutenin 30 min (0.5:1)	2.05 ± 0.10	1.15 ± 0.06	18.0 ± 1.0	10.0 ± 1.0
Casein 90 min + Glutenin 30 min (1:1)	1.87 ± 0.09	0.93 ± 0.05	20.0 ± 1.0	18.0 ± 1.0
Casein 90 min + Glutenin 30 min (2:1)	1.34 ± 0.07	0.28 ± 0.01	24.0 ± 1.0	29.0 ± 2.0

As can be seen, shorter enzymolysis increased the proportion of low-molecular-weight fractions (under 15 kDa) in casein hydrolysates. After 90 min, the proportion of high-molecular allergenic fractions (over 25 kDa) was less than 20%. Therefore, this time is sufficient to obtain hypoallergenic casein hydrolysates. According to literature [41], deep hydrolysis of casein results in the formation of bitter peptides. In our study, the degree of protein hydrolysis did not exceed 45%. Therefore, we can expect a minimal amount of bitter peptides.

For further tests, we selected the samples of three hydrolysates (one for each protein), namely:
– casein hydrolysate (90 min);
– glutenin hydrolysate (30 min); and
– gliadin hydrolysate (60 min).

The hydrolysates of casein and glutenin had a high emulsifying capacity, while the gliadin hydrolysate had a high foaming capacity. All of them had a low level of allergenicity.

One of the significant disadvantages of wheat proteins is their low content of certain amino acids.

Table 6 Amino acid score of hydrolysates

Amino acid	Amino acid score, %								
	Hydrolysates								
	Casein, 90 min	Gliadin, 60 min	Glutenin, 30 min	Casein, 90 min +					
				Gliadin 60 min, casein:gliadin ratio			Glutenin 30 min, casein:glutenin ratio		
				0.5:1	1:1	2:1	1:2	1:1	2:1
Lys	149 ± 7	13 ± 1	22 ± 1	58 ± 3	82 ± 4	85 ± 4	64 ± 3	107 ± 5	104 ± 5
Met+Cys	89 ± 4	191 ± 10	136 ± 7	157 ± 8	140 ± 7	151 ± 8	171 ± 9	131 ± 7	123 ± 6
Ile	153 ± 8	128 ± 6	93 ± 5	135 ± 7	140 ± 7	123 ± 6	113 ± 6	133 ± 7	145 ± 7
Leu	131 ± 7	120 ± 6	104 ± 5	124 ± 6	126 ± 6	119 ± 6	113 ± 6	123 ± 6	127 ± 6
Thr	123 ± 6	55 ± 3	68 ± 3	78 ± 4	90 ± 5	95 ± 5	85 ± 4	105 ± 5	100 ± 5
Phe+Tyr	188 ± 9	110 ± 6	100 ± 5	102 ± 5	97 ± 5	92 ± 5	95 ± 5	88 ± 4	92 ± 5
Trp	120 ± 6	100 ± 5	108 ± 5	54 ± 3	73 ± 4	110 ± 6	110 ± 6	110 ± 6	82 ± 4
Val	144 ± 7	104 ± 5	90 ± 5	118 ± 6	124 ± 6	118 ± 6	108 ± 5	126 ± 6	130 ± 7

Particularly, gliadin is low in lysine, threonine, tryptophan, arginine, histidine, cystine, and methionine, whereas glutenin is low in lysine, although its content is higher than in gliadin. Therefore, mixtures of casein hydrolysates and wheat protein hydrolysates are of particular interest due to their balanced amino acid composition and good functional properties. Such mixtures can be used as ingredients in functional food products.

Finally, we studied mixtures of casein hydrolysates with gliadin and glutenin hydrolysates in various ratios. We determined their amino acid composition and functional properties (Tables 4 and 5).

Mixing plant protein hydrolysates with casein hydrolysate enriches them with such amino acids as alanine, valine, serine, methionine, aspartic acid, lysine, and tyrosine (Table 4). At the same time, the contents of other amino acids change insignificantly, except for glutamic acid, which somewhat decreases, but remains quite high.

The mixtures of gliadin/glutenin and casein hydrolysates had better foaming and emulsifying properties, which improved with a higher proportion of casein. Thus, we found it worthwhile to mix hydrolyzed wheat proteins with casein to obtain improved functional properties and hypoallergenicity.

Table 6 shows the amino acid score for the obtained mixtures of protein hydrolysates. As can be seen, the score was high in the mixtures of casein and gliadin hydrolysates (2:1), as well as casein and glutenin (2:1). These mixtures also showed high emulsifying and foaming capacities. Therefore, they can be considered the most promising for obtaining hypoallergenic ingredients for functional food products.

CONCLUSION

The most effective enzyme preparations for the enzymolysis of casein, gliadin, and glutenin were Protex 6L for gliadin and chymotrypsin for glutenin and casein;

We recommend 40 u/g of substrate as an optimal amount of enzyme preparations to provide the maximum degree of hydrolysis;

The study of functional properties of casein, glutenin, and gliadin hydrolysates (15, 30, 60, and 90 min) showed that all the samples had relatively low water- and fat-holding capacities. The highest emulsifying capacity was observed in casein and glutenin hydrolysates, while the highest foaming capacity was found in gliadin hydrolysates;

The enzymolysis of all the proteins significantly decreased their allergenicity. Therefore, their hydrolysates can be used to obtain functional additives for hypoallergenic food products;

The mixtures of casein hydrolysate (90 min) with gliadin (60 min) or glutenin (30 min) hydrolysates had a balanced amino acid composition and a high amino acid score. In addition, they retained high emulsifying and foaming capacities; and

Our study proved the need for mixing wheat protein hydrolysates with casein hydrolysate to obtain improved functional properties and hypoallergenicity.

CONTRIBUTION

D.V. Prikhodko carried out the experiments, as well as processed and interpreted experimental data. A.A. Krasnoshtanova supervised the research, reviewed experimental data, and formulated conclusions.

CONFLICT OF INTEREST

The authors declared no conflict of interests regarding the publication of this article.

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Effect of dihydroquercetin on the toxic properties of nickel nanoparticles

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Abstract:
Dihydroquercetin (3,5,7,3',4'-pentahydroxy-flavanone) is known for its powerful antioxidant, organ-protective, and anti-inflammatory activities that can be applied to heavy-metal intoxication. The present research objective was to evaluate the possible protective potential of dietary dihydroquercetin in a rat model of subacute (92 days) intoxication with nickel nanoparticles.

The experiment involved five groups of twelve male Wistar rats in each. Group 1 served as control. Other groups received nickel nanoparticles as part of their diet. Groups 2 and 4 received nickel nanoparticles with an average diameter of 53.7 nm (NiNP1), while groups 3 and 5 were fed with nanoparticles with an average diameter of 70.9 nm (NiNP2). The dose was calculated as 10 mg/kg b.w. Groups 4 and 5 also received 23 mg/kg b.w. of water-soluble stabilized dihydroquercetin with drinking water.

After the dihydroquercetin treatment, the group that consumed 53.7 nm nickel nanoparticles demonstrated lower blood serum glucose, triglycerides, low-density lipoprotein cholesterol, and creatinine. Dihydroquercetin prevented the increase in total protein and albumin fraction associated with nickel nanoparticles intake. The experimental rats also demonstrated lower levels of pro-inflammatory cytokines IL-1β, IL-4, IL-6, and IL-17A, as well as a lower relative spleen weight after the treatment. In the group exposed to 53.7 nm nickel nanoparticles, the dihydroquercetin treatment increased the ratio of cytokines IL-10/IL-17A and decreased the level of circulating FABP2 protein, which is a biomarker of increased intestinal barrier permeability. In the group that received 70.9 nm nickel nanoparticles, the dihydroquercetin treatment inhibited the expression of the fibrogenic *Timp3* gene in the liver. In the group that received 53.7 nm nickel nanoparticles, dihydroquercetin partially improved the violated morphology indexes in liver and kidney tissue. However, dihydroquercetin restored neither the content of reduced glutathione in the liver nor the indicators of selenium safety, which were suppressed under the effect of nickel nanoparticles. Moreover, the treatment failed to restore the low locomotor activity in the elevated plus maze test.

Dihydroquercetin treatment showed some signs of detoxication and anti-inflammation in rats subjected to nickel nanoparticles. However, additional preclinical studies are necessary to substantiate its prophylactic potential in cases of exposure to nanoparticles of nickel and other heavy metals.

Keywords: Nanoparticles, nickel, dihydroquercetin, rats, detoxification, cytokines, intestinal barrier permeability

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INTRODUCTION

Nickel nanoparticles are components of catalysts that hydrogenate vegetable fats in the food industry [1]. They can be used in theranostics, insecticides, and cosmetics [2–4]. Miners, chemists, and metallurgists may inhale aerosol of nickel-containing nanoparticles [5]. Nickel and its oxide nanoparticles are toxic: they cause oxidative stress, apoptosis, and fibrotic changes [6–8]. Nickel derivatives are also known for their reproductive toxicity and allergenicity [9]. The International Agency for Research on Cancer classified nickel and its compounds as possibly carcinogenic to humans (Group 2B) [10].

Strict hygienic standards can reduce the harmful effects of nickel-containing nanoparticles on human health. However, the food industry is not always able to limit or ban their use. Therefore, food science needs new research on the dietary prevention of (nano)nickel toxicity, including cases of work-related exposure. Some nutritional substances possess antioxidant and bioprotective properties that can reduce the consequences of nickel intoxication, e.g., epigallocatechin-3-gallate, cinnamic acids, ascorbic acid, vitamin E, glycine, monosodium glutamate, etc. [6, 7, 9, 11].

Bioflavonoids are of particular scientific interest as substances capable of inhibiting the toxicity parameters of nickel nanoparticles. Dihydroquercetin (3,5,7,3',4'-pentahydroxyflavanone) was first isolated from Siberian larch (*Laric Sibirica* L.). It possesses a powerful antioxidant, organ-protective, and detoxifying effect, as well as a low toxicity [12, 13]. Unfortunately, natural dihydroquercetin is notorious for its low solubility in water, which affects its bioavailability [13]. However, nanotechnology makes it possible to produce dihydroquercetin in a stabilized water-soluble and more bioavailable form [14].

The research objective was to study the effect of stabilized water-soluble dihydroquercetin on the vital signs of rats exposed to nickel nanoparticles in a 92-day experiment.

STUDY OBJECTS AND METHODS

Nanomaterials. We used two preparations of nickel nanoparticles (Nanostructured & Amorphous Materials Inc., USA) with article numbers 0282HW and 0283HW. The sizes of primary nickel nanoparticles were 20 and 40 nm, as declared by the manufacturer. A transmission electron microscopy showed that the actual average diameter of nickel particles was 53.7 ± 2.9 (M ± m) and 70.9 ± 3.3 nm, respectively. The particles had a spherical shape. The nickel content was ≥ 99 %, according to the energy dispersive spectroscopy. The two preparations differed by more than two times in the content of particles with a diameter of ≤ 50 nm: 55.5 and 24.0% of the total amount, respectively. Both preparations were dispersed in an aqueous suspension with ice cooling by ultrasound for 15 min at a frequency of 44 kHz and a specific power of 2 W/cm^3 before given to the experimental animals.

Dihydroquercetin. Dihydroquercetin was administered to the rats as a water-soluble stabilized form of Taxifolin-Aqua (Prodvinutye Tekhnologii LLC, Russia). This product was registered in Russia as dietary supplements (No. RU.77.99.11.003.E.003036.07.18). A high performance liquid chromatography analysis (Guideline R.4.1.1672-2003) showed that the content of the active substance was 3 mg/mL. Taxifolin-Aqua included some approved food additives, polyvinylpyrrolidone (E1201) as a stabilizer, and potassium sorbate (E202) as a preservative.

Animals and experimental diets. The experiment involved six-week-old male Wistar rats with an average initial body weight of 170 ± 10 g obtained from the Scientific Center for Biomedical Technologies of the Federal Medical and Biological Agency of Russia. The research followed the rules of proper laboratory practice and international recommendations for the humane treatment of animals in accordance with the EU Council Directive 2010/63/EU (Directive 2010/63/EU on the protection of animals used for scientific purposes) and Recommended Practices 1.2.2520-09 on the Toxicological and Hygienic Assessment of Nanomaterial Safety. The design was approved by the Ethics Committee of the Federal Research Center of Nutrition and Biotechnology, Protocol 7, September 17, 2021. The rats were kept in pairs in polycarbonate cages at 21–24°C, 30–60% relative humidity, 12 light/12 dark cycle.

The animals were divided into five groups of twelve with the same initial body weight ($p > 0.1$, one-way ANOVA test). For 92 days, the rats received a balanced semi-synthetic diet according to AIN-93G with minor modifications and had unlimited access to drinking water [15]. The average estimated intake of nickel as a natural component of the diet was 0.03 mg/kg b.w.

Group 1 served as control and received no additives with the diet and drinking water. Starting with day 1, groups 2 and 3 received smaller nickel nanoparticles with an average diameter of 53.7 nm and larger nickel nanoparticles with an average diameter of 70.9 nm, respectively. The amount was calculated based on the weight of the daily consumed food as 10 mg/kg b.w. per day. Groups 4 and 5 received the same nanoparticles as groups 2 and 3, respectively. Additionally, they received a water-soluble stabilized form of dihydroquercetin with drinking water at 23 mg/kg b.w. based on anhydrous dihydroquercetin. The weight of food and water consumed by the rats was recorded daily to adjust the daily intake of nickel and dihydroquercetin, if necessary. The average concentration of dihydroquercetin was 0.2 mg/mL in drinking water in groups 4 and 5.

The cognitive function was assessed using the Conditioned Passive Avoidance Reflex test on days 65, 66, and 86. The level of anxiety-like functions and locomotor activity was tested on day 57 using the elevated plus maze (Panlab Harvard Apparatus, Spain). The methodology followed the pattern developed by Mzhelskaya *et al.* [15]. The rats were decapitated under ether anesthesia on day 93 after a 16-h fast.

Blood was collected in two portions in tubes with tripotassium EDTA salt as anticoagulant to determine the hematological profile and in dry sterile tubes to obtain blood serum. The internal organs, i.e., liver, kidneys, spleen, and ileum, were separated into sections with sterile surgical instruments and weighed on an electronic balance (± 1 mg). The liver sample was divided into three parts. Part 1 was homogenized in 0.1 M Tris-KCl buffer pH 7.4 in a Potter-Elway homogenizer in a ratio of 1:4 by weight to determine the thiol content. Part 2 was immediately frozen at –80°C for the genetic analysis. Part 3 was fixed in a chemically pure 3.7% formaldehyde solution in 0.1 M Na-phosphate buffer pH 7.0 for morphological studies.

Laboratory research methods. The leukocyte blood formula was determined using a Coulter AC TTM 5 diff OV hematological analyzer (Beckman Coulter, USA) with a standard set of staining reagents (Beckman Coulter, France). The biochemical parameters of blood serum involved glucose, triglycerides, total and LDL-cholesterol, total protein, albumins, globulins, creatinine, urea, uric acid, activity of alanine, and aspartate aminotransferases. They were determined using a Konelab 20i biochemical analyzer (Thermo Fischer Scientific, Finland) as instructed by the manufacturer. The content of reduced thiols in the liver tissue homogenate was measured spectrophotometrically using the Ellman's reagent. We determined the selenium concentration in urine and blood serum by the microfluorimetric method with 2,3-diaminonaphthalene. The content of fatty acid binding protein (FABP2) in blood serum was obtained by enzyme immunoassay (Cloud-Clone Corp., China). Cytokines Il-1β, IL-4, IL-6, IL-10, and IL-17A were tested using a multiplex immunoassay Luminex 200 device (Luminex Corpotion, USA). The xMAP technology was provided by Luminex xPONENT Version 3.1 software. A BioPlex Pro™ Reagent Kit V contained Bio-Plex Pro™ Reagents: Pro-Rat 33-Plex Standards, Rat Cytokine IL-1β Set, Rat Cytokine IL-4 Set, Rat Cytokine IL-6 Set, Rat Cytokine IL-10 Set, Rat Cytokine IL-17A Set (Bio-Rad Laboratories Inc., USA). Fibrosis genes *Timp1*, *Timp3*, *MMp2*, and *MMp9* in the liver were determined by the real-time reverse transcription polymerase chain reaction (RT-PCR). The primers and probes were provided by DNA Synthesis (Russia). We used a CFX 96 instrument (Bio-Rad Laboratories, Inc.) as amplifier. The gene expression was assessed by the value of the cycle threshold (Ct – cycle threshold) and normalized by the conditionally constitutive comparison genes *Actb* and *Gapdh* using the 2-ΔΔCt method as described by Trusov *et al*. [16].

The morphological examination of liver, kidney, and ileum tissue occurred after they were dehydrated in alcohols and xylene, embedded in paraffin, cut into 5-μm sections with a microtome, and subjected to Van Gieson staining with hematoxylin-eosin and fuchsin-picric acid. The study involved an Axio Imager Zl microscope (Zeiss, Germany) with a digital camera at 100×, 200×, and 400× magnifications.

The statistical processing determined the sample mean M with the standard mean square error m. We tested the hypothesis about the heterogeneity of the distribution of values across experimental groups using a one-factor ANOVA test. The pairwise differences between the experimental groups were established using the nonparametric Mann-Whitney test. The differences were considered significant at $p < 0.05$.

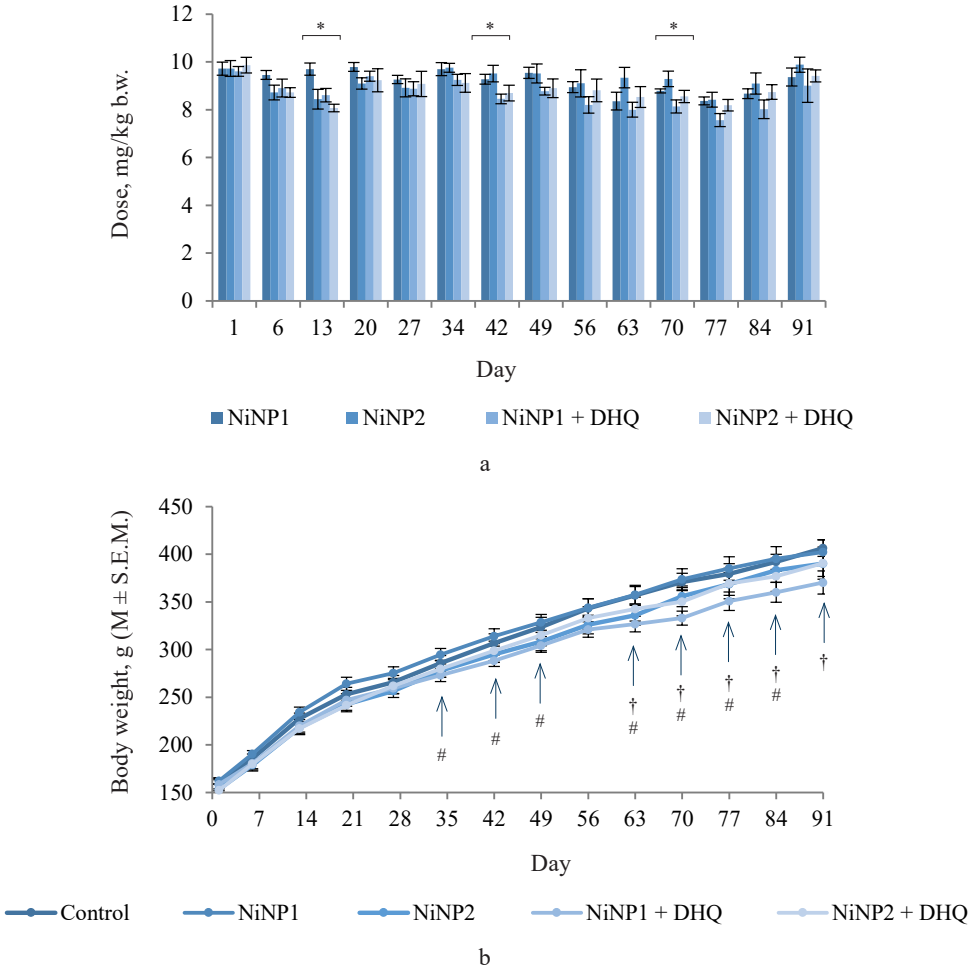
RESULTS AND DISCUSSION

Figure 1a illustrates the actual consumption of nickel nanoparticles in groups 2–5. The differences fell within the statistical error, except for days 13 and 42 (± 15–17%). These differences were completely compensated during the last five weeks of the experiment. The consumed amount of dihydroquercetin was maintained the same with the mean square error (s.d.) of ± 8%.

All animals gained body weight (Fig. 1b). However, group 4, which consumed smaller nickel nanoparticles and dihydroquercetin, had a much lower weight. The decline became significant compared to the control (group 1) on day 35 and compared to group 2, which involved smaller nickel nanoparticles, on day 63.

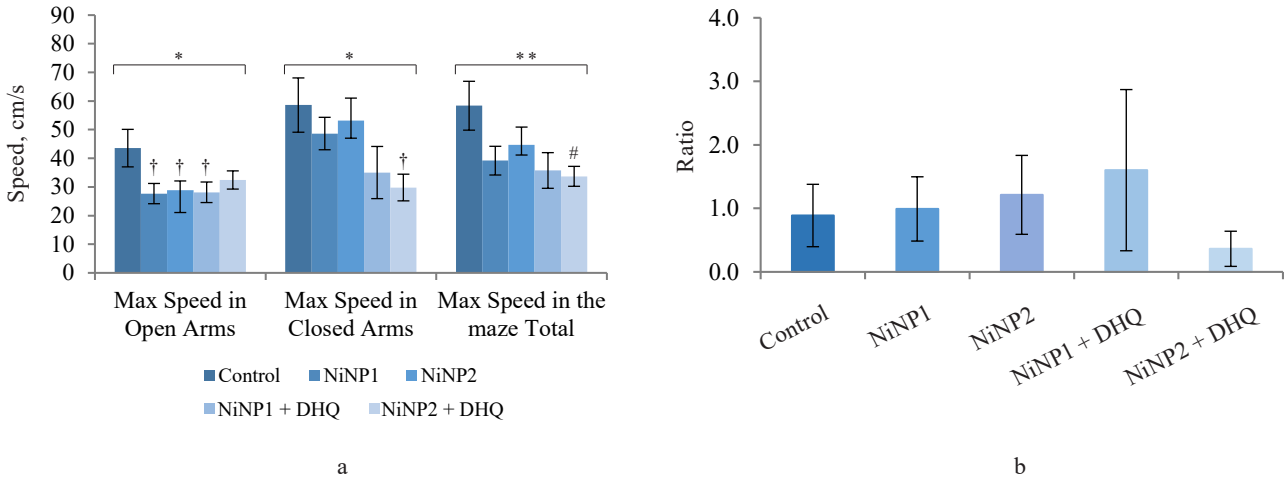
The conditioned reflex of passive avoidance test revealed no differences in anxiety-like and cognitive functions in all experimental groups. In the elevated plus maze test, all experimental groups showed poor locomotor activity, especially in terms of the maximal speed in the open and closed arms (Fig. 2a). Dihydroquercetin treatment did not improve the situation: group 5, which consumed larger nickel nanoparticles and dihydroquercetin, demonstrated a further decrease in locomotor activity compared to group 3, which consumed larger nickel nanoparticles. The measured anxiety-like functions level depended on the ratio of the times spent in the closed and open arms. The elevated plus maze test revealed no significant differences in anxiety-like functions between the groups. However, the lowest anxiety-like functions index was observed in group 5, which received larger nickel nanoparticles and dihydroquercetin (Fig. 2b).

After the experiment, the rats in groups 2 and 3, which received nickel nanoparticles of different average diameters, showed a significant decrease in the total reduced thiols (Fig. 3a) in the liver. In rats, it is usually manifested by reduced GSH glutathione. The dihydroquercetin treatment did not affect this indicator compared to the groups that received only nickel nanoparticles. However, the difference between the groups that received dihydroquercetin and the control remained insignificant. The mean urinary selenium excretion (Fig. 3b) decreased sharply compared to the control in all groups of rats treated with nickel nanoparticles. Group 3 with larger nickel nanoparticles was the only exception due to an abnormally high level of excretion in three rats. The dihydroquercetin treatment did not affect this indicator. However, the level



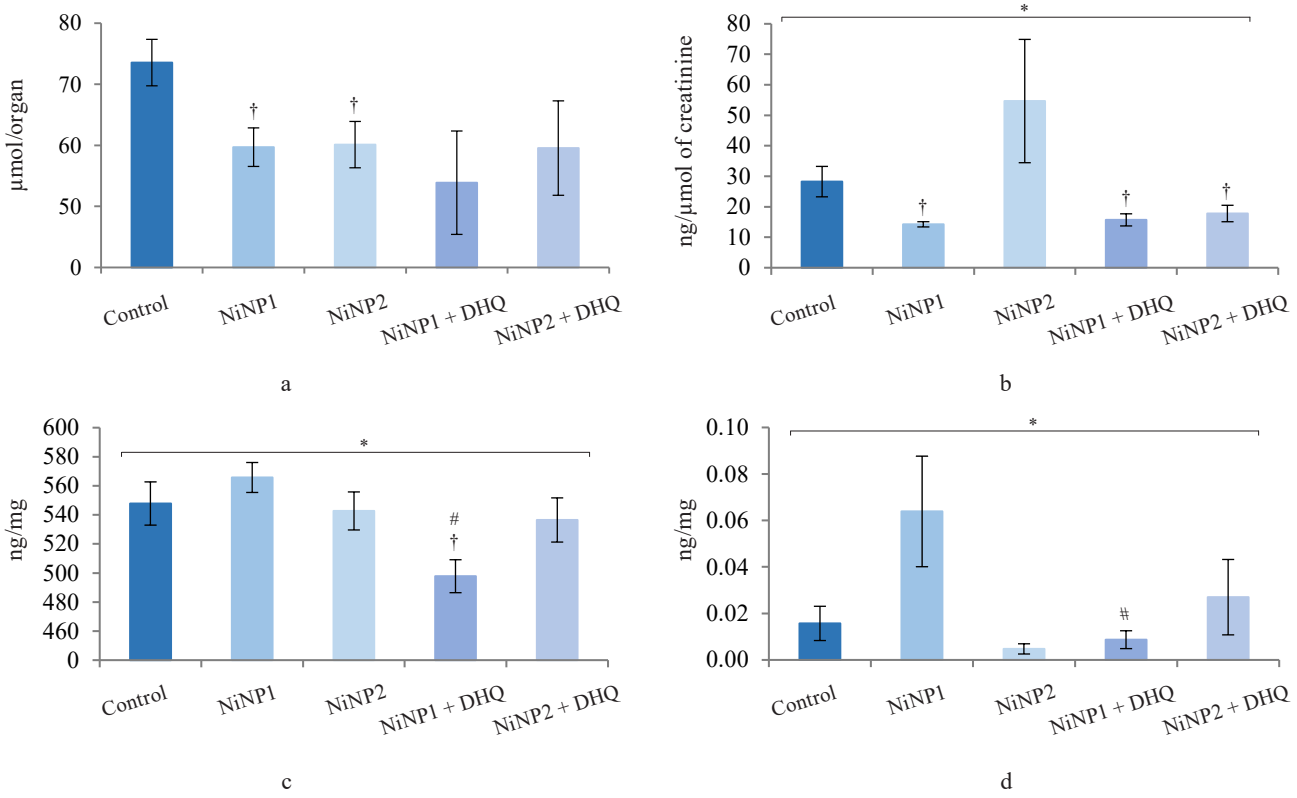
Heterogeneous distribution in four groups; *$p < 0.05$, one-way ANOVA test; † – significant difference between groups 1 and 4; # – significant difference between groups 2 and 4 ($p < 0.05$); n = 12 – number of animals in each group

Figure 1 Integral indicators of rats (M ± m): the actual dose of nickel consumed in groups 2–5 (a); average body weight of rats in groups 1–5 (b) during the experiment. NiNP1 – smaller nickel nanoparticles; NiNP2 – larger nickel nanoparticles; NiNP1 + DHQ – smaller nickel nanoparticles + dihydroquercetin; NiNP2 + DHQ – larger nickel nanoparticles + dihydroquercetin



Heterogeneous distribution in groups 1–5; * $p < 0.1$; ** – $p < 0.05$ (one-way ANOVA test); † – significant difference with group 1 (control); # – significant difference with group 3 ($p < 0.05$); n = 12 – number of animals in each group

Figure 2 Performance in the elevated plus maze test: a – locomotor activity: maximal speed in an open arms, a closed arms, and the maze in general; b – anxiety-like functions: time spent in a closed arms ratio to time spent in an open arms. NiNP1 – smaller nickel nanoparticles; NiNP2 – larger nickel nanoparticles; NiNP1 + DHQ – smaller nickel nanoparticles + dihydroquercetin; NiNP2 + DHQ – larger nickel nanoparticles + dihydroquercetin



Heterogeneous distribution in groups 1–5; * $p < 0.05$ (one-way ANOVA test); † – significant difference with group 1 (control); # – significant difference with group 3 ($p < 0.05$); n = 6 (a); n = 8 (b–d) – number of animals in the group

Figure 3 Biochemical parameters of rats demonstrating the state of the glutathione system, selenium satiety, and the integrity of the intestinal barrier: a – reduced glutathione in liver; b – urinary selenium excretion; c – selenium concentration in blood serum; d – circulating fatty acid-binding protein (FABP2) concentration in blood serum. NiNP1 – smaller nickel nanoparticles; NiNP2 – larger nickel nanoparticles; NiNP1 + DHQ – smaller nickel nanoparticles + dihydroquercetin; NiNP2 + DHQ – larger nickel nanoparticles + dihydroquercetin

of selenium in the blood (Fig. 3c) was slightly (by 10% in absolute value) but significantly reduced in group 4, which consumed smaller nickel nanoparticles and dihydroquercetin, compared with the control.

The level of circulating fatty acid-binding protein (FABP2) is a biomarker of impaired permeability of the small intestine barrier [17]. In blood serum (Fig. 3d), it demonstrated a significantly heterogeneous distribution in groups 1–5 ($p = 0.002$, ANOVA) with a pronounced tendency ($p = 0.056$) to increase in group 2, which involved smaller nickel nanoparticles. In this group, the dihydroquercetin treatment led to a significant decrease in this indicator (p2/4 = 0.012) with an almost complete normalization in absolute value. For animals treated with larger nickel nanoparticles, we registered no changes in the intestinal permeability indicator for circulating fatty acid-binding protein, regardless of the dihydroquercetin administration.

The biochemical analysis of blood serum (Table 1) showed that group 2, which received smaller nickel nanoparticles, had a significantly greater content of total protein, albumin and globulin fractions, glucose, and low-density lipoprotein cholesterol compared to the control, while the level of uric acid went down. The dihydroquercetin treatment in group 4, which received smaller nickel nanoparticles, led to a significant decrease in total protein, albumin and glucose. The content of creatinine and triglycerides in group 4 decreased compared with group 2, which received smaller nickel nanoparticles. The activity of alanine aminotransferase in group 4 was higher compared to group 2. However, it remained within the upper limit for rats of this age (101–161 IU/mL) and did not differ significantly from the control (group 1). Group 4 demonstrated the highest de Ritis ratio, i.e., serum aspartate aminotransferase ratio to alanine aminotransferase, which was almost twice as big as in group 2. In combination with data on glucose, triglycerides, and low-density lipoprotein cholesterol, this result shows that dihydroquercetin boosted the catabolic processes in rats exposed to nickel nanoparticles [18].

Unlike group 2, group 3, which consumed larger nickel nanoparticles, demonstrated less changes in biochemical profile. However, group 3 had aspartate aminotransferase activity below the bottom limit of the norm. Albumin and creatinine activity also slowed down in this group, whereas globulin fraction increased compared to the control ($p < 0.05$). The dihydroquercetin treatment administered to group 5 failed to reverse these changes, with the only exception

Table 1 Biochemical profile of rats

Group	1	2	3	4	5	ANOVA
Preparation	Control	NiNP1	NiNP2	NiNP1 + DHQ	NiNP2 + DHQ	
Indicator, units						
Alanine aminotransferase, ME/L	157 ± 22	103 ± 25	91 ± 20†	162 ± 9#	132 ± 12	
Alanine aminotransferase, ME/L	65.5 ± 4.1	62.3 ± 5.3	55.0 ± 3.2	46.9 ± 1.4†	49.5 ± 3.5†	*
Aspartate aminotransferase ratio to alanine aminotransferase	2.55 ± 0.36	1.83 ± 0.44	1.79 ± 0.45	3.47 ± 0.18#	2.77 ± 0.33	*
Total protein, g/L	73.6 ± 1.5	80.5 ± 1.2†	72.2 ± 0.7	71.5 ± 0.8#	65.6 ± 1.3†#	*
Albumin, g/L	39.4 ± 0.4	41.7 ± 0.4†	35.3 ± 0.4†	34.3 ± 0.6†#	32.1 ± 1.3†#	*
Globulins, g/L	35.1 ± 0.9	38.8 ± 1.0†	43.3 ± 0.6†	43.4 ± 0.7†#	40.2 ± 1.2†#	*
Glucose, mmol/L	6.27 ± 0.30	7.45 ± 0.31†	6.45 ± 0.14	6.43 ± 0.12#	6.24 ± 0.22	*
Triglycerides, mmol/L	1.65 ± 0.17	2.17 ± 0.24	1.24 ± 0.25	1.36 ± 0.13#	1.39 ± 0.18	*
Cholesterol, mmol/L	0.81 ± 0.06	0.92 ± 0.05	0.82 ± 0.04	0.81 ± 0.06	0.78 ± 0.06	
Low density lipoprotein cholesterol, mmol/L	0.25 ± 0.02	0.37 ± 0.03†	0.21 ± 0.02	0.19 ± 0.01†#	0.19 ± 0.02	*
Creatinine, μmol/L	65.1 ± 1.9	67.7 ± 1.6	55.7 ± 1.5†	48.6 ± 2.3†#	40.4 ± 2.9†#	*
Uric acid, μmol/L	118 ± 7	95 ± 5†	83 ± 5†	83 ± 2†	79 ± 6†	*
Urea, μmol/L	5.52 ± 0.23	6.14 ± 0.22	6.16 ± 0.33	5.88 ± 0.48	4.68 ± 0.25†#	*

NiNP1 – smaller nickel nanoparticles; NiNP2 – larger nickel nanoparticles; NiNP1 + DHQ – smaller nickel nanoparticles + dihydroquercetin; NiNP2 + DHQ – larger nickel nanoparticles + dihydroquercetin

* – $p < 0.05$ in groups 1–5 (one way ANOVA test);

† – significant difference with group 1 (control);

– significant difference with the group without dihydroquercetin

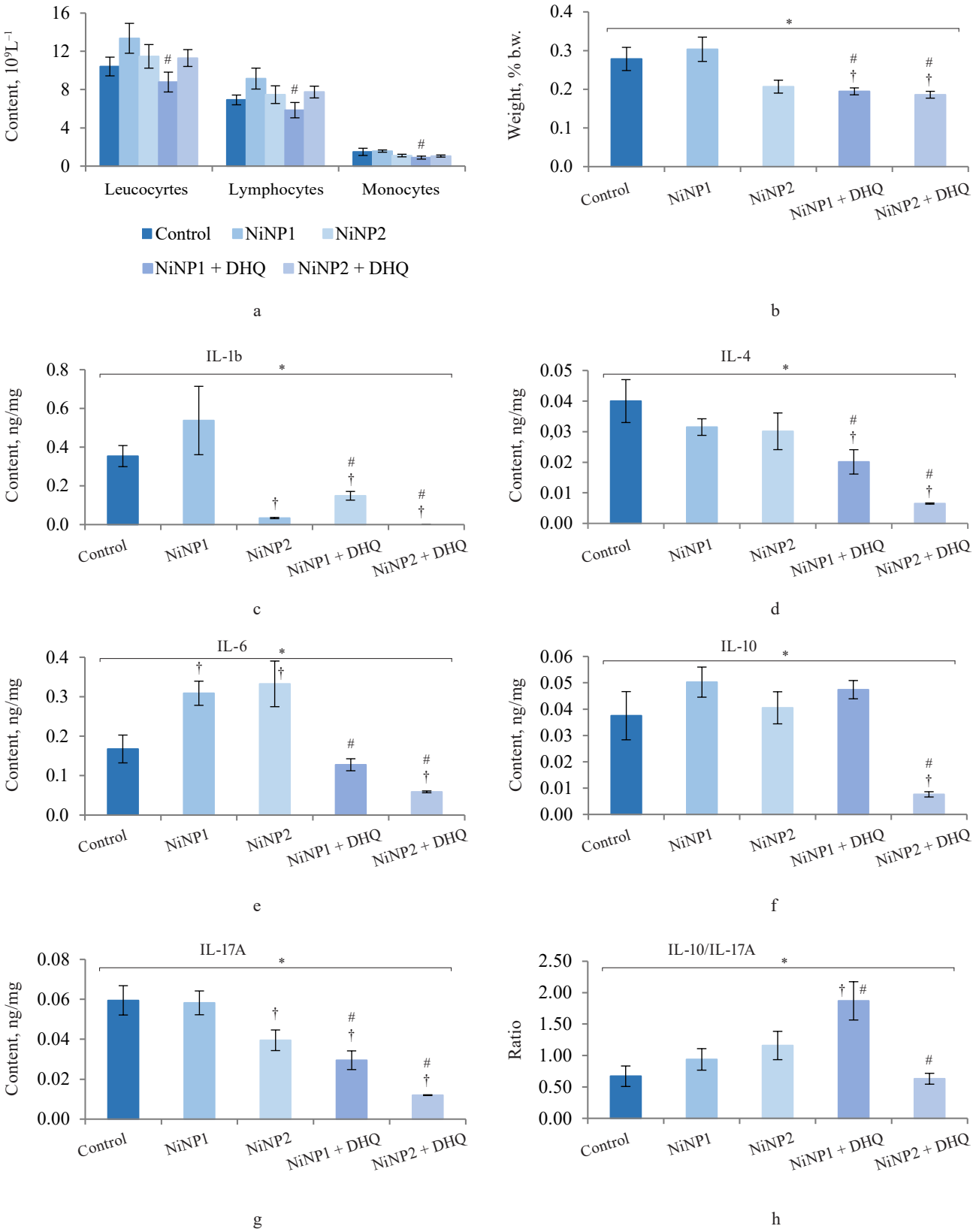
of aspartate aminotransferase. Group 5 also showed a very low urea content compared to the control.

When we introduced dihydroquercetin into the diet of rats treated with nickel nanoparticles, it sometimes affected their immune system (Fig. 4). Group 4, which received smaller nickel nanoparticles together with dihydroquercetin, demonstrated a much lower content of total leukocytes, monocytes, and lymphocytes (Fig. 4a), as well as a lower spleen weight (Fig. 4b), compared to group 2. The change in spleen weight was also registered in group 5. Unlike group 2, group 4, which received smaller nickel nanoparticles and dihydroquercetin, had lower levels of pro-inflammatory cytokines IL-1*β*, IL-4, and IL-17A (Fig. 4c–h). Group 4 also had the maximal ratio of IL-10 and IL-17A, which indicated that tolerogenic Treg lymphocytes were more active than immunostimulatory subpopulations Th1 and Th17. The production of the pro-inflammatory cytokine IL-6 was much higher in groups 2 and 3, which received nickel nanoparticles, compared with the control. The dihydroquercetin treatment abolished this effect. These results indicate that dihydroquercetin probably had an anti-inflammatory effect in our model. Group 5, which consumed larger nickel nanoparticles, also experienced the anti-inflammatory effect of dihydroquercetin: the levels of pro-inflammatory IL-1*β*, IL-4 and IL-17A dropped, although the ratio of IL-10 and IL-17A remained low. Thus, different sizes of nickel nanoparticles might have been responsible for different immune disorders in rats.

Figure 5 illustrates the data obtained on the expression of some fibrogenic genes in rat liver. Nickel nanoparticles obviously increased the expression of fibrogenesis markers *MMp2* and *MMp9* in group 2 and *Timp3* and *MMp9* in group 3. Group 5, which received larger nickel nanoparticles, demonstrated a lower *Timp3* expression after the dihydroquercetin treatment. In rats that consumed smaller nickel nanoparticles, dihydroquercetin had no effect on fibrogenic genes. A morphological study of liver tissue with Van Gieson collagen staining revealed that fibrous elements accumulated mainly in the perivascular region (Fig. 6a–e). However, we could not assess the effect of dihydroquercetin on their number.

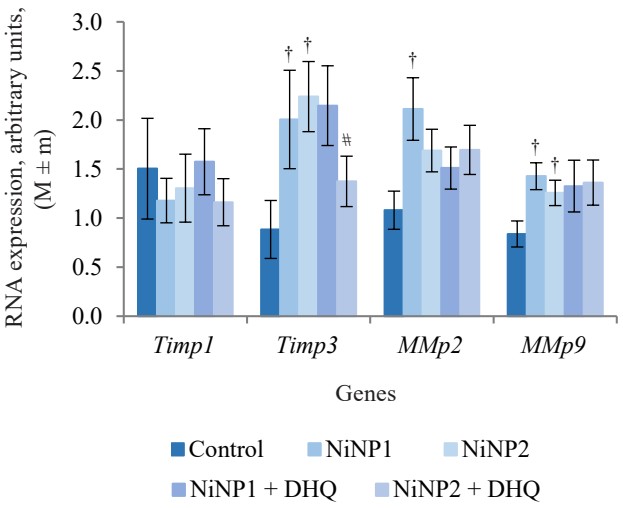
We used light-optical microscopy to study the structure of liver, small intestine, and kidneys stained with hematoxylin-eosin (Table 2). Under the effect of both sizes of nickel nanoparticles, the liver tissue showed a decrease in the average diameter of cell nuclei, as well as an increase in the number of binuclear cells and cells that contained nuclei with broken perikarotic membrane. The small intestine mucosa had a greater ratio of the lengths of villi and crypts. The kidney tissue revealed glomerular edema with a lower ratio of the diameter of the Shumlyansky-Bowman capsules to that of the glomeruli. The dihydroquercetin treatment neutralized the reducing effect on the diameter of the nuclei on the minor axis. In the group that received smaller nickel nanoparticles, the dihydroquercetin treatment also averted the increase in the number of damaged nuclei in the liver and glomerular edema in the kidneys. Other morphological changed caused by nickel nanoparticles demonstrated no normalizing effect of the dihydroquercetin treatment.

The results allow us, in general, to conclude that the dihydroquercetin treatment produced no



Heterogeneous distribution in groups 1–5, * $p < 0.05$ (one-way ANOVA test); † – significant difference with control, # – significant difference with the group without dihydroquercetin ($p < 0.05$); n = 12 (b) – number of animals; n = 8 (other indicators) – in each group

Figure 4 Immunological indicators in experimental rats: a – leukocytes and their populations in the blood; b – relative spleen weight; c–h – serum concentration of cytokines (c – IL-1*β*; d – IL-4; e – IL-6; f – IL-10; g – IL-17A; h – IL-10 ratio to IL-17A). NiNP1 – smaller nickel nanoparticles; NiNP2 – larger nickel nanoparticles; NiNP1 + DHQ – smaller nickel nanoparticles + dihydroquercetin; NiNP2 + DHQ – larger nickel nanoparticles + dihydroquercetin



† – significant difference with control; # – difference with the group without dihydroquercetin ($p < 0.05$); n = 6 – number of animals in each group

Figure 5 Expression of fibrogenic genes in liver. NiNP1 – smaller nickel nanoparticles; NiNP2 – larger nickel nanoparticles; NiNP1 + DHQ – smaller nickel nanoparticles + dihydroquercetin; NiNP2 + DHQ – larger nickel nanoparticles + dihydroquercetin

antitoxic effect in terms of the locomotor activity in the elevated plus maze test and their antioxidant status, e.g., GSH content and selenium content. However, we registered certain favorable changes in the biochemical markers of blood serum, which indicated some hypolipidemic and hypoglycemic effect of dihydroquercetin, at least in relation to smaller nickel nanoparticles. In group 4, exposed to smaller nickel nanoparticles, the dihydroquercetin treatment decreased the level of pro-inflammatory cytokines, as well as improved the functioning of the immune system in terms of integral (spleen mass) and hematological parameters. It also reduced the level of the biomarker of impaired intestinal permeability FABP2, the edema of the glomeruli in the kidneys, and the number of cells with impaired nuclear structure in the liver. In the case of rats exposed to larger nickel nanoparticles, the anti-inflammatory effect of dihydroquercetin was less manifested. However, we observed a decrease in the expression of one fibrogenic gene in the liver. The dihydroquercetin treatment increased the ratio of aspartate aminotransferase to alanine aminotransferase, which indicated the activation of catabolic processes.

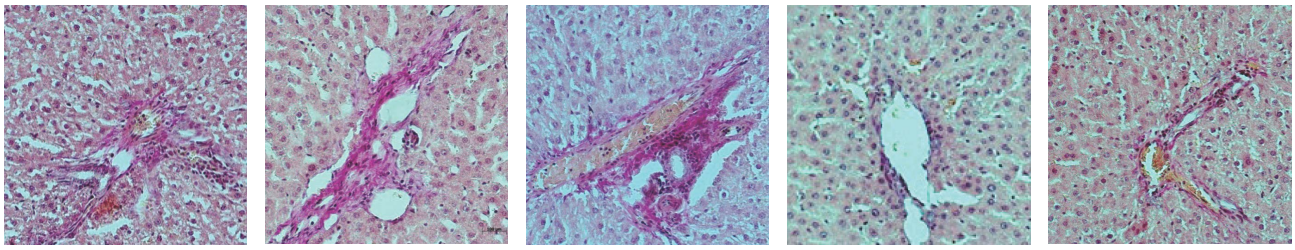


Figure 6 Light optical micrographs of rat liver sections, Van Gieson staining, 200× magnification: a – control; b – smaller nickel nanoparticles (NiNP1); c – larger nickel nanoparticles (NiNP2); d – smaller nickel nanoparticles + dihydroquercetin (NiNP1 + DHQ); e – larger nickel nanoparticles + dihydroquercetin (NiNP2 + DHQ)

Table 2 Morphometric parameters of the internal organs of rats

Group		1	2	3	4	5	ANOVA
Preparations		Control	NiNP1	NiNP2	NiNP1 + DHQ	NiNP2 + DHQ	
Indicators, units							
Liver	Core diameter along minor axis, μm	21.2 ± 0.4	19.3 ± 0.3†	18.7 ± 0.2†	20.6 ± 0.6	20.2 ± 0.5#	*
	Core diameter along major axis, μm	24.1 ± 0.4	21.6 ± 0.3†	21.0 ± 0.4†	22.5 ± 0.5†	22.5 ± 0.5†	*
	Binucleated cells, per 100 nuclei	4.2 ± 0.7	9.8 ± 2.2†	14.5 ± 2.4†	11.3 ± 2.1†	8.1 ± 1.6†	*
	Cells with disintegrated nuclei, per 100 nuclei	52.7 ± 4.6	94.0 ± 8.7†	59.0 ± 9.9	67.8 ± 4.6†#	57.8 ± 3.8†	*
Small intestine	Villus length, μm	1087 ± 46	1473 ± 151	1331 ± 76†	1672 ± 72†	1377 ± 103†	*
	Crypt length, μm	546 ± 37	466 ± 32	430 ± 31	427 ± 34†	387 ± 42†	*
	Villus to crypt ratio	2.1 ± 0.2	3.2 ± 0.2†	3.3 ± 0.4†	4.0 ± 0.3†#	3.6 ± 0.2†	*
Kidney	Capsule diameter ratio to glomerular diameter	1.26 ± 0.03	1.12 ± 0.04†	1.12 ± 0.04†	1.22 ± 0.05	1.14 ± 0.04†	*

NiNP1 – smaller nickel nanoparticles; NiNP2 – larger nickel nanoparticles; NiNP1 + DHQ – smaller nickel nanoparticles + dihydroquercetin; NiNP2 + DHQ – larger nickel nanoparticles + dihydroquercetin

* – $p < 0.05$ for groups 1–5 (one-way ANOVA test);

† – significant difference with control ($p < 0.05$);

– significant difference with the group without dihydroquercetin ($p < 0.05$)

As for the unfavorable effects of dihydroquercetin, it inhibited the total body weight gain in rats treated with larger nickel nanoparticles.

We retrieved no publications on the antitoxic effect of dihydroquercetin on (nano)nickel and its compounds. This research might be the first to report the effects listed above. However, the beneficial effects of dihydroquercetin for heavy metals are well-known. In rats exposed to excess iron, dihydroquercetin inhibited peroxide processes in the liver tissue, weakened histopathological changes, and decreased the expression of caspase-3, IL-1*β*, and IL-6 [19]. The cytoprotective effect of dihydroquercetin was registered in a model of human keratinocytes treated with cadmium salt [20].

The anti-inflammatory effect of dihydroquercetin in the liver of rats intoxicated with alcohol was associated with the activation of the PI3K/Akt signaling pathway with a corresponding suppression of NF-κB expression [21]. The ability of dihydroquercetin to inhibit the processes of fibrous degeneration of the liver tissue was demonstrated in a model of rats intoxicated with CCl4 [22]. Dihydroquercetin inhibited pathological tissue changes in mice with renal fibrosis induced by TGF-*β*1 [23]. It averted the increase in spleen mass in rotenone-treated mice [24]. In mice with dextran-sulfate-induced colitis, dihydroquercetin decreased the permeability of the intestinal barrier by stimulating the expression of claudin-1 and occludin intercellular contact proteins, while reducing the levels of IL-1*β* and IL-6 [25].

The anti-inflammatory and lipid-lowering effect of dihydroquercetin as part of a natural plant extract was demonstrated on 3T3-L1 differentiating adi-pocytes [26]. In type 2 diabetic mice model of the KK-Ay/Ta line, dihydroquercetin decreased the level of glucose, serum insulin, and the HOMA index [27].

Most publications reported the ability of dihydroquercetin to affect the production of various cytokines *in vitro* and *in vivo*. For instance, dihydroquercetin activated the AMPK/Nrf2/HO-1 signaling axis and decreased the levels of IL-6 and IL-10 in mice with endotoxemia caused by bacterial lipopolysaccharide [28]. In *in vitro* systems, dihydroquercetin reduced the production of IL-6 and LTC4 and suppressed the activity of type 2 cyclooxygenase by inhibiting intracellular calcium mobilization [29]. Dihydroquercetin inhibited pyroptosis and the production of IL-1*α*,*β*, and IL-18 in rat myoblasts of the H9C2 cell line treated with hydrogen peroxide [30]. In a mouse psoriasis model, dihydroquercetin suppressed the expression of IL-17A and the activity of CD3+ cells, especially the *γδ*T subpopulation. The effect was presumably associated with the activation of the PPAR*γ* signaling pathway [31].

Our research proved the anti-inflammatory, cytokine-modulating, hypolipidemic, hypoglycemic, and, possibly, antifibrogenic effects of dihydroquercetin on rats intoxicated with Ni-containing nanoparticles. As seen from the review above, our results are consistent with the data of numerous studies performed on alternative *in vitro* and *in vivo* models.

CONCLUSION

In this research, we introduced bioflavonoid dihydroquercetin into the diet of rats subjected to the toxic effect of nickel nanoparticles. The dihydroquercetin treatment led to some beneficial effects, e.g., it lowered systemic inflammation, normalized individual indicators of liver and kidneys, improved the level of proinflammatory cytokines, restored the biochemical parameters of blood serum, suppressed one fibrogenic marker, and decreased the intestinal barrier permeability. However, dihydroquercetin failed to restore such parameters as behavioral reactions, selenium status, intestinal mucosa morphology, and glutathione in the liver. In fact, it inhibited weight gain under certain conditions. Thus, the obtained results demonstrate certain prospects for the dietary use of water-soluble stabilized dihydroquercetin against nanonickel intoxication and, potentially, other heavy metals. Further extensive preclinical studies are needed to substantiate these data.

CONTRIBUTION

I.V. Gmoshinski: research concept, methodology, validation, formal analysis, research, data curation, manuscript, visualization. M.A. Ananyan: research concept, review, manuscript. V.A. Shipelin: research concept, methodology, validation, formal analysis, research, data curation, manuscript, visualization, review. N.A. Rieger: methodology, validation, formal analysis, research. E.N. Trushina: methodology, validation, formal analysis, research. O.K. Mustafina: methodology, validation, formal analysis, research. G.V. Guseva: methodology, validation, formal analysis, research. A.S. Balakina: methodology, validation, formal analysis, research. A.I. Kolobanov: methodology, review, research. S.A. Khotimchenko: research concept, methodology, manuscript, proofreading, project management, obtaining funding. D.Yu. Ozherelkov: research, review.

CONFLICT OF INTEREST

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Artemisia jacutica Drob. essential oil as a source of chamazulene: primary introduction and component analysis

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Abstract:
Artemisia jacutica Drob. is a valuable source of chamazulene, which has anti-inflammatory and antioxidant properties. We experimentally introduced this plant in the climatic conditions of Buryatia and compared the compositions of the essential oils produced from both cultivated and wild plants.
The reserves of *A. jacutica* and the laboratory/field germination of seeds were assessed by standard methods. Macro- and microscopic features were determined in line with general pharmacopoeia monographs. The composition of the essential oil obtained by hydrodistillation was analyzed by gas chromatography–mass spectrometry. The resulting data were processed by the principal component method. The antiradical activity was measured by the DPPH test.
The reserves of *A. jacutica* were determined in the Yeravninsky district of Buryatia. The laboratory germination of *A. jacutica* seeds was 75.00 ± 5.35%, while the field germination was only 11–23%. Planting with seedlings showed a good survival rate of 67–80%. In the first year of cultivation, *A. jacutica* plants had similar macro- and microscopic features to those of wild plants. The soils from the experimental plots were superior to the soils of *A. jacutica*'s natural habitat in terms of fertility. The essential oils from cultivated and wild plants contained 51 components. The content of chamazulene, the dominant component, was 59.22–66.60% in the cultivated plants and only 15.98–47.77% in the wild plants. The essential oil of *A. jacutica* exhibited high antiradical activity (IC_{50} = 49.47 μL/mL).
The primary introduction of *A. jacutica* showed good prospects for its cultivation in Buryatia. The macro- and microscopic features and dominant components found in the essential oil of *A. jacutica* grown on the experimental plots were similar to those found in the wild plants. Two chemotypes of *A. jacutica*, Yakutian and Buryatian, were identified according to the oil composition, with the chemotypes preserved in the cultivated plants. The oil's high antiradical activity and a high content of chamazulene make *A. jacutica* a valuable material for the cosmetic, pharmaceutical, and agricultural industries.

Keywords: *Artemisia jacutica* Drob., cultivation, laboratory and field germination, essential oil, chamazulene, antiradical activity, chemotype

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INTRODUCTION

Traditional healing systems (Chinese, Tibetan, Japanese, etc.) have been in the focus of international science in recent years. These systems are based on the use of natural bioactive substances, primarily of plant origin. Scientific advances in this area highlight the need for using secondary metabolites to create effective drugs [1–4]. Russia's Pharma-2030 Program aims to increase the market of domestic innovative pharmaceutical products, including those based

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on natural compounds. In addition, the National Technological Initiative's Healthnet Roadmap intends to involve more regions in cultivating medicinal plants and creating a whole medicinal plant sector.

The Republic of Buryatia is part of the Baikal unique ecosystem with rare and endemic plant species. Among them are the medicinal plants of the *Artemisia* L. (*Asteraceae* family) genus used as ethnopharmacological poly-target agents. For example, the antimalarial drug Artemisinin, which is based on *Artemisia annua* L., exhibits antiparasitic, antitumorous, anti-inflammatory, antioxidant, antiangiogenic, and immunomodulatory effects. Another example is the anticancer drug Arglabin based on *Artemisia glabella* L. [5].

The aromatic and pharmacological properties of *Artemisia* plants are largely due to their essential oils based on mono- and sesquiterpenoids. Multiple components of plant essential oils determine their antioxidant and anti-inflammatory properties. For example, the essential oil of *Matricaria chamomilla* L. contains chamazulene with anti-inflammatory action. In addition, chamazulene or chamazulene-containing oils have an apoptotic effect on the A375 human malignant melanoma cells, as well as strong antioxidant properties and a photoprotective effect [6–8]. Other sources of chamazulene-containing essential oils are the plants that are systematically close to the pharmacopoeial species *Artemisia absinthium* L., including *Artemisia sieversiana* L., *Artemisia jacutica* Drob., *Artemisia macrocephala* L., and others. Among these species only *A. sieversiana* and *A. jacutica* grow within the territory of Buryatia, the latter having the highest content of chamazulene [9].

A. jacutica Drob. is an East Siberian endemic used in Yakut traditional medicine to treat gastrointestinal diseases. In addition, this species has selective antifungal activity and is used for helminthic invasions in cattle. Previously, the essential oil of *A. jacutica* was shown to exhibit a wound-healing effect on the napalm-caused burn. *A. jacutica* was first introduced in the Yakutsk Botanical Garden. From 1991 to 1998, scientists studied the agrotechnical methods of growing *A. jacutica* in the Siberian Botanical Garden [10]. Kucharova *et al.* were the first to obtain strains of *A. jacutica* callus cells with stable growth parameters *in vitro* [11].

We aimed to assess a possibility of introducing *A. jacutica* in the natural and climatic conditions of Buryatia and to compare the essential oil of cultivated plants with the oil of wild plants.

STUDY OBJECTS AND METHODS

We studied the aerial parts and seeds of *Artemisia jacutica* Drob. collected in the flowering and fruiting phases, respectively, in Yeravninsky district of Buryatia in 2018–2019. The voucher samples are kept in the herbarium of the Institute of General and Experimental Biology (*A. jacutica* – UUH019308).

The yield was determined on specific thickets by the quadrat method. Fifteen plots of 1 m^2 were evenly distributed over the thickets. The area of the thickets was calculated in m^2. The method's error did not exceed 15% [12].

To determine the germination of *A. jacutica* seeds in the laboratory conditions, we used four samples of 100 seeds. The seeds were spread evenly on moistened filter paper in Petri dishes and germinated at 29–30°C. The filter paper was checked for moisture on a daily basis and, if necessary, wetted with water at room temperature to prevent overwetting. The lids of the Petri dishes were opened for several minutes every day for ventilation. The samples were protected from direct sunlight during seed germination. The germinated seeds were counted during 12 days. The seeds with primary leaves were classified as germinated, whereas hard seeds that had not swelled or changed in appearance were classified as non-germinated.

The macro- and microscopic features of raw materials were determined by standard methods in line with the General Pharmacopoeia Monographs (General Pharmacopoeia Monograph.1.5.3.0003.15 and General Pharmacopoeia Monograph.1.5.1.0002.15).

The essential oil was obtained from *A. jacutica*'s aerial parts by hydrodistillation according to the GPhA (General Pharmacopoeia Monograph.1.5.3.0010.15) using a modified Clevenger nozzle. The oil's components were determined by gas chromatography–mass spectrometry (GC-MS) on an Agilent 6890 gas chromatograph (Agilent Technologies, USA) equipped withan HP 5973N mass selective detector (Hewlett-Packard, USA) and an HP-5MS capillary column (30 m×0.25 mm×0.2 μm; Hewlett-Packard) [13].

For visualization, the data on the oil's composition were processed by the principal component method (PCM analysis, Sirius version 6.0, Pattern Recognition Systems, a/s, Norway).

The antiradical activity of the essential oil was determined by the DPPH test (using 2,2-diphenyl-1-picrylhydrazyl). For this, a solution of DPPH (0.006% in 95% ethanol) was added to *A. jacutica* oil (3.9–31.25 μL/mL in ethanol) and incubated for 30 min in the dark at room temperature. The antiradical activity (% inhibition) was measured spectrophotometrically on a ClarioStar Plus multimodal plate reader at 517 nm and calculated as:

$$\% \text{ inhibition of DPPH-radicals} = (A_0 - A_1)/A_0 \times 100 \quad (1)$$

where A_0 is the absorbance in the control and A_1 is the absorbance of the samples [14, 15].

The IC_{50} index was determined by regression analysis.

RESULTS AND DISCUSSION

Determination of *Artemisia jacutica* Drob. reserves. The reserves of *A. jacutica* were determined in the vicinity of Shiringa village (Yeravninsky district, Buryatia) in 2019. The plant's recovery period is 2 years.

Table 1 Laboratory and field germination (survivability) of *Artemisia jacutica* Drob. seeds

Laboratory germination of seeds				
Sample No.	No. 1	No. 2	No. 3	No. 4
Number of germinated seeds in 12 days, pcs.	71	76	79	74
Field germination (survivability) of seeds				
Experimental plot No.	No. 1		No. 2	
Seed sowing, %	23		11	
Seedling planting, %	80		67	

A thicket of *A. jacutica* occupies a small area of only 500 m^2. According to standard estimations, the average mass of *A. jacutica* collected from one plot was 26.38 g, the dispersion of the result was 209.01 g, and the standard deviation was 3.77 g. Thus, the yield of *A. jacutica* was estimated as 26.38 ± 3.77 g/m^2, its biological reserve was 16.92 kg, the operational reserve was 9.46 kg, with a possible annual harvest of 3.15 kg.

Thus, we proposed to introduce *A. jacutica* into the culture taking into account its limited potential reserves in Buryatia and high medicinal value as a source of bioactive compounds.

Laboratory and field germination. At the first stage, we determined the germination of *A. jacutica* seeds in the laboratory. The seeds were obtained from wild intact plants collected in the fruiting phase in 2018–2019. The oblong, dark brown achenes were 1.0–1.2 mm long, 0.4–0.5 mm wide, and covered with a golden film. The seeds began to germinate in 2–4 days. The primary introduction of *A. jacutica* was carried out on two experimental plots: in Oreshkovo settlement, the Republican Ecological and Biological Center (plot No. 1) and in Sotnikovo settlement (plot No. 2), the Republic of Buryatia (Table 1).

As can be seen, the laboratory germination of *A. jacutica* seeds was 75.00 ± 5.35%, with a standard deviation of 8% [12]. Our results were consistent with the previous studies [16, 17], where this indicator amounted to 70–100% without primary dormancy. The authors classified *A. jacutica* seeds as slow germination seeds with a maximum number of germinated seeds at the beginning of germination.

Since we did not use fertilizers or artificial irrigation on the experimental plots, the differences between the cultivated and the natural plants were associated with soils. The plots had a chestnut soil type. Nothing was grown on them before the experiments and no chemicals (pesticides) were used. The plots were periodically plowed up to get rid of weeds. The main agrochemical indicators of soil fertility were analyzed by standard methods. The reaction of the soil solution was determined in an aqueous extract. The soil samples were analyzed for carbon and humus contents by the Tyurin spectrophotometric method (The Standard Operating Procedure for Soil Organic Carbon. The Tyurin spectrophotometric method), for total nitrogen by the Kjeldahl method (General Pharmacopoeia Monograph.1.2.3.0011.15), for mobile phosphorus and potassium by the Chirikov method (State Standard 26204-91), and for exchangeable calcium and magnesium by the complexometric method (trilon method) (State Standard 26487-85). The results are presented in Table 2.

The acidity of the soils from Buryatia's Yeravninsky district (natural habitat of *A. jacutica*) and from the experimental plots was close to neutral. However, the pH of the water extract of the experimental soil was in the alkaline region of 7.3–7.6, compared to 6.5 for the soil from the natural habitat. However, the soils from the experimental plots were superior to the soils from the natural habitat in the main indicators of fertility.

As we know, soil formation is largely determined by organic matter. The activity of plants, animals, and microorganisms leads to the accumulation of organic carbon in the form of humus. The processes of humus formation and accumulation are significantly affected by climatic conditions. Humic substances contribute to an optimal soil structure for plants and are an important reserve of ash elements. Thus, they determine a number of soil's physical and chemical characteristics. Soil organic matter contains nitrogen. The accumulation of nitrogen, along with carbon, is part of soil formation determined by the cycle of matter. Organic nitrogen is taken as total nitrogen in the soil since mineral nitrogen is found in insignificant amounts. Our analysis showed that plot No. 1 had the most fertile soil rich in easily digestible nutrients. Plot No. 2 was only superior in the content of mobile phosphorus P_2O_5.

A. jacutica was planted with seeds and seedlings (Table 1). The seeds were sowed in early June in strips of 100 seeds. Shoots appeared in mid-June. The seedlings

Table 2 Agrochemical indicators of soils from the experimental plots and places of *Artemisia jacutica* Drob. natural growth

Sample	Plot No. 1	Plot No. 2	Shiringa village, Yeravninsky district
pH of water extract	7.3	7.6	6.5
Total carbon, %	1.58 ± 0.05	1.36 ± 0.05	0.58 ± 0.03
Total humus, %	2.72 ± 0.09	2.35 ± 0.08	0.99 ± 0.04
Total nitrogen, %	0.140 ± 0.006	0.120 ± 0.003	0.040 ± 0.001
Mobile phosphorus P_2O_5, mg/kg	58.03 ± 2.88	76.10 ± 2.37	9.73 ± 0.52
Mobile potassium K_2O, mg/kg	525.7 ± 24.9	244.3 ± 12.7	120.8 ± 5.2
Exchange cation Ca^{2+}, mgEq/100 g	22.67 ± 1.03	15.60 ± 0.66	5.89 ± 0.28
Exchange cation Mg^{2+}, mgEq/100 g	3.97 ± 0.14	3.90 ± 0.25	1.60 ± 0.11

(208 plants) were planted in rows (10 plants per 1 m^2) with a 50 cm distance between them.

The laboratory germination of *A. jacutica* seeds reached 75.00 ± 5.35%, while the field germination on both plots was very low, ranging from 11 to 23%. Further planting with seedlings showed a good survival rate: 80% on plot No. 1 and 67% on plot No. 2. The highest germination of seeds and the best survival of seedlings was observed in the plot with more fertile and less alkaline soil (pH 7.3). Thus, planting with seedlings is the best way to cultivate *A. jacutica* under the natural and climatic conditions of Buryatia.

Macro- and microscopic features of *A. jacutica*. Macro- and microscopic features were determined for both the wild and the cultivated plants of *A. jacutica*. We found that the morphological features of the plants did not change during cultivation, so the cultivated plants had most macro- and microscopic features similar to those of the wild plants. *A. jacutica* stems were under 25 cm long and had whole or leafy tops. Some plants had ribbed stems, simple or branched. Basal and middle stem leaves had long petioles, half the length of the leaf blade, with simple or pinnate ears at the base. The leaves had narrowly linear, or almost filiform, pointed terminal lobes. Dense hairs gave them a gray felt color. The stems were grayish-brown or greenish-gray, and leaves were grayish-green. The plants had a strong, peculiar smell. The water extract had a spicy and bitter taste (Fig. 1).

The microscopic analysis of the leaf revealed slightly sinuous cells of the upper epidermis and strongly sinuous cells of the lower epidermis. Both the upper and the lower epidermis had an anomocytic stomatal apparatus. The hairs were of two types: T-shaped and capitate. The leaves were densely covered with T-shaped thin-walled hairs, consisting of a multicellular stalk and a long transverse cell with narrowed ends. There were also some capitate hairs consisting of a unicellular stalk and a multicellular oblong head. Numerous essential oil glands consisted of 6–8 excretory cells arranged in 2 rows and 3–4 tiers. Above were glands with a septum, covered with a cuticle (Fig. 2).

Figure 1 *Artemisia jacutica* Drob. on experimental plot No. 1

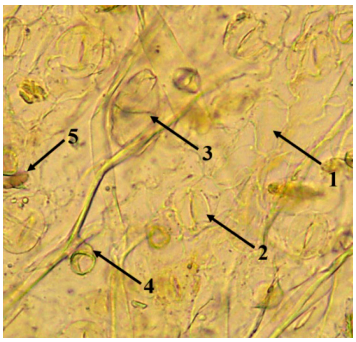

Figure 2 Microscopy of the leaf epidermis: 1 – epidermal cells; 2 – stomata; 3 – essential oil gland; 4 – T-shaped hair; and 5 – capitate hair

***A. jacutica* essential oil composition**. Essential oils were isolated from the aerial parts of the cultivated and wild plants of *A. jacutica* collected in late July of the same year. They were dark blue liquids with a characteristic odor. The oil yield was determined in terms of air-dry raw material. From the cultivated plants (aerial parts), the yield was 0.6 and 0.5% from plot No. 1 and plot No. 2, respectively. From the wild plants, the oil yield was 0.9, 1.4, and 0.7% in the budding, flowering, and fruiting phases, respectively. The composition of essential oils was studied by GC-MS (Table 3).

Table 3 The composition of the essential oils from wild and cultivated *Artemisia jacutica* Drob. plants

Component	Retention index	Wild plants			Cultivated plants	
		Budding	Flowering	Fruiting	Plot No. 1	Plot No. 2
Acyclic monoterpenoids						
β-myrcene	991	0.32	0.18	–	0.98	0.32
geranyl butanoate	1456	–	–	6.37	–	–
Monocyclic monoterpenoids						
α-phelandrene	1004	0.35	0.03	–	0.44	–
α-terpinene	1010	0.27	–	–	0.13	–
p-cymol	1024	0.41	0.11	–	0.26	–
γ-terpinene	1058	0.58	0.18	–	0.37	0.07
terpineol-4	1177	1.14	0.59	0.58	0.36	0.07
α-terpineol	1191	0.89	0.63	0.70	0.40	0.12
β-ionone	1488	–	–	–	0.31	0.22

Continuation of Table 3

Component	Retention index	Wild plants			Cultivated plants	
		Budding	Flowering	Fruiting	Plot No. 1	Plot No. 2
Bicyclic monoterpenoids						
3-thuyene	926	0.18	0.04	–	0.18	–
α-pinene	932	0.34	0.11	–	0.34	0.06
sabinene	973	0.19	0.05	–	0.18	–
β-pinene	975	–	0.05	–	0.08	–
2-karen	1000	–	0.12	–	–	0.06
1.8-cineole	1031	4.97	2.05	1.19	2.66	1.21
Acyclic sesquiterpenoids						
lavandulyl acetate	1292	–	–	1.17	–	–
β-farnesene	1458	0.38	–	0.63	1.61	0.23
nerylisobutanoate	1492	–	0.11	–	–	–
α-farnesene	1496	–	–	–	0.35	–
neryl-2-methylbutanoate	1579	7.59	3.64	10.45	4.71	3.42
neryl-3-methylbutanoate	1585	13.12	0.69	–	4.40	5.80
geranyl-2-methylbutanoate	1604	1.49	–	–	–	–
geranyl-3-methylbutanoate	1610	3.08	2.08	–	0.80	1.01
nerylpentanoate	1636	–	6.92	14.30	–	0.65
lavandulyl hexanoate	1657	4.33	–	–	1.74	2.70
nerylhexanoate	1721	0.40	1.78	–	–	0.20
Monocyclic sesquiterpenoids						
γ-curcumene	1482	2.33	0.25	–	2.60	1.24
germacrene D	1484	1.30	–	2.06	2.49	0.83
β-curcumen	1513	–	0.05	–	–	–
elemol	1553	–	0.89	–	–	–
α-bisabolol	1688	–	–	1.84	–	–
Bicyclic sesquiterpenoids						
α-bergamotene	1416	–	–	–	0.29	–
caryophyllene	1422	1.17	–	–	1.48	0.20
β-guayene	1441	–	–	1.27	–	–
humulene	1456	–	0.06	–	–	–
9-epi-caryophyllene	1469	–	0.61	–	–	–
dehydrosesquicineol	1471	1.89	0.40	0.63	1.67	0.93
selina-4,11-diene	1488	1.54	0.96	1.56	1.14	0.37
cadina-4,11-diene, cis-	1496	–	0.08	–	–	–
bicyclogermacrene	1500	0.28	0.15	–	0.52	0.20
3,6-dihydrochamazulene	1530	–	–	–	0.76	1.55
Δ-amorphous	1553	–	0.12	–	0.31	–
γ-eudesmol	1633	9.07	25.39	31.66	3.22	0.91
amorph-4-en-7-ol	1636	–	–	–	2.90	–
caryophyll-4-en-13-al	1644	–	0.70	–	1.26	–
chamazulene	**1730**	**41.17**	**47.77**	**15.98**	**59.22**	**66.60**
dehydrochamazulene	1800	0.95	–	–	1.84	1.86
Tricyclic sesquiterpenoids						
β-cubeben	1392	–	0.53	–	–	–
Σ acyclic monoterpenoids		0.32	0.18	6.37	0.98	0.32
Σ monocyclic monoterpenoids		3.64	1.54	1.28	2.27	0.48
Σ bicyclic monoterpenoids		5.68	2.42	1.19	3.44	1.33
Σ acyclic sesquiterpenoids		30.39	15.22	26.55	13.61	14.01
Σ monocyclic sesquiterpenoids		3.63	1.19	3.90	5.09	2.07
Σ bicyclic sesquiterpenoids		56.07	76.24	51.10	74.61	72.62
Σ tricyclic sesquiterpenoids		–	0.53	–	–	–
Σ monoterpenoids		**9.64**	**4.14**	**8.84**	**6.69**	**2.13**
Σ sesquiterpenoids		**90.09**	**93.18**	**81.55**	**93.31**	**88.70**
Unidentified constituents		**0.27**	**2.68**	**9.61**	**–**	**9.17**

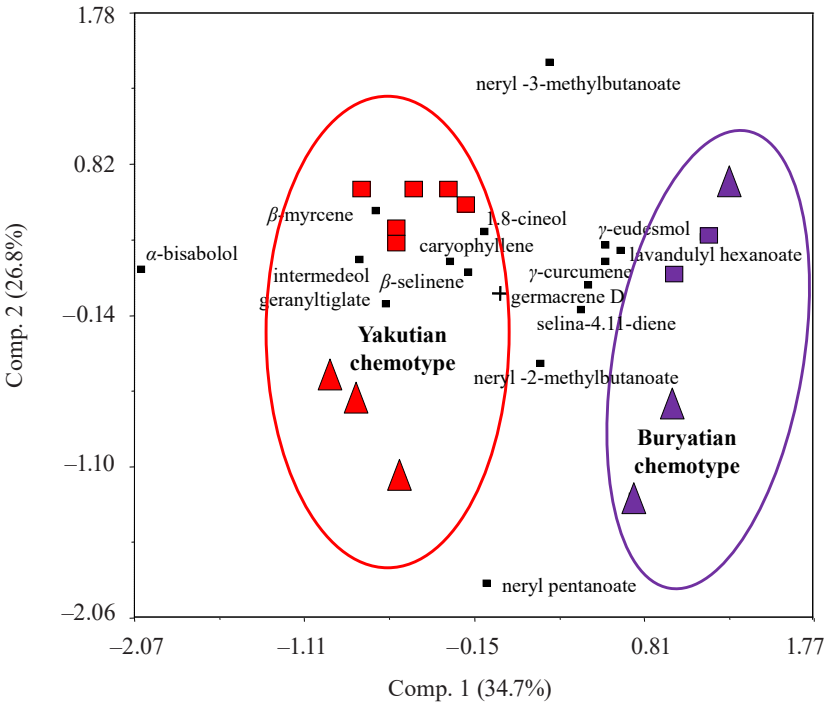

Figure 3 Principal component method. Biplot (PC1-PC2) of the composition of *Artemisia jacutica* Drob. essential oil. In the figure: triangles – wild plants, squares – cultivated plants; purple – experimental data according to Table 3, red – literature data [10]; black squares – oil components

A total of 51 components were identified in the essential oil samples. A number of components (*γ*-terpinene, terpineol-4, *α*-terpineol, *α*-pinene, 1,8-cineol, *β*-farnesene, neryl-2-methylbutanoate, neryl-3-methylbutanoate, geranyl-3-methylbutanoate, *γ*-curcumene, germacrene D, caryophyllene, chamazulene) were found in the oils from both the cultivated and wild plants at all, or almost all, development phases. Several compounds (*β*-ionone, *α*-bergamotene, Δ-amorphene, amorph-4-en-7-ol, and 3,6-dihydrochamazulene) were found only in the oil from the cultivated plants.

The oils from cultivated and wild plants were similar in composition by group. The composition of the oil from wild plants was most diverse in the flowering phase (up to 32 components). In this phase, the oil had the highest content of chamazulene (47.77%), the dominant component, compared to its lowest content in the fruiting phase (15.98%). The oil obtained from wild plants in the budding phase had higher contents of 1,8-cineol (4.97%) and neryl-3-methylbutanoate (13.12%), while in the oil obtained in the fruiting phase, neryl-2-methylbutanoate (10.45%), nerylpentanoate (14.30%), *γ*-eudesmol (31.66%), and germacren D (2.06%) prevailed. Our analysis showed a high content of geraniol and a wide variety of its derivatives, as well as its isomer nerol. However, neryl isobutanoate and geranyl-2-methylbutanoate were found only in the essential oil from wild plants (Table 3).

The highest content of chamazulene was found in the oil from cultivated plants (59.22 and 66.60% on plots No. 1 and No. 2, respectively), which was higher than its content in the oil from wild plants both in the budding (41.17%) or flowering phase (47.77%). The oil from plants cultivated on plot No. 1 was more diverse (33 components) that the one from plot No. 2 (25 components). Despite the higher survival rate of *A. jacutica* in the more fertile plot, the highest content of chamazulene was found in the oil from the less fertile plot with a higher content of mobile phosphorus P_2O_5. Apparently, phosphorus increases the activity of enzymes that control the biosynthesis of proazulene substances [18]. The total content of geraniol derivatives and nerol was also higher in the oil from the plants cultivated on plot No. 2 (11.01%), compared to plot No. 1 (9.91%). In the essential oils of wild plants, it varied from 15.11 to 25.68%, depending on the phase of plant development.

We compared the oils from *A. jacutica* cultivated in Buryatia and in the Siberian Botanical Garden (Tomsk) and found that chamazulene was their main component [10]. However, its content was lower in the plants grown in Tomsk (up to 43.37%), compared to the plants cultivated in Buryatia (up to 66.60%) or wild-growing plants in Yakutia (up to 45.75%) and Buryatia (up to 47.77%). We analyzed our results and literature data by the principal component method and identified two chemotypes, “Yakutian” and “Buryatian”. The chemotypes were preserved in the cultivated plants (Fig. 3).

The components identified in *A. jacutica* essential oil can be divided into three groups:

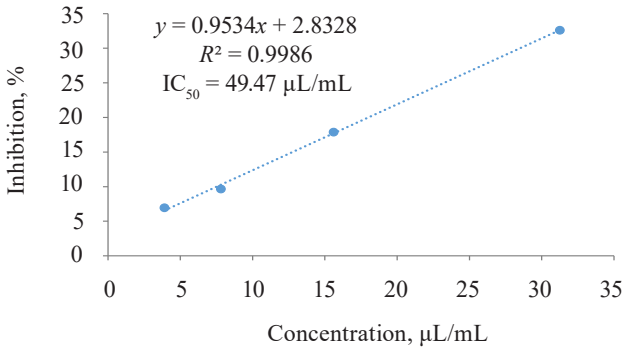

Figure 4 DPPH-antiradical activity of *Artemisia jacutica* essential oil

1) Components found in the essential oils of both wild and cultivated plants, regardless of chemotype. This group includes dominant (chamazulene, γ-eudesmol, neryl-2-methylbutanoate, 1,8-cineol) and minor (β-myrcene, α-terpineol, geranyl-3-methylbutanoate, caryophyllene) components. The acyclic sesquiterpenoid neryl-pentanoate was found in noticeable amounts only in the wild plants of the Yakutian (up to 14.59%) and Buryatian (up to 14.30%) chemotypes, and in small amounts in the cultivated plants of the Buryatian (0.65%) chemotype;

2) Components found in the essential oils of both wild and cultivated plants of either Yakutian or Buryatian chemotype. For example, the Yakutian plants had a noticeable content of α-bisabolol (6.07–24.75%), geranyl tiglat (0.69–3.05%), and intermediol (0.25–2.78%), while the Buryatian plants contained some compounds that were absent in the Yakutian chemotype, namely γ-terpinene, α-pinene, β-farnesene, γ-curcumene, lavandulyl-hexanoate, and others;

3) Components found in the essential oils from cultivated plants of the Yakutian chemotype and from both wild and cultivated plants of the Buryatian chemotype: terpineol-4, neryl-3-methylbutanoate, germacren D, and seline-4,11-diene. Sesquiterpene hydrocarbon β-selinene (0.21–0.41%) was identified only in the introduced plants of the Yakutian chemotype.

Antiradical activity of *A. jacutica* essential oil. The antiradical potential of *A. jacutica* essential oil was determined by the DPPH test (2,2-diphenyl-1-picryl-hydrazyl free radical inhibition). We found that the oil exhibited high antiradical activity (IC_{50} = 49.47 µL/mL) (Fig. 4).

The antiradical potential of *A. jacutica* oil was higher than that of *A. annua* – 50.63 µg/mL, *A. gmelinii* – 2400 µg/mL, or *A. alba* – 1.50 mg/mL [19–21]. It could be due to synergistic and antagonistic interactions between individual components of essential oil as a complex system. Azulenes, including chamazulene, are known to have significant antioxidant activity [22].

CONCLUSION

The primary introduction of *Artemisia jacutica* Drob. showed good prospects for cultivating this plant in the natural and climatic conditions of Buryatia. The macro- and microscopic features and dominant components found in the essential oil of *A. jacutica* grown on the experimental plots were similar to those found in the wild plants. Two chemotypes of *A. jacutica*, Yakutian and Buryatian, were distinguished according to the oil composition. Notably, the chemotypes were preserved in the cultivated plants. The oil's high antiradical activity and a high content of chamazulene make *A. jacutica* a valuable material for the cosmetic, pharmaceutical, and agricultural industries.

CONTRIBUTION

E.P. Dylenova developed the research concept and design, analyzed the data, and wrote the first draft. S.V. Zhigzhitzhapova collected and analyzed the data and edited the article. D.B. Goncharova collected and analyzed the data. Zh.A. Tykheev edited the article. D.G. Chimitov conducted field work and edited the article. L.D. Radnaeva edited the article.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Textural and antioxidant properties of mozzarella cheese fortified with dehydrated oyster mushroom flour

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Abstract:
As a good source of natural anti-oxidants, oyster mushroom flour can be incorporated in dairy products. However, very few scientific publications provide formulations for dairy products fortified with oyster mushroom flour. This research featured the physicochemical and antioxidant properties of oyster mushroom flour pretreated with 0.5% citric acid solution. Three samples of mozzarella cheese were incorporated with 1, 2, and 3% oyster mushroom flour and tested for physicochemical properties, total phenolic content, total flavonoid content, and 2, 2-diphenyl-1-picrylhydrazyl (DPPH). The mushroom flour had 11.09 ± 0.88% moisture content, 20.70 ± 0.74% protein, 3.25 ± 0.13% ash, 7.43 ± 0.35% crude fiber, 3.31 ± 0.51% fat, and 54.20 ± 0.81% carbohydrate. The DPPH was 87.00 ± 0.15 mg GAE/g DM, the total phenolic content was 2.09 ± 0.68 mg GAE/g DM, and the total flavonoid content was 1.67 ± 0.27 mg QE/g DM. The texture and water holding capacity of the mozzarella cheese samples fortified with oyster mushroom flour decreased as the proportion of mushroom flour increased. The color (L^* lightness, b^* redness, and a^* yellowness) was significantly lower than in the control (cheese without oyster mushroom flour). The test samples contained significantly ($p \leq 0.05$) higher amount of DPPH and phenolic compounds than the control. The sensory attributes were assessed by 30 semi-trained panelists, who gave the highest score to the sample fortified with 1% oyster mushroom flour. As a natural antioxidant, oyster mushroom flour proved to be an excellent component for functional cheese products.

Keywords: Mozzarella cheese, mushroom, drying, textural properties, antioxidant, sensory analysis

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INTRODUCTION

The food industry produces bio-functional foods supplemented with plant ingredients to meet consumer's high demand for healthy lifestyle [1]. Novel cheese products are an important part of this strategy because cheese is extremely rich in bio-active compounds. Natural cheeses can be fortified with both dairy and non-dairy ingredients, e.g., mushrooms, wheat fiber, vegetables, meat, egg protein, fruit juices and pulp, oats, nuts, etc. [2].

Mushrooms are classified as healthy food because they are low in fat but rich in proteins and dietary fibers [3]. They are often included in highly nutritive soups or mixed with herbs as a strengthening agent. Mushrooms are also a good source of energy: 454 g of fresh mushrooms provide 120 kcal. They are rich in iron, copper, calcium, potassium, vitamin D, folic acid, zing, etc., which makes it possible to use them as capsules or extracts [4].

In fact, researchers compare mushrooms with milk and non-vegetarian foods such as eggs and meat [5]. Most edible mushrooms belong to the *Agaricaceae* family of the *basidiomycetes* class [6]. This group includes about 14 000 different species of mushrooms, of which at least 1450 species are edible and 25 species are included in daily diets worldwide [7].

Unfortunately, mushrooms have a very short shelf life, which can be extended by various processing methods, e.g., freezing, drying, canning, sterilization,

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pickling, etc. As a result, the market value of mushrooms is rising every year due to their monetary potential, nutritional constituents, and the growing demand for a healthy life style.

Cheese is a fermented milk-based food product obtained by milk coagulation and draining. It is a consolidated curd of milk solids in which milk fat is entrapped by coagulated casein [8]. Cheese is one of the oldest fermented foods in human history. Nowadays, it exists in thousands of varieties. This diversity is a result of multiple factors, one of which is the type of milk (cow, buffalo, goat, sheep, etc.).

Mozzarella cheese gained a worldwide popularity as a salad ingredient or as a ready-to-eat meal. Mozzarella cheese has a wide application in the food industry due to its nutritional value and diversity [9]. For instance, mozzarella cheese contains a lot of calcium, which contributes to weight loss, as well as protects from breast cancer and metabolic disorders associated with cardiovascular diseases [10]. Mozzarella cheese is a rich source of proteins, short-chain fatty-acids, vitamins, e.g., riboflavin, thiamin, vitamin B_{12}, etc., and minerals, e.g., calcium and phosphorus [11]. Milk is an important source of such macro-nutrients as fat, proteins, and sugar (lactose), as well as micro-nutrients, represented mostly by various minerals [12]. The textural properties of cheese depend on the composition and pretreatment of milk, fortification of cheese, etc.

Various studies report better textural properties and nutritional functionality of processed cheese by fortification with spice extracts, vegetable oil, and oat flour [13–15]. However, very few publications feature mozzarella cheese fortified with mushroom powder. The present work aims at utilizing oyster mushroom powder as a source of additional nutrients for functional cheese. We tested the effect of different concentrations of mushroom flour on the textural and nutritional properties of mozzarella cheese.

STUDY OBJECTS AND METHODS

Collecting the raw materials. The instant full-cream milk powder was purchased from the local market in the vicinity of the Hajee Mohammad Danesh Science and Technology University (Bangladesh). The oyster mushrooms (*Pleurotus ostreatus* L.) came from the Horticulture Center in the city of Dinajpur (Bangladesh). The chemicals and the starter culture were purchased from Chr. Hansen (Hoersholm, Denmark). The experiments were conducted on the premises of the Department of Food Engineering and Technology, Hajee Mohammad Danesh Science and Technology University.

Preparing the mushroom flour. Fresh oyster mushrooms were rinsed under running water and sliced into 3-mm pieces. After that, we soaked 250 g of mushroom in 1 L of 0.2% citric acid solution for 30 min. Subsequently, the slices were spread over plastic trays and allowed draining. Then, they were dried in a cabinet dryer at 60°C with hot air at a flow rate of 0.305 m/s. The dry mass was grounded into fine flour in a CM/L-7360065 blender (Japan). The resulting oyster mushroom flour was sieved and packed in airtight high-density polyethylene (HDPE) bags. The flour remained in desiccators at room temperature until further use.

Compositional analysis of mushroom flour. The contents of moisture, ash, protein, fat, and fiber were determined as recommended by the Association of Official Analytical Chemists for mushroom flour [16]. The carbohydrate content was expressed by subtracting the resulting protein, fat, ash, moisture, and fiber from 100.

Preparing sample extracts from mushroom flour. The extraction of all samples followed the procedure described in [17]. We mixed 5 g of sample with 50 mL of 80% methanol at a solid to liquid ratio of 1:10, extracted it at room temperature, and kept it in a hot plate, shaking it for 3 h. The procedure was followed by centrifugation using an MF 300 General Centrifuge at 4000 rpm for 15 min. The supernatant extraction was filtered using Whatman No. 1 filter paper. The remaining residue was extracted again in the same procedure to collect the supernatant. This procedure was repeated twice. The supernatants were combined and made up to 30 mL by adding methanol. All the samples underwent the same procedure. The supernatants served as extract to determine the total phenolic content, the total flavonoid content, and the antioxidant properties. All the tests were done in triplicates in immediate succession, and the samples were stored at 5°C until further use.

Antioxidant analysis via DPPH assay. The antioxidant profile was investigated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical as a free radical scavenging model. The free radical scavenging ability was measured using a modified version of the DPPH assay described by Islam *et al.* [18]. We combined 100 μL of extract and 1.9 mL of 0.30 mM of DPPH solution in a test tube. The resulting mix was stored at room temperature for 30 min. A spectrophotometer (UV/VIS, UV1800) was used to measure the absorbance at 520 nm. Methanol served as control. The antioxidant activity, %, was calculated as a percentage suppression of the DPPH radical by the following Eq. (1):

$$\text{Antioxidant activity} = 1 - (A_s/A_o) \times 100 \quad (1)$$

where A_s is the absorbance of the sample and DPPH solution and A_o is the absorbance of the control.

Determining the total phenolic content. The total phenolic content of each sample was evaluated using the Folin-Ciocalteu assay, which was modified slightly from the standard described by Kabir *et al.* [17]. After mixing 0.5 mL of the sample extract with 0.5 Folin-Ciocalteu solutions, we added 1 mL of sodium bicarbonate (7.5% solution) to the mix, which was diluted with distilled water to obtain 10 mL of solution. The mix was vortexed for a few seconds. The solutions were centrifuged for 10 min at 4000 rpm after spending 35 min at room temperature in the dark. After that, the absorbance was detected using a spectrophotometer (UV–VIS,

UV-1800) at 725 nm. The standard curve was calibrated with gallic acid (0–200 L M). The results were presented as milligrams of Gallic acid equivalent per gram of dry solids (mg GAE/g DM).

Determining the total flavonoid content. The colorimetric approach reported by Islam *et al.*, with a few adjustments, helped to determine the total flavonoid content [18]. We mixed 1 mL of the extracts with 4 mL of distilled water and 0.3 mL of a 5% $NaNO_2$ solution in 15-mL falcon tubes. The tubes were then allowed standing for 5 min before adding 0.3 mL of 10% $AlCl_3$ to the mix and left to stand for 1 min. After that, 2 mL of 1 M NaOH and 2.4 mL of distilled water were poured together and mixed thoroughly. The tubes were maintained in a dark place at room temperature for 15 min following 10 min of centrifugation at 4000 rpm. The absorbance was measured at 510 nm against a blank made in the same way but with methanol instead of water. A standard curve of quercetin was used to quantify the total flavonoid content. From a quercetin standard curve, the total flavonoid content was determined, and the results were represented as milligrams of quercetin equivalent per gram of dry sample (mg QE/g DM).

Preparing mozzarella cheese with mushroom flour. Four mozzarella cheese samples were prepared by the method described by Shams *et al.*: control cheese (without mushroom flour), cheese with 1% mushroom flour, cheese with 2% mushroom flour, and cheese with 3% mushroom flour [19]. Table 1 illustrates the formulations.

Physical properties analysis of mozzarella cheese. We defined the physical properties of the mozzarella cheese fortified with mushroom flour. Its textural profile included such parameters as hardness, adhesiveness, cohesiveness, resilience, chewiness, and springiness. The analysis involved a penetration test using a texture analyzer equipped with a 2-kg load cell. The viscosity was calculated after mixing the sample at 4°C for 60 s using a viscometer (Brookfield, USA). The samples were analyzed using a number 2 spindle at 12 rpm. The resulting viscosity was recorded in centipoise (Cp).

Table 1 Mozzarella cheese with oyster mushroom flour: formulations

Ingredients, g	Samples			
	Control	With oyster mushroom flour		
		1%	2%	3%
Milk	100	99	98	97
Oyster mushroom flour	0	1	2	3
Starter culture	0.1	0.1	0.1	0.1
$CaCl_2$	0.62	0.62	0.62	0.62
NaCl	1	1	1	1
Rennet	0.0025	0.0025	0.0025	0.0025

NB: The optimal proportions were obtained by trial and error

Guzman *et al.* developed a centrifuge technique to assess the water holding capacity of cheese [20]. We centrifuged 5 g of cheese (*C*) for 30 min at 1250×g. The precipitant was weighed after removing the ejected whey (*WE*). The water holding capacity (*WHC*, %) was determined as follows:

$$WHC = \frac{C - WE}{C} \times 100 \quad (2)$$

The syneresis of the mozzarella cheese was assessed through the curd drainage test at room temperature based on a modified version of the procedure developed by Harwalkar *et al.* and Mahomud *et al.* [21, 22]. We cut the cheese in a beaker with a steel knife and transferred it into a funnel fitted with a stainless-steel screen. After that, the whey was drained into a graduated cylinder. The syneresis, %, percentage was calculated as the weight of the expelled water divided by the weight of the initial sample multiplied by 100:

$$\text{Syneresis} = \frac{\text{weight of supernatant}}{\text{weight of cheese}} \times 100 \quad (3)$$

The color of the mozzarella cheese was analyzed using a BIOBASE Colorimeter (BC-110/200, China). The color attribute was determined by the *L**, *a**, and *b** system, where *L** indicated lightness (100 – white, 0 – black), *a** denoted redness (+)/greenness (–), and *b** indicated yellowness (+)/blueness (–) [23].

Sensory evaluation of mozzarella cheese. The sensory properties of the mozzarella cheese were valued according to the hedonic rating test as recommended by Roessler *et al.* [24]. Randomly coded samples were presented to 30 semi-trained panelists, who were asked to rate the color, flavor, texture, taste, mouthfeel, and overall acceptability on a 9-point scale, where 1 = dislike extremely, 2 = dislike very much, 3 = dislike moderately, 4 = dislike slightly, 5 = neither like nor dislike, 6 = like slightly, 7 = like moderately, 8 = like very much, 9 = like extremely.

Statistical analysis. All experiments were done in triplicates, and the results were provided as the mean ± SD. Statistical software (SPSS for Windows Version 26.0) was used to calculate all the experimental data. The Duncan's Multiple Range Test was employed to determine the significant differences between the means of textural, sensory, and antioxidant properties of the mozzarella cheese at $p \leq 0.05$.

RESULTS AND DISCUSSION

Table 2 shows the proximate composition of the oyster mushroom flour. In this experiment, the moisture content of the mushroom flour equaled 11.09%. This value was lower than the value reported by Das *et al.*, which was 12.33% [25]. This value was consistent with 11% moisture content of the mushroom flour. The amount of ash in the mushroom flour was 3.25%. This result was also lower than the 5.54% reported by Prodhan *et al.* [26]. In the present study, the protein

content of the oyster mushroom flour was determined as 20.70%. This value was similar to the 20.0% reported by Charles *et al.* [27]. As for the protein content, Maray *et al.* and Farooq *et al.* obtained higher results: 21.9 and 28.69%, respectively [28, 29].

In our research, the fat content of the mushroom flour proved to be 3.31%, which was similar to the findings reported by Ozturk *et al.*, who detected 3.05% of fat in mushroom flour [30]. We determined 7.43% of crude fiber, which approximated the value of 7.90% published by Salehi *et al.* [31]. However, our result for crude fiber was higher than the 5.6% reported by Gonzalez *et al.* [32]. The carbohydrate of the mushroom flour appeared to be 54.20%, which exceeded the result published by Painuli *et al.*, i.e., 52.74% [33].

In this research, the experimental value of the mushroom flour was lower than that obtained by Maray *et al.*, who reported 58.76% [28]. Finally, the proximate composition analysis of the mushroom flour revealed much protein and little ash. Proteins are essential for life: every cell in the body is made up of protein. The basic structure of protein is an amino acid chain. A protein-rich diet helps human body repair and replace cells. Protein is especially important for children, adolescents, and pregnant women.

Antioxidant properties of mushroom flour. The antioxidant properties of the mushroom flour were examined by extracting the oyster mushroom flour with an 80% methanol solution. The total phenolic content of the mushroom flour was estimated by the Folin-Ciocalteu method (Table 3). The methanol extracts obtained from the mushroom flour had the total phenolic content of 2.09 mgGAE/g DM. A similar total phenolic content was reported by Rashidi *et al.* as 2.21 mg/g [34]. Zalewska *et al.* obtained a greater total phenolic content of mushroom flour of 9.48 mg/g [35]. Radzki *et al.* claimed that hot-air drying can significantly reduce the total phenolic compounds in mushrooms [36].

As for the total flavonoid content in this research, it was 1.67 mg/g, which approximated the 1.16 mg/g obtained by Mutukwa *et al.* [37]. However, Nguyen *et al.* reported a much higher value of 3.28 mg/g [38].

The effect of heat on the cell wall released the cell bound flavonoids, which affected the total flavonoid content [39]. According to the DPPH radical scavenging assay, the DPPH of the mushroom flour was 75.96% in this experiment. Much lower DPPH values were reported as 57% by Chirinang *et al.* and 58.13% by Reis *et al.*, while Tsai *et al.* obtained an even greater DPPH of 77.37% [40–42]. The difference in DPPH might be attributed to different factors, e.g., extraction methods, species peculiarities, blanching conditions, etc. Nevertheless, oyster mushrooms could serve as a prospective source of natural antioxidants.

Table 2 Proximate composition of mushroom flour

Components, %	Mushroom flour
Moisture	11.09 ± 0.88
Ash	3.25 ± 0.13
Protein	20.70 ± 0.74
Fat	3.31 ± 0.51
Fiber	7.43.00 ± 0.35
Carbohydrate	54.20 ± 0.83

All data is the mean standard deviation of three replicates

Table 3 Antioxidant properties of mushroom flour

Properties	Mushroom flour
Total phenolic content, mg GAE/g DM	2.09 ± 0.68
Total flavonoid content, mg QE/g DM	1.67 ± 0.27
DPPH, %	75.96 ± 3.00

All values are mean ± standard deviation of at least three replicates (n = 3)

Physical properties of mozzarella cheese fortified with oyster mushroom flour. Table 4 summarizes the physical properties of mozzarella cheese. The textural properties of the mozzarella cheese were determined as 0.37 ± 0.02 kg for the control sample without mushroom flour and 0.42 ± 0.05 kg for the cheese sample with 3% mushroom flour. The texture of S_1 was the lowest and that of S_4 was the highest. The texture increased slightly together with the mushroom flour amount. The hardness correlated with the texture of cheese according to Ozturk *et al.* [30].

The viscosity of mozzarella cheese ranged from 2.317 ± 5.56 to 2.479 ± 9.53 Cp. The experimental values

Table 4 Physical properties of mozzarella cheese fortified with oyster mushroom flour

Parameter	Control	Samples with oyster mushroom flour		
		1%	2%	3%
Texture, kg	0.37 ± 0.02^{b}	0.38 ± 0.10^{b}	0.39 ± 0.01^{b}	0.42 ± 0.05^{a}
Viscosity, Cp	2379.66 ± 67.68^{b}	2317.00 ± 5.56^{b}	2448.00 ± 33.80^{a}	2479.00 ± 9.53^{a}
Water holding capacity, %	98.42 ± 0.07^{a}	98.43 ± 0.25^{a}	98.46 ± 0.56^{a}	98.90 ± 0.10^{a}
Syneresis, %	2.03 ± 0.06^{c}	2.20 ± 0.10^{b}	2.26 ± 0.15^{b}	2.50 ± 0.10^{a}
*L**	40.46 ± 3.01^{a}	39.72 ± 1.80^{a}	38.65 ± 0.14^{a}	38.04 ± 0.53^{a}
*a**	2.84 ± 0.24^{a}	2.58 ± 0.35^{ab}	2.39 ± 0.09^{ab}	2.24 ± 0.13^{b}
*b**	9.77 ± 0.85^{a}	8.93 ± 0.27^{a}	7.74 ± 0.31^{b}	6.70 ± 0.44^{c}

All values are the mean ± SD of three replicates. The test values in the same row have different superscripts for each parameter that is significantly different ($p \leq 0.05$)

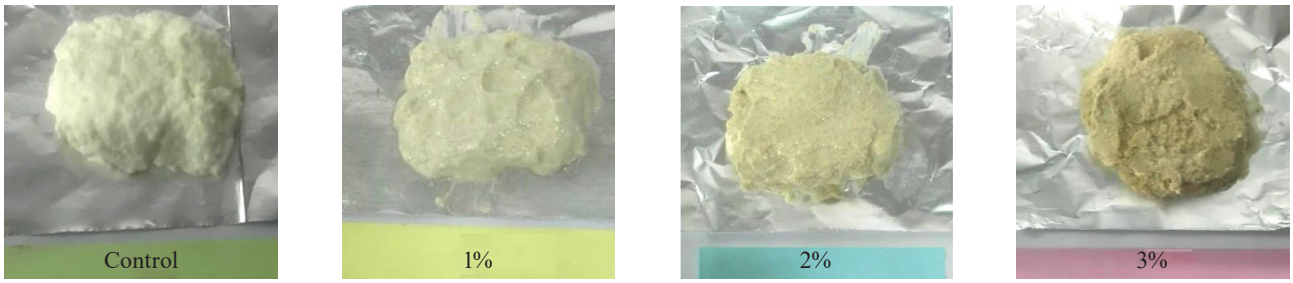

Figure 1 Physical appearance of mozzarella cheese fortified with oyster mushroom flour

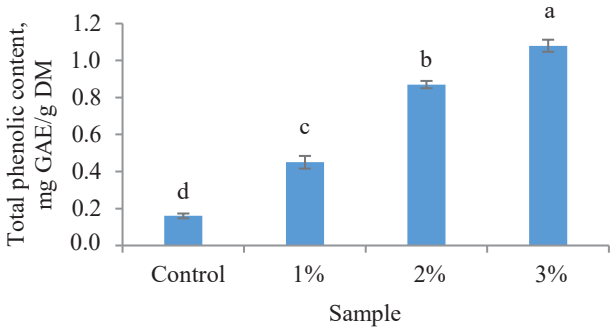

Figure 2 Total phenolic content of mozzarella cheese fortified with oyster mushroom flour

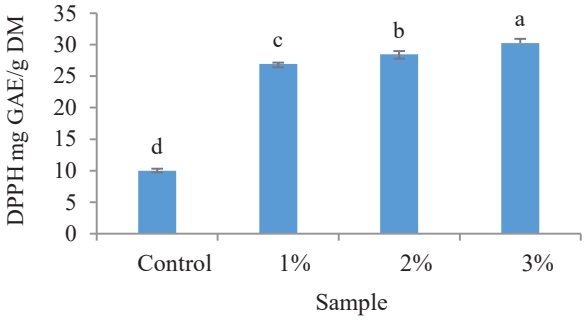

Figure 3 DPPH of mozzarella cheese fortified with oyster mushroom flour

of the control sample and the one with 1% mushroom flour were significantly similar ($p \leq 0.05$) whereas those of the samples with 1 and 3% mushroom flour were significantly different. The viscosity increased moderately with the incorporation of mushroom flour.

The water holding capacity of the mozzarella cheese increased slightly with increasing in an oyster mushroom amount (Table 4). The test values were significantly similar ($p \leq 0.05$) in all the samples. The water holding capacity increased slightly with the oyster mushroom flour proportion.

The physical values of mozzarella cheese varied significantly ($p \leq 0.05$) in the control the sample with 1% mushroom flour, and the sample with 3% mushroom flour. The syneresis was at its highest in the sample with 3% mushroom flour and equaled 2.50 ± 0.10% compared to the control sample which was 2.03 ± 0.06%. The syneresis of the control mozzarella cheese was lower than that of the other samples. This property increased together with the percentage of the oyster mushroom flour.

The color characteristics of the cheese samples are presented in Table 4. The test values of L^* were significantly similar ($p \leq 0.05$) in all the samples. However, the a^* and b^* values were significantly different ($p \leq 0.05$) in all the samples. The control and the sample with 1% mushroom flour showed higher L^*, a^*, and b^* values. The extra mushroom flour affected the color of the cheese, which decreased slightly in the sample with 2% and the sample with 3% mushroom flour. The semi-dark color of the experimental cheese might have been caused by the enzymatic browning during the mushroom flour processing (Fig. 1). Enzymatic browning is known to deteriorate the color because ambient oxygen oxidates the mono-phenolic substances and polyphenol oxidase in mushroom matter [43].

Antioxidant properties of mozzarella cheese fortified with oyster mushroom flour. Figure 2 illustrates the total phenolic compounds of the mozzarella cheese fortified with various amounts of oyster mushroom flour. The total phenolic content varied from 0.16 to 1.08 mg GAE/g DM. The experimental values were significantly different ($p \leq 0.05$) in every sample The highest total phenolic content was found in the sample with 3% of mushroom flour. The results were expressed as milligram of gallic acid equivalents per gram (mg GAE/g DM) of mozzarella cheese. Phenolic compounds are important for fruit because they have antioxidant properties to prevent the breakdown of hydro peroxides into free radicals or inactivate lipid free radicals [44].

The DPPH of the mozzarella cheese ranged from 9.97 to 30.25 mg GAE/g DM (Fig. 3). The DPPH was the highest in the sample with 3% mushroom flour and the lowest in the control. The DPPH of the mozzarella cheese increased gradually as the percentage of the mushroom flour increased. The results for the control and the test samples were significantly different. The values were significantly different ($p \leq 0.05$) in all the samples. Higher DPPH levels indicated a higher antioxidant activity. On the other hand, lower DPPH values indicated a lower antioxidant activity [45]. The total flavonoid content was measured three times for

Table 5 Sensory attributes of mozzarella cheese fortified with oyster mushroom flour in different amounts

Sample	Color	Flavor	Texture	Taste	Mouth feel	Overall acceptability
Control	7.90 ± 0.44[a]	8.00 ± 0.32[a]	7.75 ± 0.44[a]	7.80 ± 0.52[a]	8.10 ± 0.55[a]	7.85 ± 0.48[a]
1%	7.75 ± 0.55[a]	7.65 ± 0.48[b]	7.35 ± 0.58[b]	7.30 ± 0.47[b]	7.50 ± 0.60[b]	7.50 ± 0.60[a]
2%	6.85 ± 0.36[b]	7.00 ± 0.56[c]	6.50 ± 0.51[c]	6.40 ± 0.50[c]	6.55 ± 0.51[c]	6.75 ± 0.44[b]
3%	6.40 ± 0.50[c]	6.30 ± 0.65[d]	6.05 ± 0.60[d]	5.85 ± 0.58[d]	6.05 ± 0.68[d]	6.05 ± 0.68[c]

All values are the mean ± SD of three replicates. The test values in the same column carry different superscripts for each parameter that is significantly different ($p \leq 0.05$)

each sample, but the values were so low that they could be neglected. The absence of flavonoids in the fortified mozzarella cheese might be attributed to the very small amount of total flavonoids detected in the oyster mushroom flour.

Sensory evaluation of mozzarella cheese fortified with mushroom flour. Table 5 demonstrates the effect of mushroom flour on the properties of mozzarella cheese. The sample with 1% mushroom flour was well accepted by the panelists due to its superior color, flavor, texture, taste, and mouthfeel compared to those of other mozzarella cheese. The sample with 1% oyster mushroom flour increased the hedonic acceptability of the mozzarella cheese because it had a more appealing appearance and texture. The samples with 2 and 3% of mushroom flour received a lower score because of their texture, flavor, and color. Therefore, oyster mushroom flour can be fortified with skim milk powder to produce mozzarella cheese with good sensory attributes.

CONCLUSION

The research revealed the physicochemical and antioxidant properties of dehydrated oyster mushroom flour, as well as the effect of its different proportions on the textural, nutritional, antioxidant, and sensory properties of mozzarella cheese. The total antioxidant activity was 75.96 ± 3.00%, the total phenolic content was 2.09 ± 0.68 mg GAE/g DM, and the total flavonoid content was 1.67 ± 0.27 mg QE/g DM. The antioxidant properties and the total phenolic content of the experimental mozzarella cheese fortified with oyster mushroom flour were superior compared to the control. The best sensory evaluation score belonged to the sample fortified with 1% oyster mushroom flour. This amount can be recommended for functional mozzarella cheese since this percentage had no negative effect on the sensory properties of the mozzarella cheese. The samples with 2 and 3% mushroom flour demonstrated better antioxidant properties and a greater phenolic content, but the extra percentage of mushroom flour reduced the consumer acceptability. The new functional cheese proved safe and beneficial for health. Oyster mushroom flour possesses properties that can be used in the food industry. The obtained results reinforce the importance of investments in the development of innovative products with oyster mushroom flour. Economically, these attempts may result in gains for both agricultural practice and the food industry.

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 interests.

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Factors affecting consumer preference for healthy diet and functional foods

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Abstract:
An inadequate diet and lifestyle are major causes of various diseases. A healthy diet can prevent diseases and maintain a good health status. The present research objective was to test young consumers for their preference for healthy diets and their intention to purchase functional foods, as well as to identify factors that affect their attitudes.
The study involved 720 consumers (average 20 years old), a structured questionnaire, and a specially-developed scientific methodology. The results were grouped based on sex and body mass index. The analysis focused on the points where consumers' interest in healthy diet overlapped with health-related diet needs and how each of them corresponded with their knowledge on the following spheres: nutrition and health risks, labelled data use in food choice, preference for foods fortified with vitamins, minerals, dietary fibers, or functional foods with a poor sensory quality. The data were processed using descriptive statistics, Z-test, and correlation tests.
All the consumers demonstrated healthy diet preferences and a significant positive relationship ($p < 0.01$) with the factors. They made an informed choice based on the labelled data and preferred foods fortified with vitamins, minerals, and dietary fibers, as well as functional products with a lower sensory quality. All respondents, regardless of sex and body mass index, demonstrated nutrition and health risk awareness. The health-related diet needs also affected their food preferences ($p < 0.05$), with some exceptions.
The young consumers possessed sufficient basic knowledge on food quality, nutrition, and health. Their attitudes depended on their preference for healthy diets and functional foods. The novel methodology can be applied to other studies of consumer preferences.

Keywords: Consumer preference, influencing factors, healthy diet, food product quality, labeling, fortified food

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INTRODUCTION

Basic dietary habits are established early in childhood but continue to form under the effect of various environmental factors. An inadequate diet and sedentary lifestyle are major causes of some specific diseases, morbidity, and mortality. Obesity is responsible for such noncommunicable diseases as cardiovascular disease, type II diabetes, hypertension, osteoporosis, and certain cancers [1–5]. They increase health care costs, decrease treatment efficiency, and cause unemployment, as well as major lifestyle changes [6]. Some of these conditions, however, can be prevented by radical and timely changes in individual lifestyle and eating habits.

Food consumption is usually based on a few ingredients or food products that can satisfy intake standards if combined. Nutritionists recommend to optimize the traditional daily nutrition model with balanced nutrients and bioactive compounds to prevent diseases and maintain a healthy life [3]. Young consumers remain a challenge for the food industry as their nutrient intake recommendations and diet improvement require interaction between different sectors, e.g., food producers, marketing experts, and nutritionists [7–11]. Fortified foods seem to be the most appropriate preventive approach so far. However, its success depends on consumer preferences.

Before product formulation and modeling, producers identify food properties that can be assigned as beneficial for target consumers [12–14]. A healthy diet is popular when it includes tasty foods with acceptable sensory quality. Food producers have an obligation to

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improve the availability and attractiveness of healthy food products if they want to gain consumers' trust [4, 9, 15, 16].

Different researches confirmed that consumer habits on the target market could be sources of innovative ideas for novel foods [12, 17]. To choose from available products, consumers rely on their eating habits, preferences, or expectations, which usually differ according to life-style, interest, and motivation to eat some particular type or category of food [18, 19]. This study featured young consumers, their preferences for healthy diets, and their intention to purchase food products with improved nutritive quality, as well as to identify factors that affect their attitudes.

Hypotheses and research design. Food producers are food market participants that shape the assortment. They can use the preferences of their target consumers for a healthy diet and functional foods to determine food quality parameters or ideas for novel functional foods [4, 20–23]. Most consumers are familiar with foods with added value or fortified with vitamins, minerals, or nutritive fibers. However, factors that affect food choice or interest in nutrients and food intake could differ within groups of consumers even inside the country or region [10, 12, 24–26]. Therefore, new consumer-oriented health-focused products require surveys of choice factors and their interaction. Body mass index is calculated based on human body weight and body height and expressed in kg/m^2. It can serve as an indicator of diet-related risk factors. An improper diet and inability to follow recommendations cause health issues manifested as increased or insufficient body mass.

Various studies that featured young consumers included an analysis of their preferences for a healthy diet, their intention to purchase functional foods, and the factors that shaped their consumer habits [12, 26, 27]. Consumer preferences for a healthy diet and specific food quality can be the factors that define their attitudes. We formulated them as questions (Qx). Before the experiment, we came up with two main hypotheses and two additional hypotheses to be tested.

Respondents do not differ in consumer attitude toward a healthy diet and functional foods:
H1: All respondents have the same consumer attitude regardless of sex;
H2: All respondents have the same consumer attitude regardless of body mass index;
H2a: All male respondents have the same consumer attitude regardless of body mass index; and
H2b: All female respondents have the same consumer attitude regardless of body mass index.

The null hypothesis could be tested by crossing the grouped answers based on respondents' sex (men and women) and body mass index (normal and inadequate) for each question and a Z-test.

The three main hypotheses (H 3–5) were formulated for correlation testing for each group of respondents (all, men, women) or for all respondents, men, and women grouped according to body mass index. The first group was marked as Hxa-f, while the second was marked as BHxa-f, where x stood for the hypothesis number, and letters a-f were related to the dependent variables, i.e., questions. All the hypotheses were focused on the research objective. Hypotheses 4 and 5 were subdivided into six additional hypotheses, related to the dependent variables, i.e., questions Qx:
B/H3: Consumer interest in healthy diet (Q1) is not connected with individual health-related diet needs (Q8);
B/H4: Consumer interest in healthy diet (Q1) is not connected with B/H4a-f; and
B/H5: Individual health-related diet needs (Q8) are not connected with BH5a-f,

Where B/Hxa-f are:
B/H4/H5/a: food labels (Q2);
B/H4/H5/b: labels use in food choice (Q3);
B/H4/H5/c: purchasing foods fortified with vitamins and minerals (Q4);
B/H4/H5/d: purchasing foods fortified with dietary fibers (Q5);
B/H4/H5/e: products with an improved nutritive content but a poor sensory quality (Q6); and
B/H4/H5/f: nutrition and health risk awareness (Q7).

STUDY OBJECTS AND METHODS

Participants' profile. Different ideas for innovations in food development or reformulation of existing products could be identified by researching the target market. However, before food producers make the final decision on the type of quality improvement of a completely new or existing product, they must find out about the habits and preferences of real consumers of the selected food category and the factors that affect their attitudes.

This study involved 720 young adults who live in the Republic of Srpska (Bosnia and Herzegovina). They were selected as representatives of target twenty-year-old consumers (Table 1) who are independent in their choice of food. They were identified as representatives suitable for studies regarding future activities on food product development or nutritive quality improvement. They were recruited for the research from public secondary and higher educational institutions, based on expressed individual interest for participation. The questionnaires were printed for direct self-reporting data and distributed in secondary schools and higher public educational institutions in Banja Luka, Bijeljina, East Sarajevo, Prijedor, and Zvornik.

The questionnaire started with demographic data. They were used as variables for the research and included sex, age, and education. The list also included economic status, health- induced dietary needs, body weight, and height. Table 1 illustrates the data we used to calculate the body mass index (kg/m^2).

The respondents were grouped based on the calculated body mass index (kg/m^2):
≤ 18.5 kg/m^2 – underweight;
18.5–24.9 kg/m^2 – normal;

25–29.9 kg/m^2 – overweight; and
≥ 30 kg/m^2 – obese.

Two groups of respondents were formed based on body mass index, namely normal body mass index and inadequate body mass index. The second group included underweight, overweight, and obese respondents all together as the number of respondents in each subgroup was relatively low [24].

The second part of the questionnaire contained questions regarding consumer preference for healthy diet, their intention to purchase food products with improved nutritive quality, and factors that affected their attitudes. The data were grouped based on consumers' sex, age, and body mass index.

Economic status may also have an impact on food choice, so we included this question in our questionnaire. The distribution appeared to be homogeneous: all the respondents were unemployed students from public educational institutions. Based on the general economic environment, the expected economic status was satisfactory or low. The data analysis revealed that most respondents shared a satisfactory economic status. Thus, no conditions for sub-groups based economic status were revealed (Table 1).

Procedure and questionnaire design. The questionnaire for the target market research was based on an extensive review of scientific publications that featured the preferences that young consumers have for a healthy diet, their intention to purchase functional foods, and factors that affect their attitudes. All the questions that referred to personal data and preferences at the time of purchase were precise and unambiguous. The questions related to the interest in healthy diet and food products quality were general questions that required *Yes/No* answers (Table 2). They served to define the overlapping points of consumers interest in healthy diet (Q1) with individual health-related diet needs (Q8), and where each of them overlapped with

Table 1 Demographic characteristics (n = 720)

Demographic characteristics	Variable	Average age ± SD*	Frequency	Frequency, %
Sex	Male	18.50 ± 2.80	290	40.3
	Female	19.50 ± 2.73	430	59.7
Men, age	≤ 18	16.48 ± 0.67	155	53.4
	18–20	19.08 ± 0.98	55	19.0
	≥ 21	22.50 ± 1.62	80	27.6
Women, age	≤ 18	16.50 ± 0.58	147	34.2
	18–20	19.40 ± 0.87	94	21.9
	≥ 21	22.00 ± 1.63	189	43.9
Education: high school students	Male	16.72 ± 0.64	184	51.1
	Female	16.73 ± 0.63	176	48.9
Education: university undergraduates	Male	22.61 ± 2.27	106	33.1
	Female	21.69 ± 1.41	254	66.9
Economic status	Satisfactory		661	91.8
	Low		59	8.2
Needs for special health-related diet	Yes		137	19.0
	No		583	81.0
Body mass index**	Underweight (< 18.5 kg/m^2)		63	8.7
	Normal (18.5–24.9 kg/m^2)		571	79.3
	Overweight (25–29.9 kg/m^2)		76	10.6
	Obese (> 30 kg/m^2)		10	1.4

*SD = standard deviation

**Body mass index = body mass index expressed as body weight/heigh

Source: compiled by the authors based on research data

Table 2 Questions used to identify factors that affect consumers' preference in healthy diet and functional foods

Question (Qx)	*Yes/No* questions on preferences, awareness, and food choice habits
Q1	Do you prefer a healthy diet?
Q2	Do you read labeled information about food product ingredients?
Q3	Do you compare the labeled data when choosing between similar food products?
Q4	Do you prefer to purchase products fortified with vitamins and minerals?
Q5	Do you prefer to purchase products fortified with dietary fibers, e.g., integral cookies?
Q6	Would you like to buy foods that are not particularly attractive, but have an improved nutritive content?
Q7	Can inadequate nutrition affect one's health?
Q8	Do you need a special diet because of health problems?

their interest in the labeled composition (Q2), the way the used labels in their food choice (Q3), their intention to purchase products fortified with vitamins and minerals (Q4) or dietary fibers (Q5), their preference for products with nutrient-fortified content but a lower sensory quality (Q6), individual awareness of nutrition and health risks (Q7), and individual health-related diet needs (Q8) (Table 2). The respondents took about 10 min to fill in the data.

Data analysis. The descriptive statistics included means, standard deviations (SD) (Table 1), and frequencies (n; %) within each group of respondents. The responses regarding consumer interest in the healthy diet and food products were ranked in descending order based on the frequency or percentage of the possible *Yes*-responses on each of the eight questions for all respondents (n = 720) covered by survey and for respondents grouped based on sex.

The data base for the research and analysis was formed by grouping affirmative responses as follows: all participants; participants grouped based on sex (men and women); participants grouped based on body mass index (participants with normal body mass index, participants with inadequate body mass index); participants grouped based on body mass index and sex (male participants with normal body mass index, male participants with inadequate body mass index; female participants with normal body mass index, female participants with inadequate body mass index).

The null hypothesis that predicted no significant difference in the consumer attitude towards specified information was tested by crossing the grouped data and performing the Z-test ($p < 0.05$). The data from the questionnaires were grouped to analyze preferences for healthy diet and intention to purchase functional foods. The descriptive statistics and correlation tests (Pearson's correlation coefficient r) were used to test the relationship between selected variables and identify factors that affected consumer attitudes ($p < 0.05$). The data processing involved the statistical software *3BStat* Gold Edition, Version 1.01 [28].

RESULTS AND DISCUSSION

Healthy diet and food preferences. Safe consumption has a long history. It covers nutrients that are essential for human well-being when consumed within a certain range or at the levels recommended in balanced human diets. Recommendations for a healthy diet usually cover optimal nutrients, energy, and water intake. A varied diet provides sufficient amounts of proteins, vitamins, and minerals, while balancing the amount of carbohydrates, dietary fiber, and fat. These substances should be bioavailable for absorption or accessibility to normal metabolic and physiological processes [1, 4, 29].

People develop their own individual criteria for quality parameters which are processed in food choice. They include sensory properties, food origin, and its possible effect on their health [8, 9, 14, 26]. Food producers can improve the nutrient profile of their products by introducing healthy ingredients or reducing the content of some other ingredients. The amount of a nutrient which must be consumed on a regular basis to maintain good health are indicated by dietary reference values. They serve as scientific bases for nutrition recommendations, dietary guidelines, diet assessment and planning, or reference values in food labelling [29]. Food science and technology offer a new framework for health-focused product design or reformulation within the areas of food physics, food storage, preservation, nutrient restoration, and fortification [6].

Young people's lifestyle is a complex of behavior patterns and opinions, which must be analyzed and incorporated into research and development together with cultural interpretations [7, 10, 27]. For example, eating habits of young consumers usually include a lot of energy-rich bakery products with a poor fiber content and a lot of simple sugars and fats, which may cause obesity [30–32].

Different factors may affect consumer behavior and interest in specific product characteristics [5, 8, 14, 26, 20]. Our study examined the consumer interest in healthy diet, nutrition and health risk awareness, food labeling, and preference for functional foods. The difference in preferences for a healthy diet and foods was investigated by comparing questionnaire responses grouped according to sex or body mass index. We also analyzed consumer intention to purchase foods with improved nutritive quality and some factors that affect their attitudes, which were included in the group of questions related to the food quality and healthy diet.

Consumer interest in a healthy diet and food products ranking. Optimal nutrition means that the diet is healthy and able to ensure one's well-being. The respondents' interest in a healthy diet and functional foods was determined by ranking their responses to each question with specified data and comparing the total number (n) and percentage of affirmative answers on each of the eight questions for all respondents (n = 720) and respondents grouped according to sex (Table 3).

The analysis of affirmative responses for all respondents (Table 3) confirmed that most of them possessed some basic knowledge on the causal relationship between inadequate nutrition and health risks (Q7). They indicated individual healthy diet preferences (Q1) as their good purchasing behavior, which ranked second in total answers. Consumers have access to product information, but the risk of misinformation remains quite significant. Practical application of scientific findings needs more appropriate activities than recommendations for nutrient intake. The food industry may offer health benefits by modeling food products and nutrient content [33].

Whole-grain bakery products are recommended as part of a healthy diet. They can be consumed in large quantities with basic bakery products, e.g., cakes or cookies. Whole-grain flour contains carbohydrates and dietary fibres, which are inherent in a healthy diet. Also,

Table 3 Affirmative responses for all respondents (n = 720), men (n_M = 290), and women (n_F = 430)

Question (Q_x)	*Yes*-responses for all respondents (n = 720)		*Yes*-responses for men (n = 290)		*Yes*-responses for women (n = 430)	
	n_{Qx} [a]	% [b]	n_{MQx} [a]	% [b]	n_{FQx} [a]	% [b]
Q1	617	85.81	251	86.55	367	85.35
Q2	521	72.46	191	65.86	331	76.98
Q3	296	41.17	136	46.90	161	37.44
Q4	588	81.78	229	78.97	360	83.72
Q5	362	50.35	116	40.00	246	57.21
Q6	444	61.75	166	57.24	279	64.88
Q7	689	95.83	273	94.14	417	96.98
Q8	137	19.05	56	19.31	82	19.07
[c]$Sum_{1–7}$	3517	69.78	1362	27.02	2161	42.88

[a] Number of affirmative responses (n_{Qx}) to each question (x = 1–8)

[b] Percentage of affirmative responses to each question (x = 1–8) calculated based on the possible number of affirmative responses

[c] Percentage of affirmative responses to all questions (x = 1–7) calculated based on 5040 *Yes*-responses for all 720 respondents

Source: calculated by the authors based on the research data

it is source of proteins, fats, vitamins, and mineral. Its optimal quantity depends on the row grain type and flour processing method [13]. Dietary fibres possess numerous positive physical and physiological properties and are valuable ingredients in human nutrition. Like vitamins or minerals, they are recommended as components of healthy diet.

The attitudes for fortified and health-promoting foods ranked third in this research based on the responses to questions regarding the sufficient intake of foods that are rich in vitamins and minerals (Q4). A higher fiber content was known to improve consumers' diet and meet their expectations [31, 32]. In our study, respondents were less interested in dietary fiber intake regarding such products as integral cookies (Q5), which ranked sixth. General awareness of and preference for particular subjects and habits in food choice were investigated using other questions. Consumer interest in information labeled on packed foods, and especially in product composition and ingredients, proved to be valuable data in food quality evaluation and food choice for healthy diet planning.

Affirmative responses regarding the attitude to labeled data in food choice (Q3) were on the seventh place. The respondents confirmed a greater interest in labeled information (Q2), which ranked fourth (Table 3). Other studies that used questionnaires to investigate consumer knowledge on nutrition and labeled data on functional foods confirmed different levels of dietary awareness, labeled data use, motivation, and ability to process and understand them [23, 24, 34–36].

The technological and sensory quality of a food product depends on the type and amount of ingredients, formulation, and processing methods. Healthy foods often have a poor sensory profile, which limits consumer appeal. We analyzed attitudes to buying functional foods with a poor sensory quality (Q6): half of the respondents accepted the compromise while the other half did not, ranking it fifth.

The respondents were additionally asked about their health-related special diet needs (Q8) to identify factors that affected their interest in a healthy diet and functional foods (Table 3). Nutrients improve human health and can prevent chronic disease, if optimally ingested. Correct attitudes to improving individual diets and healthy activities are very important for disease prevention. Individual diets should be based on recommended dietary intake, individual needs, and personal goals. They should be adapted to a particular lifestyle, which, in its turn, interacts with numerous external factors [5, 37, 38].

Differences in attitude between all respondents, men, and women. Various competent institutions play an important role in food control and legislation. They are responsible for food quality, safety, packaging, and labeling [22, 23, 34]. Labeling means a mandatory indication of specific information on packaged food products. This information appears in a standardized format on food labels or packages. It is consumer-friendly and guides consumers in their search for particular foods and nutrients. Labels give information on the ingredients, technological and nutritive quality, recommended or restricted intake of nutrients, etc. They may also contain various nutritional and health claims that emphasize some special characteristics of the product that are important for diet planning. However simple this information might be, consumers should have at list some basic knowledge to be able to use it for adequate choices.

Table 3 analyzes the affirmative responses for all participants grouped based on sex. It tested hypothesis H1, which predicted no difference in consumer attitude toward a healthy diet and functional foods regardless of sex. All respondents (affirmative responses to Q 1–7) were grouped based on sex. The results indicated an obvious statistically significant difference between men and women ($Z = 3.5681$; $p < 0.05$). As a result, hypothesis H1 was rejected.

Both men and women (Z-test) were familiar with the principles of proper nutrition and claimed very good knowledge on health and nutrition (Q7). They also preferred a healthy diet (Q1) and healthy foods fortified with vitamins and minerals (Q4). No statistically significant difference ($p > 0.05$) was recorded between male and female responses to questions Q7, Q1, and Q4.

Similar studies show a widespread consumer interest in labeled nutritive quality. Most consumers understood common labeling formats and knew how to use labeled data on nutrition [4, 9]. Another research confirmed these findings and reported that consumers liked it when products were visually different and had attractive labels. However, men were less interested in personalized nutrition than women [19]. According to some publications, most consumers analyze and compare nutrient status, fiber, fat, and calorie content labeled on foods [30–32, 37, 39].

In our research, most respondents read labeled composition data (Q2), but women demonstrated a significantly higher interest in it ($Z = 3.2759$; $p < 0.05$). An opposite relationship with significant difference ($Z = 2.5275$; $p < 0.05$) was observed between male and female affirmative answers (Table 3): men were more likely to compare labeled data on similar food products (Q3) before making the final choice. However, all respondents demonstrated little interest in comparing labeled data, only 41.17% of all respondents included in the research. Each consumer had individual preferences for specific food products, depending on their approach and motivation. They processed the labelled information depending on their knowledge and awareness in the sphere of healthy foods.

More than a half of all consumers would buy functional foods with a lower sensory quality (Q6), but women gave more affirmative responses than men, with a significant difference between them ($Z = 2.0700$; $p < 0.05$). About half of all respondents (50.35%) were interested in products fortified with nutritive fibers (Q5). Again, women expressed more interest than men, with a significant difference ($Z = 4.5297$; $p < 0.05$), which indicated their eagerness to meet the recommended higher intake of whole-grain and fiber-fortified foods. Studies that determine how consumers make decisions and what affects their food choice can yield solutions to some important food industry problems [2, 40]. Consumers are often prone to the so-called impulsive purchasing, which can be corrected by educating them in the matters of healthy nutrition and functional foods [41].

Differences in attitude between respondents grouped based on body mass index. Hypothesis H2 also tested the difference in attitude toward a healthy diet and functional foods. The Z-test ($p < 0.05$) involved crossing the answers of all respondents with normal and inadequate body mass index (Table 4). Hypothesis H2a tested the difference for all men with normal body mass index and inadequate body mass index (Table 5). Hypothesis H2b tested the difference for women with normal body mass index and inadequate body mass index (Table 6).

Table 4 illustrates attitudes toward specific information related to healthy foods expressed with affirmative answers. It revealed no statistically significant difference ($Z = 0.0923$; $p > 0.05$) between respondents with normal body mass index and inadequate body mass index. However, not enough evidence was obtained to reject hypothesis H2. Another analysis revealed no statistically significant difference ($Z = 0.3855$; $p > 0.05$) between affirmative responses given by all consumers and those with normal body mass index (Table 4) or inadequate body mass index ($Z = 0.8596$; $p > 0.05$).

Table 4 Affirmative responses for all respondents (n = 720), respondents with normal body mass index ($n_N = 572$), and respondents with inadequate body mass index ($n_I = 148$)

Question (Q_x)	*Yes*-responses for all respondents (n = 720)		*Yes*-responses for normal body mass index ($n_N = 572$)		*Yes*-responses for inadequate body mass index ($n_I = 148$)	
	n_{Qx} [a]	% [b]	n_{NQx} [a]	% [b]	n_{IQx} [a]	% [b]
Q1	618	85.83	490	85.66	128	86.49
Q2	522	72.50	419	73.25	103	69.59
Q3	297	41.25	238	41.61	59	39.86
Q4	589	81.81	473	82.69	116	78.38
Q5	362	50.28	285	49.83	77	52.03
Q6	445	61.81	360	62.94	85	57.43
Q7	689	95.83	548	95.80	142	95.95
Q8	138	19.17	108	18.88	30	20.27
[c]$Sum_{1–7}$	3522	69.88	2813	55.81	710	14.08

[a] Number of affirmative responses (n_{Qx}) to each question (x = 1–8)

[b] Percentage of affirmative responses to each question (x = 1–8) calculated based on the possible number of affirmative responses

[c] Percentage of affirmative responses to all questions (x = 1–7) calculated based on 5040 *Yes*-answers for all 720 respondents

Source: calculated by the authors based on research data

Table 5 Affirmative responses for all men (n_M = 290), men with normal body mass index (n_{NM} = 217), and men with inadequate body mass index (n_{IM} = 73)

Question (Q_x)	*Yes*-responses for all male respondents (n_M = 290)		*Yes*-responses for men with normal body mass index (n_{NM} = 217)		*Yes*-responses for men with inadequate body mass index (n_{IM} = 73)	
	n_{Qx} [a]	% [b]	n_{NQx} [a]	% [b]	n_{IQx} [a]	% [b]
Q1	251	86.55	190	87.56	61	83.56
Q2	191	65.86	142	65.44	49	67.12
Q3	136	46.90	104	47.93	32	43.84
Q4	229	78.97	173	79.72	56	76.71
Q5	116	40.00	86	39.63	30	41.10
Q6	166	57.24	122	56.22	44	60.27
Q7	273	94.14	203	93.55	70	95.89
Q8	56	19.31	43	19.82	13	17.81
[c]$Sum_{1–7}$	1362	67.09	1020	50.25	342	16.85

[a] Number of affirmative responses (nQx) to each question (x = 1–8)

[b] % of affirmative responses to each question (x = 1–8) calculated based on the possible number of affirmative responses

[c] of affirmative responses to all questions (x = 1–7) calculated based on 2030 Yes-responses for 290 male respondents

Source: calculated by the authors based on research data

Table 6 Affirmative responses for all women (n_F = 430), women with normal body mass index (n_{NF} = 355), and women with inadequate body mass index (n_{IF} = 75)

Question (Q_x)	*Yes*-responses for all female respondents (n_F = 430)		*Yes*-responses for women with normal body mass index (n_{NF} = 355)		*Yes*-responses for women with inadequate body mass index (n_{IF} = 75)	
	n_{Qx} [a]	% [b]	n_{NQx} [a]	% [b]	n_{IQx} [a]	% [b]
Q1	367	85.35	300	84.51	67	89.33
Q2	331	76.98	277	78.03	54	72.00
Q3	161	37.44	134	37.75	27	36.00
Q4	360	83.72	300	84.51	60	80.00
Q5	246	57.21	199	56.06	47	62.67
Q6	279	64.88	238	67.04	41	54.67
Q7	417	96.98	345	97.18	72	96.00
Q8	82	19.07	65	18.31	17	22.67
[c]$Sum_{1–7}$	2161	71.80	1793	59.57	368	12.22

[a] Number of affirmative responses (n_{Qx}) to each question (x = 1–8)

[b] % of affirmative (*Yes)* responses to each question (x = 1–8) calculated based on the possible number of affirmative responses

[c] % of affirmative (*Yes)* responses to all questions (x = 1–7) calculated based on 3010 *Yes*-responses for 430 female respondents

Source: calculated by the authors based on research data

Two additional hypotheses were tested by crossing the answers divided according to body mass index on order to analyze the difference between men (hypothesis H2a) (Table 5) and women (hypothesis H2b) (Table 6). However, the analysis revealed no significant difference ($Z = 0.0923$; $p > 0.05$) between men with normal body mass index and inadequate body mass index in attitude related to healthy foods. Similarly, no difference was recorded between women with normal body mass index and women with inadequate body mass index ($Z = 0.9520$; $p > 0.05$). Not enough evidence made in impossible to reject hypotheses H2a and H2b.

Identifying factors that affect attitudes towards healthy diets and functional foods. The relationship between respondents' interest in healthy diet (Q1), their health-related special diet needs (Q8), and some variables (Q2–7) made it possible to identify factors that affected their attitudes. Correlation was chosen as a method that can reveal the relationship between two variables. Pearson's correlation coefficient (*r*) was used to measure the strength of the association between two variables. Differences in correlation intensity or existence were analyzed for all consumers and for each group based on sex and body mass index for all respondents and for men and women grouped according to body mass index.

This analysis tested main hypotheses H3–5 for each group of respondents (all, men, women). It indicated Hxa-f for all respondents and BHxa-f for men and women grouped according to body mass index, where x stood for the ordinal number of hypotheses, and letters a-f indicated dependent variables.

Attitudes of consumers grouped based on sex. Hypothesis H3–5 was tested to identify factors that affected consumer attitudes related to healthy diet and functional foods and the significance of their relationships. Pearson's correlation coefficient (*r*) was applied to the data selected as an independent variable for further research (Q1 and Q8). It was analyzed by crossing consumer interest in healthy diet (Q1) with individual health-related diet needs (Q8) for all respondents (n = 720) and those grouped based on sex (Table 7). Individual health-related diet needs (Q8) were selected for the second independent variable in the correlation analysis, because a relatively larger part of respondents (Table 1) confirmed it.

We examined the relationship between responses regarding consumer interest in a healthy diet (Q1) as an independent variable and health-related diet needs (Q8). The analysis revealed a significant moderate positive correlation ($p < 0.05$) for all respondents and a very high monotonic correlation ($p < 0.01$) for men and women (Table 7). As a result, we rejected hypothesis H3 that regarded health-related diet needs as a factor that affects consumer attitude to a healthy diet. The positive correlation coefficient (*r*) revealed a significant linear relationship in the basic set and that both variables increased or decreased together.

Consumers who prefer a healthy diet usually look for food components with nutritional benefits, e.g., fortified with vitamins, minerals, fibers, and bioactive compounds. They know that the total nutritional value of a common diet may be improved by functional foods where some ingredients are partially or completely replaced with those recommended [17, 32, 35, 36].

Health-focused products have a positive effect on purchase decisions. A better product quality gives better results. This analysis tested the correlation coefficient for the relationship between an independent variable and a number of dependent variables. The independent variable was expressed in the question about consumers' interest in a healthy diet (Q1). The list of dependent variables included the questions about their interest in labeled food composition (Q2), the way they used labels in their food choice (Q3), their intention to purchase products fortified with vitamins, minerals (Q4), and dietary fibers (Q5) or nutrient-fortified products with a lower sensory quality (Q6), as well as their nutrition and health risk awareness (Q7). The analysis indicated a very high significant monotonic correlation ($p < 0.01$) for all respondents, as well as for men and women (Table 7). Therefore, hypotheses H4a-f could be rejected. The dependent variables proved to be factors that affect consumer attitudes to healthy diet. The obtained results confirmed a significant relationship between the examined indicators of consumer attitudes related to the healthy diet and functional foods.

Novel foods often fail on the market because producers skip some important activities or make decisions without appropriate research [40, 42, 43]. Numerous studies featured food product development and fortification [6, 12, 20, 44]. They confirmed the importance of integrating food science and technology with marketing in consumer-oriented healthy food development. Wonderlich-Tierney *et al.* analyzed food advertising as an influencing factor and proved that it did not increase food consumption among college-aged women and men [45]. However, the effect depended on personal preferences, and women were more susceptible to food advertising than men.

We used the question about health-related diet needs (Q8) as an independent variable to test its relationship with dependent variables defined in other questions: interest in labeled composition (Q2), the way participants used labels in their food choice (Q3), and their nutrition and health risk awareness (Q7). The analysis revealed a significant moderate positive correlation ($p < 0.05$) between them for all respondents. Therefore, health-related diet needs proved to be a factor that affect consumer preferences in healthy diet and functional foods.

Another test studied the correlation between responses regarding consumers' health-related diet needs (Q8) taken as an independent variable with

Table 7 Correlation test (Pearson's correlation coefficient *r*) and significance of relationship between variables: number of affirmative responses to each question (Q_x) for all respondents (n = 720) and respondents grouped based on sex (men $n_M = 290$, women $n_F = 430$)

Dependent variable Question (Q_x)	Independent variable (Question Q_x) and correlation coefficient (*r*)					
	Q1	Q8	Q_M1	Q_M8	Q_F1	Q_F8
Q1	1	0.6925*	1	0.8556**	1	0.8195**
Q2	0.9713**	0.5881*	0.9749**	0.7666**	0.9983**	0.8137**
Q3	0.8760**	0.6764*	0.9559**	0.8376**	0.9593**	0.8093**
Q4	0.9863**	0.7377**	0.9964**	0.8647**	0.9898**	0.8584**
Q5	0.8730**	0.4546	0.9701**	0.8130**	0.9530**	0.7093**
Q6	0.9378**	0.5134	0.9785**	0.7869**	0.9848**	0.8120**
Q7	0.9922**	0.6934*	0.9973**	0.8524**	0.9959**	0.8549**
Q8	0.6925*	1	0.8556**	1	0.8195**	1

Statistically significant correlation: moderate *$p < 0.05$, very high monotonic correlation **$p < 0.01$, perfect direct monotonic correlation = 1

Source: calculated by the authors based on research data

a number of dependent variables. The dependent variables were expressed in questions about their interest in purchasing products fortified with vitamins and minerals (Q4) and labeled composition (Q2), the way they used labels in their food choice (Q3), their preferences for foods fortified with dietary fibers (Q5) and functional foods with a lower sensory quality (Q6), and their nutrition and health risk awareness (Q7). The analysis revealed a very high significant monotonic correlation ($p < 0.01$) for all respondents, as well as for those grouped according to sex (Table 7). These findings indicated that hypotheses H5a-f could be rejected due to a significant correlation ($p < 0.05$). Therefore, health-related diet needs proved to be a factor that affect consumer attitudes to healthy foods.

However, we revealed no correlation ($p > 0.05$) between responses of all consumers regarding health-related diet needs (Q8) and their interest in purchasing products fortified with dietary fibers (Q5) or functional foods a with lower sensory quality (Q6) (Table 7). Therefore, these factors had no effect on their food choice. Other studies confirmed that food quality parameters, nutrition awareness, and health motivation affected purchase behavior. Collecting, analyzing, translating, and providing nutrition information based on scientific evidence remains an important professional challenge for food producers [9, 33, 36]. Similar marketing studies about new food products, purchasing intention, preferences, and perception can provide information on the efforts that consumers make to improve their personal nutritional status [21].

Attitudes of consumers grouped based on body mass index. The growing concerns that consumers express about health-related nutrition are focused on the composition and quality of foods that they expect to find on the market. This situation provides an opportunity for contemporary food R&D in the area of new functional foods [6, 35]. Table 8 illustrates the correlation coefficient and significance of relationship between consumer interest in indicators of healthy diet and functional foods. The analysis tested hypothesis BH3–5 for all respondents (n = 720) with normal body mass index ($n_N = 572$) and inadequate body mass index ($n_I = 148$); men with normal body mass index ($n_{NM} = 217$) and inadequate body mass index ($n_{IM} = 73$); women with normal body mass index ($n_{NF} = 355$) and inadequate body mass index ($n_{IF} = 75$). The analysis also identified differences in correlation intensity or its existence.

One's diet depends on the basic knowledge on nutrition and individual health condition: a certain type of food or nutrient can be recommended in larger/smaller quantities or totally forbidden. Hypothesis BH3 featured health problems and their effect on purchase decisions. The analysis revealed no relationship between the independent variable of the interest in healthy diet (Q1) and individual health-related diet needs (Q8) for respondents grouped based on body mass index. The correlation was significant moderate positive ($p < 0.05$) only for all respondents and for men with normal body mass index (Table 8). Individual health-related diet needs tested as an dependent variable proved to be a factor that affected the attitudes to healthy diet for all respondents and men with normal body mass index. Therefore, hypotheses BH3 could be rejected. However, no significant correlation ($p > 0.05$) was revealed between women with normal body mass index and inadequate body mass index (Table 8), and enough evidence was obtained to reject hypothesis BH3.

We performed a correlation analysis for relationship between the independent variable of interest in healthy diet (Q1) and the number of dependent variables for consumers grouped according to body mass index. The list of dependent variables included their interest in labeled ingredients (Q2), the way they used labels in their food choice (Q3), preferences for products fortified with vitamins and minerals (Q4), dietary fibers (Q5), and functional foods with a lower sensory quality (Q6), and their nutrition and health risk awareness (Q7).

Table 8 Correlation test (Pearson's correlation coefficient *r*) and significance of relationship between variables: number of affirmative responses to each question (Q_x) for all respondents (normal body mass index $n_N = 572$, inadequate body mass index $n_I = 148$); men (normal body mass index $n_{NM} = 217$, inadequate body mass index $n_{IM} = 73$); women (normal body mass index $n_{NF} = 355$, inadequate body mass index $n_{IF} = 75$)

Dependent variable Question (Q_x)	Independent variable (Question Q_x) and correlation coefficient (*r*)							
	Q_N1	$Q_{NM}1$	$Q_{NF}1$	Q_N8	$Q_{NM}8$	$Q_{NF}8$	Q_I1	Q_I8
Q1	1	1	1	0.6538*	0.8789*	0.1429	1	0.3003
Q2	0.9637**	0.9570**	0.9996**	0.5266	0.7528	0.1506	0.1989	0.1592
Q3	0.8771**	0.9238**	0.8391*	0.6653*	0.9363**	0.2101	–0.4952	–0.7477
Q4	0.9806**	0.9987**	0.9616**	0.6870*	0.8010*	0.3111	0.1399	0.2978
Q5	0.8887**	0.9590**	0.8398*	0.4338	0.7997	–0.2026	0.7897	0.7000
Q6	0.9554**	0.9737**	0.9640**	0.5429	0.7775	0.1076	0.6714	–0.105
Q7	0.9893**	0.9952**	0.9836**	0.6652*	0.8659*	0.2955	0.2311	0.5936
Q8	0.6538*	0.8789*	0.1429	1	1	1	0.3003	1

Statistically significant correlation: moderate *$p < 0.05$, very high monotonic correlation **$p < 0.01$, perfect direct monotonic correlation = 1

Source: calculated by the authors based on research data

The analysis revealed statistically significant and very high monotonic correlation ($p < 0.01$) for all respondents, men with normal body mass index, and women with normal body mass index. The results were slightly different, with a significant moderate positive correlation ($p < 0.05$), for responses on label use in food choice (Q3) and purchasing foods fortified with dietary fibers (Q5). Based on Table 8, additional hypotheses BH4a-f could be rejected: in this case, the dependent variables really affect consumer attitudes to healthy diet. In similar studies, most respondents claimed to examine the labels when making the purchase decision and evaluate the food based on the labelled nutritional composition in order to avoid food-related diseases [4, 11, 12, 22, 39].

Our study also indicated that the simple correlation coefficient (r) was not statistically significant ($p > 0.05$) for the same relationships between separately analyzed responses for all respondents with inadequate body mass index (Table 8). We detected no linear relationship and enough evidence to reject additional hypotheses BH4a–f. No further correlation analyses were necessary to test the relationship between responses grouped based on consumers body mass index.

We tested the relationship between responses grouped based on body mass index for health-related diet needs (Q8), which served as an independent variable, and labeled data comparing between similar products in food choice (Q3). The analysis revealed a very high significant correlation ($p < 0.01$) for men with normal body mass index, while this correlation was significant moderate positive ($p < 0.05$) for all respondents with normal body mass index. A statistically significant moderate positive correlation ($p < 0.05$) was detected between health-related diet needs (Q8), preferences for functional products fortified with vitamins and minerals (Q4), and nutrition and health risk awareness (Q7). Therefore, health-related diet needs proved to affect consumer attitudes to functional foods for all respondents and men with normal body mass index. As a result, additional hypotheses H5b,c,e were rejected.

We also revealed no correlation ($p > 0.05$) between health-related diet needs (Q8) and the interest in labeled ingredients (Q2), foods fortified with dietary fibers (Q5), or functional foods with a lower sensory quality (Q6) for all respondents and men with normal body mass index. No correlation ($p > 0.05$) was registered between the independent variable of health-related diet needs (Q8) and all dependent variables for women with normal body mass index and all respondents with inadequate body mass index (Table 8). The research revealed differences in respondents' attitudes to a healthy diet and functional foods and their positive relation to healthy food habits. Food producers should do more to raise consumer preferences for healthy products and design functional foods with appropriate sensory quality [4, 10, 21, 36, 46]. Health-aware consumers prefer recommended functional foods, but their taste and smell are still important and may result in their rejecting products with a poor sensory quality.

CONCLUSION

Consumers have individual criteria and different habits that affect their behavior in food choice. However, inadequate diet and lifestyle may trigger some noncommunicable diseases. Basic nutrition and health risk awareness is important for health. We analyzed and identified factors that affect consumer preferences for healthy diet and functional foods. We grouped affirmative responses as follows: all participants; participants grouped based on sex (men and women); participants grouped based on body mass index (normal body mass index and inadequate body mass index); participants grouped based on body mass index and sex (men with normal body mass index, men with inadequate body mass index; women with normal body mass index, women with inadequate body mass index).

We identified differences in consumer preferences between various groups. The obtained data may be useful for food producers to synchronize the quality of their new functional products with the actual situation on the market. New data on factors that affect consumer preferences in a healthy diet and functional foods may help develop novel fortified foods.

This research tested a new scientific approach to food preference analysis and purchase behavior on the target market. This approach is a tool for identifying nutritive and sensory quality parameters of healthy food products that are of interest for particular target consumers. Healthy foods developed for target consumers should be based on scientific evidence integrated with recommendations on healthy nutrition. New healthy food products require appropriate technological, nutritive, and sensory quality, which depends on ingredients, formulation, and processing methods. The presented methodology of testing and processing can be applied to other marketing studies aimed at different groups of consumers and with different objectives.

CONTRIBUTION

S. Grujić developed the research concept, designed the article, and wrote the manuscript. S. Grujić and M. Grujčić reviewed scientific literature, collected and analyzed the data, read and approved the final version of 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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Effects of flaxseed on the nutritional and sensory qualities of pan and Arabic flat breads

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Abstract:
Flaxseed is a useful source of omega-3 fatty acid and many health-promoting phytochemicals. Baked products are extensively consumed in the Arabic countries. This study mainly aimed to improve the nutritional quality of pan and Arabic flat breads by adding whole and crushed flaxseed.

The study objects were pan and Arabic flat breads wholegrain and white wheat flour with whole or crushed flaxseed at different amounts. The proximate composition, texture, color characteristics, and sensory analysis were evaluated by standard methods.

The fat content increased in both the white wheat flour and in the wholegrain wheat flour pan breads with 8% of crushed flaxseed. The addition of 8% of crushed flaxseed to the white wheat flour bread also increased its specific loaf volume, while the addition of 8% of whole flaxseed significantly decreased the specific loaf volume of the wholegrain wheat flour bread. Crushed flaxseed did not affect the L^* values, but significantly increased the a^* values in the white wheat flour pan bread. Supplementing the white wheat flour pan bread with 8% of whole flaxseed decreased the compression force, thus indicating a softer texture. 8% of whole flaxseed significantly increased fat and protein contents of the white wheat and wholegrain wheat flour pan breads. Similar trends were observed for the Arabic breads, thus proving the enhancing effect of flaxseed on the nutritional quality of these baked products. The addition of crushed flaxseed to white wheat flour Arabic bread adversely affected its appearance and crust color, as well as significantly increased its ash, fat, protein, and dietary fiber contents. However, it did not have any adverse effect on the texture, flexibility, and flavor.

Flaxseed grains in the amount of 8% can be recommended to produce baked products with good sensory and nutritional qualities.

Keywords: Crushed and whole flaxseed, dietary fiber, Arabic bread, pan bread, nutritional quality

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INTRODUCTION

Eating right has been recommended to attain a relatively disease-free life. Nowadays, more and more consumers are interested in eating healthy foods not only for good taste or nutrition, but also to prevent noncommunicable diseases, such as type 2 diabetes, heart diseases, and several types of cancer [1–4]. Current nutrition research continues to identify various bioactive compounds in foods that appear to function as protectors against such diseases. In particular, consumers are advised to increase their intake of fiber-rich whole grains, plant fibers (psyllium), and flaxseed [5–7].

The flaxseed grain is a tiny, smooth, flat seed. Its color ranges from light red to reddish brown. Flaxseed has been consumed by people in many Asian countries for about 5000 years [8]. It has been shown to be a useful and powerful agent against fatty degeneration in cardiovascular diseases, cancer, and type 2 diabetes [4]. In addition to nutrition, flaxseed has a few technological benefits in producing superior quality processed food products [9, 10]. Oil and protein are flaxseed's

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major components. Flaxseed meal (after crushing) also contains non-starch polysaccharides (mucilage), cyanogenic glycosides (precursor of hydrocyanic acid and thiocyanate), phytic acid, phenolics, mammalian trypsin and chymotrypsin inhibitors, linatin (vitamin B_6 inhibitors), and lignans (diglucosides) with anti-neoplastic and anti-estrogenic properties [11].

About two-thirds of flaxseed fiber is water-insoluble and consists of non-starch polysaccharides, such as cellulose and lignin [12]. Insoluble fiber helps improve laxation and prevent constipation, mainly by increasing fecal bulk and reducing bowel transit time [13]. A water-soluble fiber fraction of flaxseed comprises about one-third of total dietary fiber. There has been research into the heterogeneous nature of a purified water-soluble neutral fraction coming from the mucilage gum of flaxseed. Water-soluble fiber helps maintain blood glucose levels and lowers blood cholesterol levels [4, 12]. High-fiber foods are also reported to offer protection against certain types of cancer [14]. A high-fiber diet helps lower blood levels of lipids and certain hormones [7]. A low-fiber, high-fat diet tends to raise blood estrogen levels, which may contribute to cancer development by stimulating tumor cell growth [15, 16].

Food processors have always attempted to incorporate newer healthy ingredients to produce a variety of health-promoting functional foods. Flaxseed is one of such promising functional ingredients that can be used in baked goods and other cereal-based products. There is a strong rationale for introducing this ingredient into our staple foods, such as Arabic flat and pan breads, to provide a safety net for those at a higher risk of noncommunicable diseases. As humans now live longer, they can benefit from consuming grains (such as flaxseed) rich in health-promoting bioactive compounds. Therefore, we aimed to study the benefits of flaxseed for enhancing the nutritional quality of Arabic flat and pan breads, which are now commonly consumed all over the world.

STUDY OBJECTS AND METHODS

Raw materials. Wholegrain wheat flour and white wheat flour samples were obtained from the Kuwait Flour Mills & Bakeries Co., Shuwaikh. Fine granulated sugar, common salt, bakery shortening (Wesson, USA), whole flaxseed, instant dry yeast, non-fat dry milk, and baking supplies were procured from the local market. The diacetyl tartaric acid ester of mono- and diglycerides was a free gift from the American Ingredients Company (Kansas City, Missouri, USA).

Optimization of pan bread formulation. The optimized straight dough bread making method (AACC method 10-10-B) was used for baking trials with a few modifications as reported earlier [6]. Every bake was repeated 3 to 4 times, with and without whole and crushed flaxseed, and the averages of two best bakes were tabulated. Wholegrain and white wheat flours were replaced with 2, 4, 6, and 8% of flaxseed for producing pan breads. The amounts of flour and flaxseed were based on the 14% moisture. After preliminary trials, the use of 4 and 8% of flaxseed with 0.5% of diacetyl tartaric acid ester of mono- and diglycerides was optimized for these bread formulations. Water absorption was optimized by adding as much water as possible while keeping the dough still manageable to handle and the water required was calculated as baking absorption percentage. The bread samples, immediately taken out of the oven, were weighed, and loaf volume was measured by the rapeseed displacement method as reported earlier [6].

Optimization of Arabic bread formulation. For Arabic bread making, wholegrain and white wheat flour were used as controls, with varying levels of flaxseed (whole or crushed). Arabic breads were made according to the method reported earlier [17]. The wholegrain and white wheat flour were replaced with 2, 4, 6, and 8% of flaxseed. The amounts of flour and flaxseed for these trials were based on the 14% moisture. After preliminary trials, the use of 4 and 8% of flaxseed with 0.5% of diacetyl tartaric acid ester of mono- and diglycerides was optimized, and the prepared Arabic bread samples were used for further analyses.

Chemical analyses. The pan and Arabic bread samples were freeze-dried and powdered in a Falling Number Mill (Model 3100, Sweden) to pass through a 100-mesh sieve. They were stored in airtight containers in a refrigerator (4°C) until further chemical analysis.

All the bread samples were analyzed for proximate composition, such as moisture (Method 44-19), crude protein (Method 46-12), total ash (Method 08-01), and crude lipids (Method 30-25) by using the standard AACC methods as reported earlier [6]. The results were expressed on a moisture-free basis. The nitrogen content determined by the Kjedalhl method was converted into a crude protein content using a conversion factor of N×5.70 for bread samples. All the analyses were conducted in triplicate, with the averages presented here. The pan and Arabic bread samples were also analyzed for insoluble, soluble, and total dietary fiber contents according to the standard AACC methods as reported earlier [17].

Instrumental texture measurement. After baking, the pan bread samples were cooled for about 2 h to reach the room temperature of 22 ± 1°C. From the middle portion of each bread sample, four slices of uniform thickness (2.5 cm) were obtained and immediately taken for objective texture measurement. The texture was measured using a Stable Micro Systems TAXT2 texture analyzer (UK) with a plastic plunger (2.5 cm diameter, 1.7 mm/s speed, peak compression force at 30% compression, 7.5 mm compression depth). The peak compression force was measured in the center of all the four slices, and the mean ± S.D. values were reported [18].

Objective color measurements. The crumb color of the pan bread samples cooled to room temperature (22 ± 1°C) was measured with a Model 545 Macbeth Color Checker, a potable spectrophotometer (Kollmorgen Instruments Corp., UK) as CIE *L* a* b** values. Under

this tristimulus color coordinate system, the *L** value (lightness) varies from 0 (black) to 100 (white), the *a** value (redness) varies from –100 (green) to +100 (red), and the *b** value (yellowness) varies from –100 (blue) to +100 (yellow). As the values of *a** and *b** rise, the color becomes more saturated or chromatic, but these values approach zero for neutral colors (white, grey, or black). The instrument settings were illuminant D_{50}, display *L** *a** *b**, and the observer angle of 2°. The instrument was calibrated with a white primary tile supplied by the manufacturer. After slicing the bread loaf into two halves, twelve readings were taken in the crumb area of both halves [19]. After discarding two most extreme readings, the remaining ten readings were averaged and reported along with their standard deviations.

Sensory analysis. The baked pan bread samples were cooled to room temperature (22 ± 1°C) and subjected to sensory analysis on the nine-point hedonic scale for crumb color, texture, flavor, and overall acceptability. A semi-trained panel consisted of 12 judges from the employees of the Kuwait Institute for Scientific Research, who gave their written informed consent [17]. Each panelist was served with a control sample along with the test samples and was asked to assign scores. A sensory score of 5 or above was rated as acceptable, and a score below 5 was considered unacceptable. The data were subjected to analysis of variance, and the average values were reported.

Statistical analysis. All of the chemical analyses were reported on a moisture-free basis. The data were subjected to analysis of variance, and the mean values were evaluated for statistical significance (p = 0.05) using the Duncan's New Multiple Range Test (SAS Program, Windows Version 6.08, ANOVA Procedure), with inferences reported at the appropriate places [6].

RESULTS AND DISCUSSION

Chemical analyses. As evident from the data in Table 1, the chemical composition of pan bread was affected by the addition of whole flaxseed to the control with white wheat flour. With 8% of flaxseed added to the white wheat flour control bread, the fat and protein contents increased from 2.72 to 6.01%, and from 13.32 to 14.11%, respectively. Similar trends in fat and protein contents were also observed with the addition of crushed flaxseed to the white wheat flour sample. With 8% of whole flaxseed added to the wholegrain wheat flour control, the fat content increased from 3.67 to 6.32% in the test samples (Table 2). A similar trend in the fat content was observed in the wholegrain wheat flour pan bread with 8% of crushed flaxseed. However, the protein content of the wholegrain wheat flour sample did not show any significant differences with the addition of 8% of crushed or whole flaxseed. Also, the ash content was not affected significantly in either the white or the wholegrain wheat flour pan breads with 8% of crushed or whole flaxseed.

Specific loaf volume and instrumental texture. Pan bread is valued not only for its loaf volume, flavor, or nutritional quality but also for its desirable physical texture. Bakers have always attempted to improve the specific loaf volume, physical texture, and shelf life of pan bread using various additives, such as dough improvers or bread softeners [18]. The pan bread loaf volume as affected by the addition of crushed or whole flaxseed was measured in terms of cc/g (Table 3). The addition of 8% of crushed or whole flaxseed to the white wheat flour control pan bread significantly increased the specific loaf volume from 4.11 to 4.51 cc/g and from 4.02 to 5.09 cc/g in the test samples, respectively. As for the wholegrain wheat flour control pan bread, crushed flaxseed did not significantly change the specific loaf

Table 1 Effect of flaxseed addition on the chemical composition* of pan bread from white wheat flour, % dry basis

Sample	Ash	Fat	Protein
White wheat flour (control)	$2.13^{a} \pm 0.02$	$2.72^{a} \pm 0.01$	$13.32^{a} \pm 0.34$
White wheat flour + 4% Crushed flaxseed	$2.45^{a} \pm 0.02$	$4.54^{b} \pm 0.01$	$13.85^{b} \pm 0.05$
White wheat flour + 8% Crushed flaxseed	$2.53^{a} \pm 0.05$	$5.63^{c} \pm 0.03$	$14.07^{b} \pm 0.05$
White wheat flour + 4% Whole flaxseed	$2.07^{a} \pm 0.08$	$4.18^{b} \pm 0.05$	$13.77^{a} \pm 0.18$
White wheat flour + 8% Whole flaxseed	$2.11^{a} \pm 0.08$	$6.04^{c} \pm 0.03$	$14.11^{b} \pm 0.08$

Values with different superscripts differ significantly in a column (p = 0.05)

*Mean ± S.D.

Table 2 Effect of flaxseed addition on the chemical composition* of pan bread from wholegrain wheat flour, % dry basis

Sample	Ash	Fat	Protein
Wholegrain wheat flour (control)	$2.84^{a} \pm 0.04$	$3.67^{a} \pm 0.01$	$12.96^{a} \pm 0.09$
Wholegrain wheat flour + 4% Crushed flaxseed	$2.85^{a} \pm 0.15$	$5.34^{b} \pm 0.01$	$13.06^{a} \pm 0.02$
Wholegrain wheat flour + 8% Crushed flaxseed	$3.01^{a} \pm 0.08$	$6.58^{c} \pm 0.02$	$13.03^{a} \pm 0.25$
Wholegrain wheat flour + 4% Whole flaxseed	$2.84^{a} \pm 0.09$	$4.92^{b} \pm 0.03$	$13.14^{a} \pm 0.06$
Wholegrain wheat flour + 8% Whole flaxseed	$2.76^{a} \pm 0.23$	$6.32^{c} \pm 0.01$	$13.27^{a} \pm 0.17$

Values with different superscripts differ significantly in a column (p = 0.05)

*Mean ± S.D.

Table 3 Effect of flaxseed addition on the specific loaf volume of pan bread

Sample	Specific loaf volume, cc/g*	
	Crushed flaxseed	Whole flaxseed
White wheat flour (control)	$4.11^{a} \pm 0.21$	$4.02^{a} \pm 0.18$
White wheat flour + 4% flaxseed	$4.36^{b} \pm 0.12$	$4.66^{b} \pm 0.22$
White wheat flour + 8% flaxseed	$4.51^{c} \pm 0.12$	$5.09^{c} \pm 0.16$
Wholegrain wheat flour (control)	$3.64^{d} \pm 0.11$	$3.83^{d} \pm 0.14$
Wholegrain wheat flour + 4% flaxseed	$3.49^{d} \pm 0.22$	$3.93^{d} \pm 0.04$
Wholegrain wheat flour + 8% flaxseed	$3.56^{d} \pm 0.11$	$3.59^{c} \pm 0.23$

Values with different superscripts for white or wholegrain wheat flour differ significantly in a column ($p = 0.05$)

*Mean ± S.D.

Table 4 Effect of whole or crushed flaxseed addition on the objective texture of pan bread

Sample	Compression force, g*	
	Crushed flaxseed	Whole flaxseed
White wheat flour (control)	$368.4^{a} \pm 47.9$	$246.4^{a} \pm 29.3$
White wheat flour + 4% flaxseed	$371.0^{a} \pm 48.7$	$200.5^{b} \pm 34.0$
White wheat flour + 8% flaxseed	$365.4^{a} \pm 34.6$	$197.7^{b} \pm 26.0$
Wholegrain wheat flour (control)	$366.3^{b} \pm 47.2$	$373.6^{d} \pm 45.2$
Wholegrain wheat flour + 4% flaxseed	$375.7^{b} \pm 56.1$	$412.9^{d} \pm 55.0$
Wholegrain wheat flour + 8% flaxseed	$306.8^{c} \pm 53.4$	$459.8^{c} \pm 46.0$

Values with different superscripts for white or wholegrain wheat flour differ significantly in a column ($p = 0.05$)

*Mean ± S.D.

Table 5 Objective color measurements** of pan breads with crushed flaxseed

Sample	*L**	*a**	*b**
White wheat flour (control)	$65.5^{a} \pm 1.7$	$-0.80^{a} \pm 0.30$	$13.5^{a} \pm 1.0$
White wheat flour + 4% crushed flaxseed	$62.6^{b} \pm 1.6$	$0.37^{b} \pm 0.20$	$13.1^{a} \pm 0.8$
White wheat flour + 8% crushed flaxseed	$59.9^{c} \pm 1.4$	$0.94^{c} \pm 0.40$	$13.0^{a} \pm 0.9$
Wholegrain wheat flour (control)	$60.5^{d} \pm 1.6$	$3.58^{d} \pm 0.50$	$21.9^{b} \pm 1.1$
Wholegrain wheat flour + 4% crushed flaxseed	$59.6^{e} \pm 1.8$	$4.53^{e} \pm 0.40$	$22.4^{b} \pm 0.8$
Wholegrain wheat flour + 8% crushed flaxseed	$56.2^{f} \pm 1.2$	$4.69^{e} \pm 0.30$	$21.2^{b} \pm 0.8$

Values with different superscripts for white or wholegrain wheat flour differ significantly in a column ($p = 0.05$)

**Mean ± S.D.

volume of the test samples, while whole flaxseed decreased the specific loaf volume of the wholegrain wheat flour bread.

The instrumental texture of the pan breads supplemented with crushed or whole flaxseed was measured as compression force values (Table 4). As can be seen, the texture was not significantly affected by the addition of crushed flaxseed to the white wheat flour bread samples. However, in case of the wholegrain wheat flour control pan bread (366.2 g), the sample with 8% of crushed flaxseed had a significantly softer texture (306.8 g). The texture of the control white wheat flour bread was harder (246.4 g) than that of the sample with 8% of whole flaxseed (197.7 g). Similar texture softening of the bread supplemented with soluble dietary fiber was reported earlier [18]. However, the wholegrain wheat flour pan bread made with 8% of whole flaxseed had a significantly harder texture (459.8 g) than the control wholegrain wheat flour pan bread (373.6 g).

Objective color measurements of pan bread crumb. The objective color of pan bread crumb affected by the addition of flaxseed was measured in terms of CIE *L** *a** *b** tristimulus values using the procedure reported earlier [19]. The results for crushed and whole flaxseed are presented in Tables 5 and 6, respectively. The white wheat flour and wholegrain wheat flour control pan breads made with 8% of crushed flaxseed had their lightness values decreasing from 65.5 to 59.9 and from 60.5 to 56.2, respectively. This indicated that the crumb color became significantly darker. Furthermore, it was darker in the wholegrain wheat flour bread than in the white wheat flour bread (Table 5). There were insignificant changes in the *b** values for both the white and wholegrain wheat flour breads with the addition of crushed flaxseed. However, there was a significant change in the *a** values for the control white wheat flour bread (–0.80) compared to the sample supplemented with 8% of crushed flaxseed (0.94).

Table 6 Objective color measurements** of pan breads with whole flaxseed

Sample	*L**	*a**	*b**
White wheat flour (control)	$66.3^{a} \pm 1.8$	$-0.86^{a} \pm 0.10$	$13.7^{a} \pm 1.1$
White wheat flour + 4% whole flaxseed	$64.2^{b} \pm 1.4$	$-0.84^{a} \pm 0.20$	$12.0^{b} \pm 0.6$
White wheat flour + 8% whole flaxseed	$63.4^{b} \pm 2.0$	$-0.71^{a} \pm 0.30$	$11.4^{c} \pm 0.8$
Wholegrain wheat flour (control)	$61.2^{d} \pm 1.4$	$3.90^{b} \pm 0.40$	$23.1^{d} \pm 1.1$
Wholegrain wheat flour + 4% whole flaxseed	$61.3^{d} \pm 1.2$	$3.70^{b} \pm 0.50$	$21.9^{e} \pm 1.1$
Wholegrain wheat flour + 8% whole flaxseed	$62.1^{c} \pm 2.1$	$3.80^{b} \pm 0.30$	$22.1^{e} \pm 0.8$

Values with different superscripts for white or wholegrain wheat flour differ significantly in a column ($p = 0.05$)

**Mean ± S.D.

Table 7 Sensory quality scores* for Arabic breads with crushed flaxseed

Sample Code	Appearance	Crust color	Texture	Flexibility	Flavor
White wheat flour (control)	$7.8^{a} \pm 0.9$	$7.8^{a} \pm 0.7$	$6.9^{a} \pm 1.1$	$7.5^{a} \pm 0.9$	$7.0^{a} \pm 1.2$
White wheat flour + 4% crushed flaxseed	$7.1^{b\text{-}a} \pm 0.9$	$7.0^{ab} \pm 1.0$	$6.9^{a} \pm 0.8$	$6.5^{a} \pm 1.0$	$6.8^{a} \pm 0.6$
White wheat flour + 8% crushed flaxseed	$6.6^{b} \pm 1.3$	$6.6^{b} \pm 0.9$	$7.1^{a} \pm 1.1$	$6.9^{a} \pm 1.2$	$7.0^{a} \pm 0.9$
Wholegrain wheat flour (control)	$7.8^{d} \pm 0.4$	$7.2^{c} \pm 0.6$	$7.3^{b} \pm 0.9$	$7.1^{b} \pm 0.5$	$7.0^{b} \pm 0.6$
Wholegrain wheat flour + 4% crushed flaxseed	$7.6^{dc} \pm 1.1$	$7.3^{c} \pm 1.2$	$7.1^{b} \pm 1.1$	$7.5^{b} \pm 0.8$	$7.1^{b} \pm 0.9$
Wholegrain wheat flour + 8% crushed flaxseed	$6.8^{c} \pm 1.1$	$6.6^{cd} \pm 1.1$	$7.1^{b} \pm 1.3$	$7.1^{b} \pm 1.2$	$7.0^{b} \pm 1.1$

Values with different superscripts for white or wholegrain wheat flour differ significantly in a column ($p = 0.05$)

**Mean ± S.D.

Table 8 Sensory quality scores* for Arabic breads with whole flaxseed

Sample Code	Appearance	Crust color	Texture	Flexibility	Flavor
White wheat flour (control)	$7.4^{a} \pm 0.9$	$6.8^{a} \pm 0.9$	$6.9^{a} \pm 0.9$	$7.2^{a} \pm 1.1$	$7.5^{a} \pm 0.9$
White wheat flour + 4% whole flaxseed	$7.4^{a} \pm 0.5$	$7.1^{a} \pm 0.7$	$7.4^{a} \pm 0.7$	$7.9^{a} \pm 0.7$	$7.6^{a} \pm 0.8$
White wheat flour + 8% whole flaxseed	$7.2^{a} \pm 0.8$	$6.9^{a} \pm 1.2$	$7.0^{a} \pm 1.1$	$7.0^{a} \pm 1.1$	$7.8^{a} \pm 1.0$
Wholegrain wheat flour (control)	$7.4^{b} \pm 1.1$	$7.2^{b} \pm 0.8$	$6.4^{b} \pm 0.8$	$6.2^{b} \pm 1.2$	$6.7^{c} \pm 0.9$
Wholegrain wheat flour + 4% whole flaxseed	$7.7^{b} \pm 0.7$	$7.6^{b} \pm 0.8$	$7.3^{c} \pm 0.9$	$7.3^{c} \pm 0.7$	$7.8^{d} \pm 0.6$
Wholegrain wheat flour + 8% whole flaxseed	$7.7^{b} \pm 0.7$	$7.9^{b} \pm 0.7$	$8.2^{d} \pm 0.4$	$8.1^{c} \pm 0.7$	$7.8^{d} \pm 0.6$

Values with different superscripts for white or wholegrain wheat flour differ significantly in a column ($p = 0.05$)

**Mean ± S.D.

This indicated that crushed flaxseed made the red color of the white wheat flour crumb significantly darker.

The effects of adding whole flaxseed to the white and wholegrain wheat flour breads on their crumb color are shown in Table 6. As can be seen, the *L** values for the white wheat flour control bread crumb (66.3) significantly decreased to 63.4 with the addition of whole flaxseed, which made it appear darker. On the other hand, the addition of whole flaxseed to the wholegrain wheat flour control bread significantly increased its crumb color *L** values from 61.2 to 62.1, making it appear lighter, probably due to the dilution effect on the wheat bran [1].

Sensory quality of pan and Arabic bread. All the Arabic bread samples made with crushed flaxseed were evaluated for various sensory attributes, such as appearance, crust color, texture, flexibility, and flavor on the 9-point hedonic scale using semi-trained panelists (Table 7). The panelists did not find any significant differences in texture, flexibility, and flavor between the white and wholegrain wheat flour Arabic control breads and those made with 8% of crushed flaxseed. However, the panelists gave slightly but significantly lower sensory scores for the appearance and crust color of the samples containing crushed flaxseed compared to the control. Interestingly, the panelists could not find any significant differences in appearance, crust color, texture, flexibility, and flavor between the white wheat flour control samples and those made with 8% of whole flaxseed (Table 8). Furthermore, they gave consistently higher sensory scores for all the attributes of the samples with whole flaxseed, compared to the control. Thus, if consumers make this Arabic bread part of their usual diet, they could increase their dietary fiber intake [20, 21].

All the white and wholegrain wheat flour pan breads made with crushed or whole flaxseed were evaluated for color, texture, flavor, and overall acceptability on the 9-point hedonic scale by semi-trained panelists (Tables 9 and 10).

Dietary fiber contents. Flaxseed is a useful source of dietary fiber that provides about 28 g of total dietary

fiber/100 g on a dry weight basis. About two-thirds of flaxseed dietary fiber is water-insoluble and the remaining one-third is water-soluble [11, 12]. The dietary fiber contents in the pan and Arabic breads made from white and wholegrain wheat flour with various amounts of crushed or whole flaxseed are presented in Figs. 1 to 9. We found a significant increase in dietary fiber with 8% of flaxseed in both the pan and Arabic bread samples (Figs. 1–9). The white wheat flour Arabic breads with crushed or whole flaxseed had higher insoluble dietary fiber contents compared to that of the control (Figs. 5 and 7).

The major components of flaxseed are oil and protein. Flaxseed meal (after crushing) also contains non-starch polysaccharides (mucilage), cyanogenic glycosides (precursor of hydrocyanic acid and thiocyanate), phytic acid, phenolics, mammalian trypsin and chymotrypsin inhibitors, linatin (vitamin B_6 inhibitors), and lignans (diglucosides) with anti-neoplastic and anti-estrogenic properties [22, 23]. Flaxseed is also rich in unsaturated fats that are useful in improving the nutritional quality of pan bread made with this ingredient [4].

Nutrition and health professionals recommend including more plant-based foods in our diets. Since flaxseed is mainly rich in superior quality fat and proteins, its incorporation into wheat flour can produce highly nutritious baked goods. Compared to our results, Daun and DeCrecq, who studied Canadian flaxseed, reported significantly higher contents of fat (41%), protein (20%), total dietary fiber (28%), moisture (7.7%), and total ash (3.4%) [24]. In another study of white wheat flour panbread supplemented with crushed flaxseed, 5%

Table 9 Sensory quality scores* for pan bread with added crushed flaxseed

Sample Code	Color	Texture	Flavor	Overall acceptability
White wheat flour (control)	$7.6^a \pm 0.7$	$7.4^a \pm 0.7$	$6.9^a \pm 0.7$	$7.1^a \pm 0.5$
White wheat flour + 4% crushed flaxseed	$7.2^a \pm 0.6$	$7.1^a \pm 0.8$	$7.3^a \pm 0.6$	$7.2^a \pm 0.6$
White wheat flour + 8% crushed flaxseed	$6.9^a \pm 0.7$	$7.1^a \pm 0.9$	$7.4^a \pm 0.6$	$7.2^a \pm 0.6$
Wholegrain wheat flour (control)	$7.2^b \pm 1.2$	$7.2^b \pm 0.6$	$7.2^b \pm 0.8$	$7.2^b \pm 0.7$
Wholegrain wheat flour + 4% crushed flaxseed	$6.8^b \pm 0.6$	$6.7^b \pm 0.8$	$6.7^b \pm 0.6$	$6.6^b \pm 0.8$
Wholegrain wheat flour + 8% crushed flaxseed	$6.6^b \pm 0.8$	$6.5^b \pm 0.7$	$6.6^b \pm 0.6$	$6.5^b \pm 0.8$

Values with different superscripts for white or wholegrain wheat flour differ significantly in a column ($p = 0.05$)

**Mean ± S.D.

Table 10 Sensory quality scores* for pan bread with added whole flaxseed

Sample Code	Color	Texture	Flavor	Overall acceptability
White wheat flour (control)	$7.1^a \pm 0.8$	$7.1^a \pm 1.2$	$6.4^a \pm 1.2$	$6.8^a \pm 1.0$
White wheat flour + 4% whole flaxseed	$7.2^a \pm 0.6$	$6.9^a \pm 1.3$	$7.4^a \pm 0.8$	$7.2^a \pm 0.7$
White wheat flour + 8% whole flaxseed	$7.1^a \pm 0.8$	$6.8^a \pm 1.7$	$7.2^a \pm 1.2$	$7.2^a \pm 0.7$
Wholegrain wheat flour (control)	$6.7^b \pm 1.3$	$6.6^b \pm 1.4$	$6.6^b \pm 1.6$	$6.9^b \pm 1.1$
Wholegrain wheat flour + 4% whole flaxseed	$6.7^b \pm 1.0$	$6.4^b \pm 1.1$	$7.0^b \pm 1.1$	$6.8^b \pm 1.1$
Wholegrain wheat flour + 8% whole flaxseed	$6.3^b \pm 1.3$	$6.7^b \pm 1.2$	$7.2^b \pm 0.9$	$6.6^b \pm 1.4$

Values with different superscripts for white or wholegrain wheat flour differ significantly in a column ($p = 0.05$)

**Mean ± S.D.

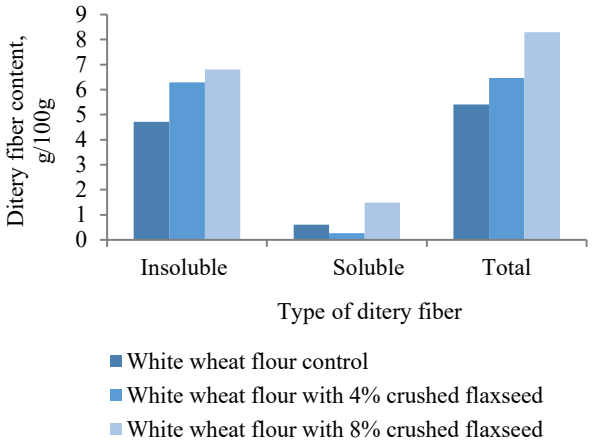

Figure 1 Dietary fiber in white wheat flour pan bread with crushed flaxseed

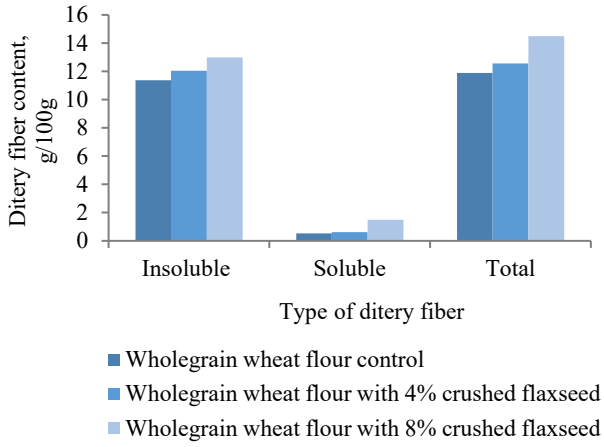

Figure 2 Dietary fiber in wholegrain wheat flour pan bread with crushed flaxseed

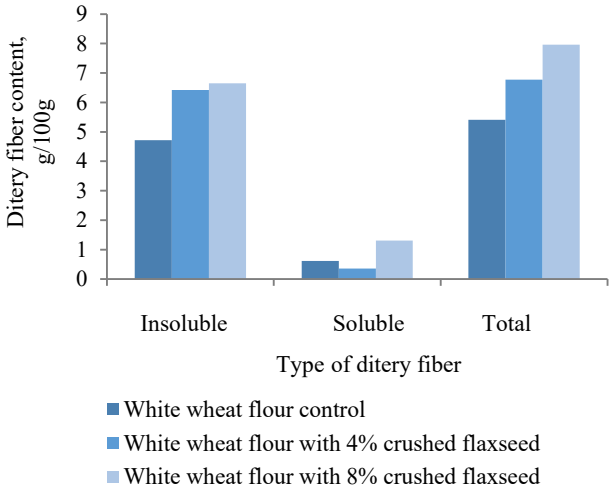

Figure 3 Dietary fiber in white wheat flour pan bread with whole flaxseed

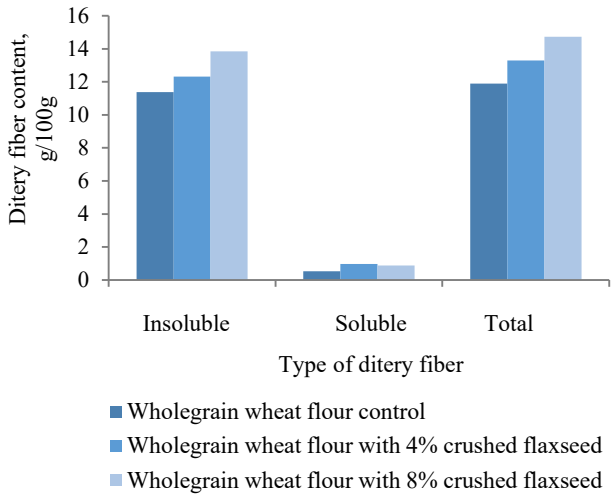

Figure 4 Dietary fiber in wholegrain wheat flour pan bread with whole flaxseed

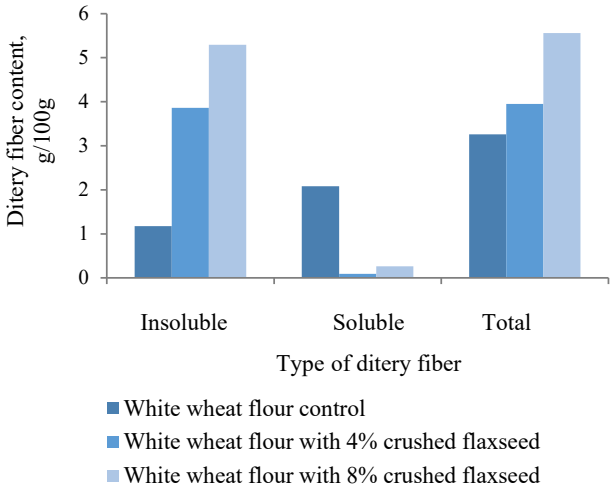

Figure 5 Dietary fiber in white wheat flour Arabic bread with crushed flaxseed

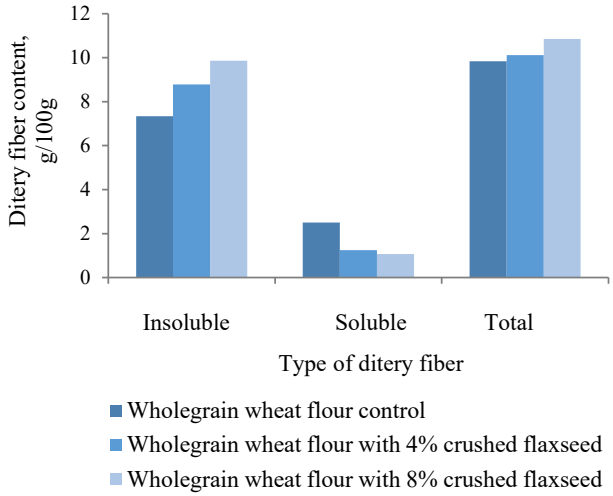

Figure 6 Dietary fiber in wholegrain wheat flour Arabic bread with crushed flaxseed

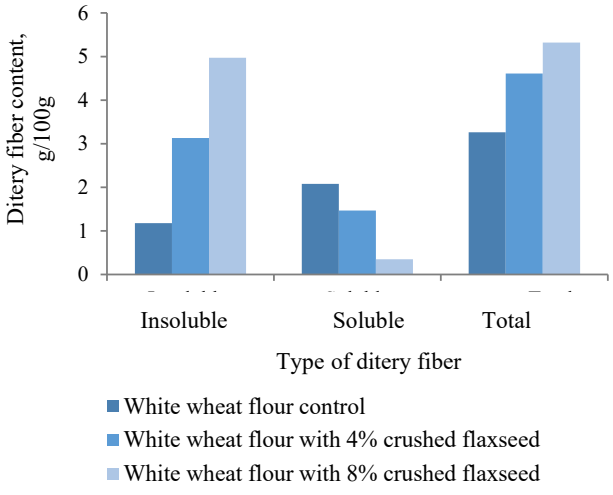

Figure 7 Dietary fiber in white wheat flour Arabic bread with whole flaxseed

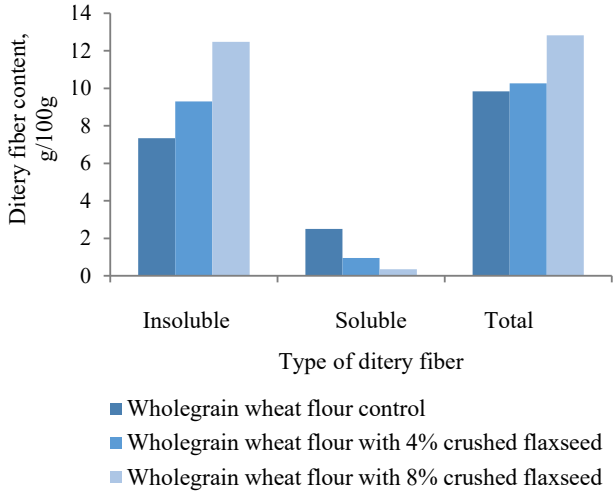

Figure 8 Dietary fiber in wholegrain wheat flour Arabic bread with whole flaxseed

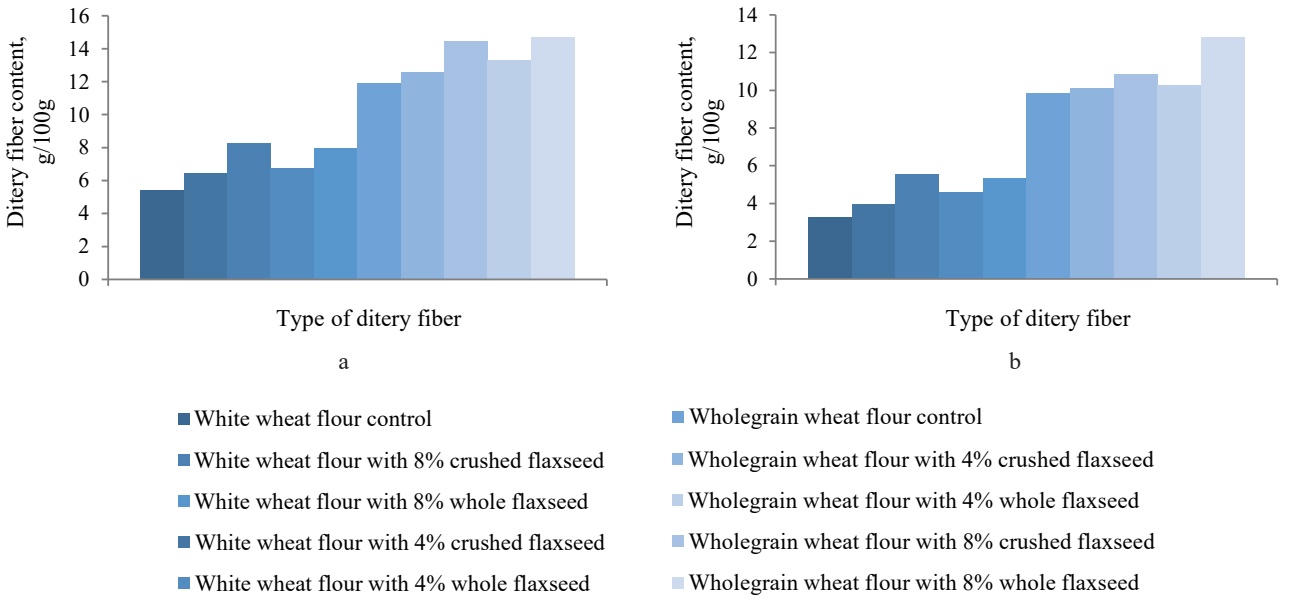

Figure 9 The total dietary fiber content in white and wholegrain wheat flour pan bread (a) and Arabic bread (b) with whole or crushed flaxseed

of flaxseed produced ash and fat contents similar to our results, but at 10% of flaxseed, the contents of ash, fat, and protein were significantly higher than in our study [25].

The addition of whole or crushed flaxseed decreased the specific loaf volume of pan bread, especially in the samples made from wholegrain wheat flour. This might be due to the dilution of gluten proteins, as well as the rupture of gas cell walls by whole flaxseeds [26, 27]. The change in specific loaf volume also significantly affected the instrumental texture of pan bread. The harder texture of the wholegrain wheat flour pan bread with 8% of flaxseed can be explained by the compact structure of this bread, mainly because of its lower specific loaf volume (Table 3). The addition of crushed or whole flaxseed (10%) has also been reported to reduce the specific loaf volume of control pan bread from 2.68 to 2.42 cc/g [25]. This decrease has been attributed to the dilution of gluten proteins and the interference of lignans and dietary fiber components in the development of gluten network [28].

The flaxseed grain coat, because of its natural yellowish brown color, was expected to affect the crumb color of pan bread. The wholegrain wheat flour control bread notably changed its crumb color with the addition of crushed flaxseed (8%), with the *a** value increasing from 3.58 to 4.69, indicative of a redder color. Interestingly, whole flaxseed did not produce significant differences in the *a** values of either wholegrain or white wheat flour pan breads. However, the *b** values of the white wheat flour bread significantly decreased from 13.7 for the control to 11.4 for the sample supplemented with whole flaxseed (8%), indicating a less chromatic or less saturated color. Similarly, the *b** values for the wholegrain wheat flour bread significantly decreased from 23.1 for the control to 22.1 for the test sample with whole flaxseed. This was possibly due to the dilution effect on the bran in wholegrain wheat flour or the yellowish brown color of the whole flaxseed cell coat. Similar effects on the objective color of white and wholegrain wheat breads were reported earlier by Marpalle *et al.* [25]. According to their study, the crumb color became darker as the level of flaxseed increased from 0 (control) to 5 and 10%, with the *a** values increasing (redder color) and the *b** values decreasing (less chromatic or less saturated color). The addition of ground flaxseed was also shown to significantly affect the crumb and crust color of bread, making it darker, possibly due to the Maillard reaction of proteins and phenolic compounds in flaxseed [29].

One of the most important signs of success for any new food product is its acceptance by the ultimate consumers. In our study, the panelists could not find any significant differences in the sensory attributes among the control pan bread and the samples containing 8% of crushed or whole flaxseed, rating all the samples as well-accepted (a score of 5 and above). Interestingly, the panelists could not find any significant differences in the appearance, crust color, flexibility, texture, or flavor between the control Arabic bread and the samples made with 8% of whole flaxseed, finding all of them acceptable.

In the study by Marpalle *et al.*, 10% of roasted crushed flaxseed was also reported to produce acceptable breads, both from white and wholegrain wheat flour [25]. Our results were also consistent with those reported by Ramcharitar *et al.*, who used 11.6% of crushed flaxseed in muffins [30]. Of all the Arabic bread samples, the highest insoluble fiber content was

found in the wholegrain wheat flour bread with 8% of whole flaxseed. Thus, supplementing white or wholegrain wheat flour breads with 8% of crushed or whole flaxseed significantly enhanced their total dietary fiber contents, which would definitely provide health benefits to the consumers.

In a recent study by Hussain *et al.*, 12% of full fat flaxseed and 16% of partially defatted flaxseed have been shown to enhance the dietary fiber content of pan bread and unleavened flat bread from 3.40 to 6.58% and from 12.64 to 17.45%, respectively [31]. The total dietary fiber contents in their study were much higher than in our work, possibly due to larger amounts of flaxseed used by them.

CONCLUSION

Flaxseed, a grain of immense nutritional value, has been utilized to produce pan bread as well as Arabic bread. Our results clearly indicate that staple baked products with good sensory and nutritional qualities can be produced using up to 8% of either crushed or whole flaxseed. Although the addition of crushed flaxseed had a slightly negative effect on the appearance and crust color of Arabic bread, it did not adversely affect its texture, flexibility, or flavor attributes. Interestingly, whole flaxseed is a better possibility since it did not adversely affect any of the sensory qualities, significantly enhancing the dietary fiber content in the bread. Thus, using flaxseed grains can be recommended to produce nutritionally superior baked products rich in dietary fiber for the benefit of the consumers.

CONTRIBUTION

All the authors contributed to this research. Ms. Jameela conceived the research idea, guided the research team, and conducted the objective color measurement. Dr. Fatima wrote the first draft of the manuscript, as well as prepared the tables and figures. Mr. Mohammad conducted the baking studies, texture measurement, and the chemical analyses. Ms. Amani conducted the statistical analyses of research data. Dr. Jiwan guided the research team and refined the last version of the manuscript. All the authors read and approved the final manuscript for submission to this journal.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest with regard to this research work.

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Molecular marker technologies in food plant genetic diversity studies: An overview

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Abstract:

Marker-assisted technologies in the field of plant biotechnology have attracted great interest of scientists seeking to determine the genetic variety and improve specific characteristics of species. Among several types, molecular markers hold great promise due to their high efficiency, adequate accuracy, and good reproducibility. This review aimed to present different molecular markers used in genetic biodiversity studies of common food plants, including potato, corn, and tomato.

We presented some of the most frequent molecular markers in terms of their methodologies, advantages, challenges, and applications. We also reviewed the latest advances in the genetic diversity studies of common food plants that contribute to agricultural activities.

According to latest progress, Simple Sequence Repeats, Sequence Characterized Amplified Region, and Single Nucleotide Polymorphism are the most common molecular markers in plant diversity studies due to their co-dominancy, high level of polymorphism, great reproducibility, and adequate specificity. Considering common food plants like potato, corn, and tomato, Simple Sequence Repeats and Single Nucleotide Polymorphisms provide detailed information about polymorphisms, resistance to pathogens or diseases, genome maps, and population dynamics. However, more research should be conducted to apply the latest and more efficient technologies, such as Next Generation Sequencing, Diversity Array Technologies, and omics, to the genetic diversity studies of plant species.

Within the scope of recent progress, this review has a strong potential in providing relevant material for further research. It can serve as a guide to adopt the latest and most efficient sequencing platforms for examining various plant species, primarily potato, corn, and tomato.

Keywords: Molecular marker, genome sequencing, Amplified Fragment Length Polymorphism (AFLP), Single Nucleotide Polymorphism (SNP), Random Amplified Polymorphic DNA (RAPD), polymerase chain reaction (PCR), potato, corn, tomato

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INTRODUCTION

Today, one of the most striking challenges that the world faces is the growth of human population at an unstoppable rate. According to the latest forecasts, the world population is expected to reach 9.7 billion in 2050 and 11.2 billion in 2100, if no action is taken [1]. Moreover, there is an increasing concern about global warming that threatens the natural habitat of living organisms. These and similar environmental and sociological problems point out the potential risks in the availability of food, energy, water, and agricultural resources in the near future. Most of these problems involve the depletion of food plant resources that will present a serious threat to human and animal nutrition. Traditional agricultural activities that could protect these plants are limited since most cultivable lands are already under cultivation or urbanized. Also, environmental pollution and stress factors, such as drought, cold, salinity, and metal toxicity, make it difficult to carry out crop production. Therefore, researchers have focused on the search for more sustainable and economic solutions through technological and scientific innovations [2, 3].

The conservation of plant biodiversity and the sustainable use of existing resources are among the t op priorities for researchers in various fields. With the recent developments in genetic engineering techniques, genetic variability and biodiversity of food plants have become a subject of molecular biology applications [4]. In particular, molecular markers are

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the key tools that are currently available to identify, conserve, and improve plant species. They represent DNA sequences that show polymorphism between individuals or populations. This technology offers an excellent opportunity for preserving the existing plant species, understanding metabolic pathways, increasing biodiversity, improving plant resistance to disease and insects, and developing new hybrid crops.

Notably, the application of marker technologies is not affected by climatic and environmental conditions, unlike agricultural practices. Some of the molecular markers include Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Sequence-Related Amplified Polymorphism (SRAP), Inter Simple Sequence Repeats (ISSR), Simple Sequence Repeats (SSRs), Sequence Characterized Amplified Region (SCAR), Restriction Fragment Length Polymorphism (RFLP), Single Nucleotide Polymorphism (SNP), Diversity Array Technologies (DArT), and Next Generation Sequencing (NGS) [5].

The application of molecular marker technologies to preserve plant diversity is now being recognized as a way of solving the problems of traditional agriculture and food security. There have been a large number of completed and ongoing projects aimed at integrating molecular marker technologies into plant biotechnology studies. They have focused on a wide range of plant species including medicinal, wild, and edible plants that are highly important for biodiversity and human population. However, edible food plants are gaining much more attention as the world's population is increasing, climatic conditions are getting worse, and humanity is facing serious food shortages [6].

Thus, a systematic review of molecular marker technologies applied to food plants can serve as a promising guide to develop novel strategies for preserving and improving these species. In this context, we aimed to present various molecular methods used in genetic biodiversity studies of some common food plants, including potato, tomato, and corn. These plants are essential and economically important vegetables among various types of crop plants in many countries. Also, they are highly preferable in the fight against food scarcity due to their nutritious and calorie-dense properties [7]. First, we summarized some of the most frequent molecular markers, their methodologies, advantages, challenges, and applications. Then, we reviewed the latest advances in genetic diversity studies of common plants that can aid agricultural activities. To our knowledge, this is one of the first reports that brings together the key aspects of the most recent progress in applying molecular marker technologies to potato, tomato, and corn plants.

STUDY OBJECTS AND METHODS

This review aimed to bring together the existing academic literature regarding the use of various molecular methods in genetic biodiversity studies of common food plants. Using relevant keywords (including "genetic diversity" and "molecular marker technology"), we searched for English-language articles mainly published in 2017–2022 and indexed by three databases, namely Web of Science, Science Direct, and Scopus. The list of publications was limited to high-quality peer-reviewed journals and the references in the retrieved papers were also screened for relevant studies.

RESULTS AND DISCUSSION

Molecular marker technologies. In the past, traditional breeding approaches to genetic diversity offered significant contributions to the identification and development of plant species. Some of the traditional methods included backcrossing, hybrid, and mutation breeding. However, these attempts were highly time-consuming and susceptible to environmental conditions. Marker technology was developed to overcome these limitations and involved morphological, biochemical, and molecular markers. Molecular marker technology has gained much more attention due to the progress in molecular biology techniques and the challenges of the other markers [3, 8, 9]. Molecular markers are employed to detect different variations derived from insertions, deletions, or duplications located on chromosomes. They have several advantages over traditional methods as they show high efficiency and accuracy in all tissues with good reproducibility. Also, they are not affected by environmental conditions and/or the growth stage of plants [10, 11].

Molecular markers are mainly divided into three categories depending on their detection techniques: polymerase chain reaction (PCR)-based, hybridization-based, and DNA sequence-based markers (Fig. 1). These classes include a variety of specific markers: Restriction Fragment Length Polymorphism (RAPD), Amplified Fragment Length Polymorphism (AFLP), Single Nucleotide Polymorphism (SNP), Sequence Characterized Amplified Region (SCAR), Inter Simple Sequence Repeats (ISSR), and Restriction Fragment Length Polymorphism (RFLP). They have their own pros and cons, which will be detailed in the next section [9].

Hybridization-based marker techniques. The RFLP marker is the only method classified in hybridization-based marker techniques. Changes in nucleotide sequences of the genomic DNA are derived from point mutations, insertions, deletions, translocations, duplications, etc., and they exhibit polymorphisms between individuals of species. These mutations can modify the restriction sites and lead to length variations in DNA fragments. When the genomic DNA is isolated and mixed with restriction enzymes, DNA is cleaved by these enzymes (restriction endonuclease, or restrictase) at cleavage sites. This is followed by the hybridization of the target DNA by a labeled probe after it is transferred to agarose gel electrophoresis and the Southern blotting.

RFLPs have been widely utilized as an efficient tool for the detection of polymorphisms, genome mapping, and population dynamic studies. For example, Kim *et al.*

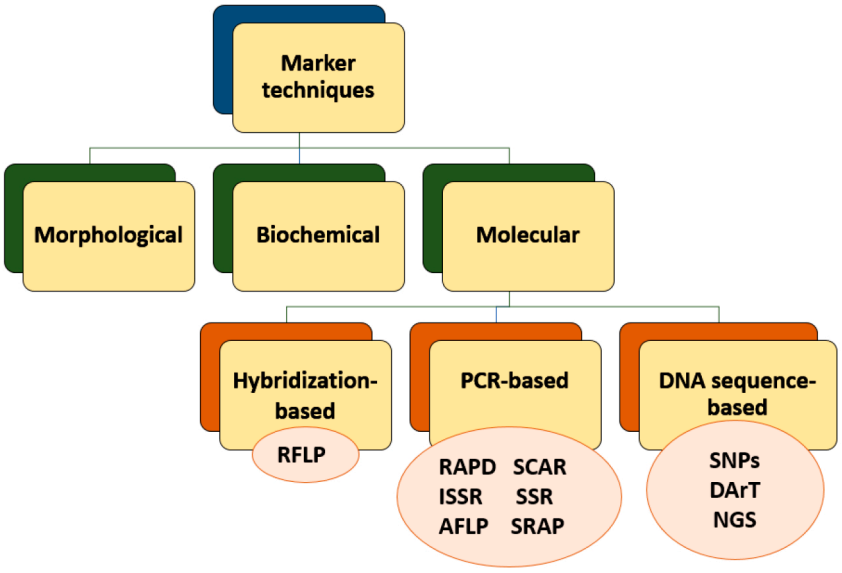

Figure 1 Classification of marker technologies

analyzed the quantitative trait locus of corn to determine its resistance to downy mildew, a major disease causing significant yield loss [12]. They used 691 SSR and 36 RFLP markers to identify polymorphisms between different lines, and reported that around one-third of the markers showed to be polymorphic. This technique has several advantages, including codominant inheritance, high reproducibility, locus specificity, and high genomic abundance. However, it requires a considerable amount of high-quality DNA, expensive toxic radioactive probes, and prior sequence information. Moreover, this method is time-consuming and costly [13, 14]. These disadvantages have guided researchers to develop other marker techniques.

PCR-based marker techniques. The PCR-based markers rely on the amplification of a particular DNA sequence in the presence of specific primers and enzymes by using the PCR technique [5, 15]. RAPD is one of the most common PCR-based marker systems that are generated with the random amplification of the genomic DNA in the presence of short arbitrary primers. After the PCR step, the separation and visualization of fragments by gel electrophoresis provide the detection of genomic polymorphisms by comparing the size of a band with the known molecular marker [16, 17]. Due to high speed, simplicity, and cost-effectiveness, the RAPD markers are highly preferred for a wide variety of plant species. For example, Sesli and Yegenoglu aimed to determine the effectiveness of RAPD and ISSR markers for the wild oil plant [18]. They reported that the RAPD marker provided reliable results about the genetic variability of this plant in a relatively shorter time than the ISSR marker.

In another study, RAPD, ISSR, and start codon-targeted (SCoT) markers were compared to characterize the molecular profiles of five kalanchoe species. The authors found the RAPD marker to be considerably efficient in identifying the genetic polymorphism due to its high polymorphism level of 50–70% [19]. Notably, this marker requires a small amount (20–40 ng) of DNA and does not need any additional information prior to experiments [20, 21]. The major challenge of this technique is that the process and the obtained results are sensitive to slight changes in reaction conditions. Therefore, the RAPD protocol should be carried out under strictly controlled conditions. Otherwise, contradictions may be observed between different experiments resulting in lower reproducibility [21]. Sharma *et al.* compared the genetic diversity in carnation genotypes/mutants with RAPD, ISSR, and SSR markers [22]. They reported that the RAPD marker showed worse reproducibility than the other markers, since it was influenced by variable factors such as primer, template quantity, and amplification profile. One of the possible strategies to solve this problem was presented by Lin *et al.*, who studied the development of a novel capillary electrophoresis-random amplified polymorphic DNA (CE-RAPD) method to improve the resolution of small molecular detection in RAPD profiling [23]. This method was defined as a promising tool for RAPD that provides standardization through repeated experiments.

The AFLP marker was developed to overcome the problems associated with the RAPD and RFLP methods. In detail, DNA is firstly digested by using two different restriction enzymes. Then, oligonucleotide adapters are ligated to the restriction fragments. Lastly, selective PCR is applied to amplify the attached fragments. Finally, the amplified fragments are checked by gel electrophoresis after they are stained with fluorescent or radioactive labelling [5].

AFLP is a dominant marker type that can use any DNA fragment regardless of its source, without requiring any prior data about the sequence. It can provide considerably reliable and reproducible results in a relatively shorter time than the RAPD and RFLP methods. Since restriction sites may be found across the whole genome, the AFLP markers can be used to simultaneously analyze a great number of polymorphisms with a single primer [24]. Therefore, this method plays a key role during the preparation of genetic and physical mapping. However, its application is limited because of some challenges, including its complexity. Moreover, it requires high molecular weight DNA and has a low ability to distinguish the homozygous from the heterozygous because of dominant characteristics. In addition to the method's complexity, the observation of band profiles is difficult because of a short length of fingerprints in agarose gel [12, 25]. In order to resolve this, researchers have developed a novel Three Endonuclease-AFLP (TE-AFLP) method to analyze the genetic diversity of an Indian tea plant. As a result, clear banding profiles produced by the TE-AFLP technique were reported to be simple to score across gels [26].

SSRs, also called microsatellites, are a type of PCR-based markers which have short tandemly repetitive sequences found in the eukaryotic genome. Microsatellite DNAs have repeat motifs up to six nucleotides that show variations within and among individuals of a particular species. These variations and the length of sequences determine the level of polymorphism which can be detected with the PCR analysis.

The advantages of the SSR markers are high genomic abundance, co-dominant inheritance, ease of automation, high polymorphism, and moderate reproducibility. In comparison to the RAPD and AFLP markers, this method requires prior sequence information about the genome of a species for DNA sequence and it is a highly expensive procedure [16, 27]. As an alternative, ISSR markers are developed by modifying SSR, namely by amplifying genomic regions located between two identical repeat sections that are oppositely oriented. Although the ISSR markers are highly applicable in plant genetic studies, their limitations are low reproducibility and homology of co-migrating amplification products [5, 28]. Several studies have been performed to enhance the identification and authentication of plant species by using the ISSR marker technology. They revealed that ISSR coupled with the high resolution melting (HRM) analysis could provide beneficial results about the plant diversity and could be used to create new cultivars with desirable traits. The HRM analysis is a powerful technique that produces sequence-related melting profiles during dissociation of double stranded DNA and provides information about the differences in the genotype at the level of a single nucleotide [29, 30].

Co-dominant SCAR markers are an improved variant of RAPDs. They have higher specificity because the primers for SCARs are designed considering nucleotide sequences established in cloned RAPD fragments. Apart from high specificity, this marker system has such advantages as reproducibility, high efficiency with low quantities of a DNA template, ease of application, and low sensitivity to experimental conditions. Despite the numerous advantages, the use of SCARs is negatively affected by the need for additional information before the PCR and the detection limits of the primers. Regarding the detection limit, a wide variety of levels from 0.1 to 25 ng were reported as possible challenges. However, increasing the sample number and using the quantitative PCR may improve the detection limits [31, 32].

Another simple PCR-based marker is SRAP, a dominant and effective system for the production of genome-wide fragments. It is based on the amplification of open reading frames by utilizing two different primers, including CCGG sequence in the forward primer and AATT in the reverse primer. This marker is robust, efficient, and inexpensive. Thus, it is widely used in different applications, including map construction, genetic variety investigation, and DNA fingerprinting [17, 33].

DNA sequence-based marker techniques. The DNA sequencing technology has gained great attention with the developments in molecular biology. SNP is one of the sequence-based marker technologies that represents the most abundant and efficient DNA fingerprinting approach. This marker detects possible polymorphisms derived from changes in a single nucleotide position due to substitution, deletion, or insertion [13]. A variety of techniques have been reported to determine SNP genotypes, which are based on performing allelic distinction techniques and detection approaches. Some of the common detection methods are cleaved amplified polymorphic sequences (CAPS), Sanger sequencing, SNP-RFLP, and single strand conformational polymorphism (SSCP) [5]. The importance of SNP markers has risen with the latest developments in high-throughput genotyping methods like NGS, DArT marker, genotyping by sequencing, and allele-specific PCR.

DArT markers are microarray-based hybridization techniques that are largely used for mapping and genetic diversity studies. This system is considered a time-saving alternative to hybridization-based markers. DArT offers excellent potential to examine plant diversity, because it enables simultaneously genotyping of several thousands of polymorphic loci in a single assay. Also, it is highly reproducible and no prior information is needed to determine the sequence. The DArT marker analysis mainly involves the construction of a genetic library and its printing on microarray chips. After that, the labelled DNA is hybridized on chips and scanned for data analysis [5, 34]. The use of microarray platforms makes the DArT markers greatly preferred due to their ability to separate very high DNA fragment densities [35].

With the development of sequencing methods and high throughput technologies, simultaneous analysis of large amounts of DNA sequences has become an

important approach. Currently, NGS techniques are becoming available for the sequencing of thousands to billions of nucleic acid sequences in one assay. They include a variety of techniques such as Illumina, Genapsys, Qiagen, Ion Torrent, and Roche 454 sequencing, which show different numbers of reads per run changing from 2 M to 1.1 billion. The massively parallel sequencing capacity of NGS provides more accurate analysis of the whole genome at a low cost [36, 37]. This technology has been widely applied to detect polymorphism, construct genetic maps, genotype mapping populations, and analyze whole genome sequences [17, 38]. However, the use of the NGS technology for plant species is still limited due to the lack of sufficient data for standard operating procedures. Also, the main challenges arise from the preparation of libraries since this technique generates massive amounts of data [39]. Therefore, there is a need for more research to accelerate plant diversity studies with the NGS technology.

Recent advances in genetic diversity of common food plants. Efforts have been made in the last years to protect some of the common food plants and improve their properties, such as resistance to pathogens, yield, and adaptation to environmental conditions. Applying genetic marker technologies to investigate plant diversity can help protect the existing plant sources and develop new strains with specific features [40, 41]. Some of the most recent molecular markers used to study variations in potato, corn, and tomato plants include RAPD, SSR, AFLP, SNP, SCAR, and DArT (Table 1). This review summarizes the latest developments in the genetic diversity studies of these three plants.

Genetic diversity of potato. Potato (*Solanum tuberosum* L.) is one of the most common food plants in the world, together with rice, corn, and wheat. It is a non-cereal staple crop which serves as a major source for millions of people worldwide. China, India, and Russia are the leaders in potato production. In order to meet the global demand, it is crucial to improve its genetic potential [57]. For many years, scientists have studied different genetic varieties of potato using multiple marker-assisted technologies. Among them, SSRs have been mostly applied to characterize potato genetics due to their reproducibility, simplicity of use, and high polymorphism. A total of 1219 potato varieties from around the world have been investigated using SSRs to determine possible diversities over time and space. No major changes were reported in the genetic diversity of this crop over the past three centuries [42]. In another study, a diverse population of 189 genotypes of *S. tuberosum* was screened with SSR markers to determine the late blight resistance, which is one of the most destructive diseases in potato production. After analyzing wild and cultivated potato germplasms, the authors found significant resistance variations among the genotypes within the species [43]. These and similar studies (Table 1) have provided useful information for the determination of potato diversity and showed the effectiveness of SSR markers.

Table 1 Recent applications of marker-assisted technologies for potato, corn, and tomato plants

Marker	Species	Plant Material	Marker Type/Size	Genetic diversity	References
Potato	*Solanum tuberosum*	30 heirloom varieties 205 old cultivars 984 modern cultivars	35 SSR markers	No loss of diversity over the past three centuries	[42]
	Wild and cultivated *Solanum* species	61 wild genotypes 32 *S. tuberosum* Andigenum group 79 varieties of *S. tuberosum* Chilotanum group	30 SSR markers	Significant variations in wild and cultivated potato genotypes Cultivated *S. tuberosum* Chilotanum showed lower genetic diversity	[43]
	Solanum tuberosum subsp. *andigenum*	120 accessions from Colombia 2 accessions from Ecuador 13 accessions from Peru 8 accessions from Bolivia	1534 polymorphic AFLP markers	High total diversity (95%)	[44]
	Solanum fendleri	94 populations of the wild *S. fendleri* from six different mountain regions in southern Arizona, USA	2094 polymorphic AFLP markers 16 adaptive markers	A core subset including 26 accessions with 96% marker diversity	[45]
	n.a.	214 TAMU potato clones (68 red-skinned, 62 russet, 32 yellow-skinned, 31 chipping, 21 purple-skinned clones)	10 106 SNP markers	Heterozygosity with an overall average of 0.59 Differentiation among breeding clones	[46]
	n.a.	73 Korean potato clones (45 commercial varieties, 28 breeding lines) 37 potato collections from different countries	6575 SNP markers	10 highly informative SNPs discriminating all 393 potatoes	[47]

Continuation of Table 1

Marker	Species	Plant Material	Marker Type/Size	Genetic diversity	References
Corn	n.a.	20 corn genotypes representing sweet corn, popcorn, yellow corn, and white corn	8 RAPD markers	Maximum gene diversity of 0.354 for yellow corn, minimum diversity of 0.254 for white corn Same genotypic diversity for all corn types	[48]
	Zea mays var. *saccharata*	39 sweet corn inbred lines	63 SSR markers	The major allele frequency between 0.42–0.79 Polymorphic information content between 0.27–0.63 High polymorphism among the inbreeds	[49]
	Zea mays	120 inbred lines obtained from the Corn Experiment Station, Gangwon Agricultural Research and Extension Services, collected in Korea	108 dominant SCAR markers (32 monomorphic, 76 polymorphic)	The overall average PIC value of 0.34 Expected heterozygosity of 0.324	[50]
	n.a.	192 F_7 families derived from B73 (susceptible) × Ki11 (resistant)	691 SSR and 36 RFLP markers and QTL analysis	12.95% of phenotypic variation, resistance to downy mildew disease associated with the upregulation of 15 genes	[12]
	Zea mays	59 corn genotypes (46 Zambian grown landraces, 10 cultivated three-way check hybrids, 3 obsolete varieties)	SNP-based DArT markers	The mean gene diversity of 0.29 Polymorphic information content of 0.23 Higher genetic variety within a population rather than between populations	[51]
	n.a.	162 early maturing yellow and white tropical inbred lines	9684 DArT-SNP markers	Gene diversity of 0.30 Polymorphic information content ranging from 0.08 to 0.38	[52]
Tomato	*Solanum lycopersicum*	48 tomato genotypes (32 from ICAR-NBPGR, India; 14 from the Tomato Genetics Resource Center, University of California; 2 superior released varieties)	130 SSR markers	Polymorphic information content between 0.12–0.93 Significant level of molecular variance	[53]
	Solanum lycopersicum	64 landraces from Campania, Sicily and Apulia 3 outgroups	7720 SNPs	Several sequence variants related to fruit maturation and resistance to stress	[54]
	n.a.	32 accessions of Hail landraces	7 SRAP primer combinations	Average polymorphic information content of 0.68 Discrimination power of 14.29 Wide range of genetic diversity at both inter and intra-variation levels	[55]
	Solanum lycopersicum	14 modern varieties, 71 landraces and 22 commercial hybrids	SSRs 2 SCAR markers	Average polymorphic information content of 0.74 Genetic loss because of breeding in the modern tomato gene pool	[56]

n.a. – not available

The AFLP marker technology is also used in potato genetic diversity studies. For example, del Rio and Bamberg aimed to analyze the minimum amount of germplasm units showing the highest plant diversity by building a core subset [45]. In their study, 144 accessions from the Potato Genebank located in the United States were discriminated from each other with genetic similarity values of 62–89%. After the selection process, a final core subset was built with a 96% marker diversity of 26 accessions.

The AFLPs are a key tool for potato studies because of high polymorphism and a small amount of

DNA required. Yet, their use is limited by high cost. The DNA sequence-based markers, especially SNPs, can become an alternative to the PCR-based marker systems. Recently, Texas A&M University (TAMU) within its potato breeding program has investigated potato varieties and advanced clones at a molecular level by performing SNP markers. Their detailed analysis highlighted the genetic diversity of potato sources and paved the way for applying SNPs in other breeding programs [46]. Jo *et al.* carried out a similar study of potato clones from the Korean potato breeding program that provided useful information about the crop's breeding history, regional adaptations, and market demands [47]. Despite the developments, the lack of sufficient data about potato proteomics and metabolomics makes it difficult to further investigate its genetic diversity and improve its quality.

Genetic diversity of corn. Corn (*Zea mays* L.) is another important crop worldwide that is consumed not only by humans but also by livestock. Its genetic diversity has been studied for many years and described in plentiful literature. Javed *et al.* applied the RAPD technology, one of the oldest marker systems, to assess different corn genotypes representing sweet corn, popcorn, yellow corn, and white corn [48]. According to their results, white corn had minimum gene diversity, while yellow corn showed the maximum level among 20 corn genotypes. Although some lines exhibited polymorphism with other groups, a total divergence was observed for all the studied types. As in potato diversity, the SSR markers have been commonly used to investigate the population structure and genetic diversities of corn [49, 58, 59].

In addition to the RAPD and SSR markers, SCARs, another PCR-based marker technology, may provide highly efficient data for the crop's allele and genetic diversity. However, SCARs have not been applied in molecular studies of plants as commonly as the previous two markers. The potential of SCARs for corn diversity was investigated by Roy *et al.*, who reported this marker's effectiveness in analyzing the structure of corn inbred population of the Korean breeding lines [50].

Despite the great developments in sequencing technologies, recent efforts in corn studies have been shifted to the use of DNA sequence-based markers, and a number of successful results have been reported. One of the studies determined the genetic diversity of corn genotypes by using the SNP-based DArT marker [51]. In another study, 162 yellow and white corn inbred lines were analyzed for clarification of heterotic groups, inter-trait relationships, and population structure with the use of the DArT-SNP markers [52]. These studies provided new insights into corn genetic diversity.

Genetic diversity of tomato. Tomato (*Solanum lycopersicum* L.), which originated in South America, is cultivated throughout the world as one of the most important vegetable crops. For example, the volume of tomato production in Russia is about 30 million tons. It has a high nutritional value due to a rich content of different metabolites such as lycopene, vitamins A and C, minerals, and dietary fiber. Therefore, researchers have used different technologies to study the genetic diversity of tomato and improve its properties.

The molecular markers that are generally used to explore tomato diversity are SSR, RAPD, SNPs, and ISSR [53, 60, 61]. For example, 48 genotypes of different exotic and indigenous sources were evaluated using 130 SSRs to determine the plant's resistance to two common tomato diseases. The analysis resulted in developing a new species resistant to tomato spotted wilt virus and fusarium wilt diseases [53]. Gonias *et al.* employed characterization analyses by using SSRs coupled with SCAR, another reliable marker, to evaluate the polymorphism level of tomato germplasms [56]. This two-stage molecular approach produced useful data about the genetic variety and resistance of tomato to two serious fungal diseases, fusarium crown and root rot, as well as late blight. As a sequence based-marker, SNPs were used to analyze the genetic diversity of 64 tomato accessions from southern Italy. They showed a variety of mutations in genes, which were associated with stress tolerance and fruit quality [54]. Recently, the SRAP markers have been applied to analyze inter- and intra-genetic variability among Hail tomato landraces. The scientists determined the uniqueness of Hail tomato landraces and presented the potential use of the SRAP markers for other tomato-breeding programs which are of plant breeders' interest to include in breeding programs for crop improvement. We assess the inter- and intra-genetic variability among 96 accessions representing three Hail tomato landrace using DNA-based marker sequence-related amplified polymorphism (SRAP) [55].

CONCLUSION

Molecular marker technologies have found a wide range of applications in plant biotechnology because of their specific advantages in terms of efficiency, accuracy, reproducibility, simplicity, detection of polymorphism, and cost. This review summarized major molecular markers that have been used to study the genetic diversity of potato, corn, and tomato plants.

We identified different types of molecular marker technologies used for genetic diversity research of plant species. These markers are classified into three groups: hybridization-, PCR-, and sequence-based technologies. The PCR-based markers are more advantageous than the hybridization-based markers because of the latter's toxicity and time-consuming characteristics. Considering the latest progress, SSRs and SCARs are highly preferred PCR-based markers due to their co-dominancy, high level of polymorphism, great reproducibility, and adequate specificity. However, rapid developments in molecular biology and biotechnology have led to a great shift towards sequence-based markers and novel marker technologies. One of such techniques are co-dominant SNPs, which show an extremely high level of polymorphism and good reproducibility. The recently

developed NGS technology is a major breakthrough in the efforts to sequence plant genomes. This sequencing method has a strong potential to be used for plant diversity studies because it can provide reliable results by sequencing billions of nucleic acid sequences simultaneously. However, further research is needed to fully understand this technique and to validate its robustness for a variety of plant species.

Potato, corn, and tomato are essential and economically important vegetables, and their genetic information has been studied by several researchers. According to these studies, SSRs and SNPs are the most extensively used markers for these three food plants, since these markers possess high genomic abundance, co-dominant inheritance, ease of automation, high polymorphism, and adequate reproducibility. These markers provide detailed information about polymorphisms, resistance to pathogens or diseases, genome maps, and population dynamics. However, the most recent methodologies like DArT and NGS should be implemented into the genetic diversity studies of these plants to improve efficiency. This review can serve as a guide for adopting the latest and most efficient sequencing platforms to examine various plant species, primarily potato, corn, and tomato.

CONTRIBUTION

Bahar Aslanbay Guler conceptualized the research and wrote the original draft. Esra Imamoglu conceptualized and supervised the research, as well as reviewed and edited the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Hyptis suaveolens L. leaf extracts in traditional health care systems

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Abstract:
Hyptis suaveolens L. is a medical and food plant that is commonly used to treat various microbial infections in humans in many countries of the world. We aimed to study the aqueous and ethanol extracts of *H. suaveolens* leaves to determine their antibacterial, *in-vitro* antioxidant, and phytochemical potentials for traditional medicine by using chemical analysis.
The aqueous and ethanol extracts inhibited the tested bacteria species with zones of 0–15 and 10–29 mm, respectively. On the typed culture isolates, the inhibition zones were 8–25 and 16–32 mm for the aqueous and ethanol extracts, respectively.
The minimum inhibitory concentrations of the aqueous and ethanol extracts were not different, while the minimum bactericidal concentrations for the aqueous extract was higher than that for the ethanol extract. The screened phytochemicals were qualitatively and quantitatively present in both extracts, except for saponins which were absent in the aqueous extract. The free radical scavenging activity in the aqueous and ethanol extracts was 1.44 ± 0.50 and 1.57 ± 1.40 mg of ascorbic acid/1 g dry leaves, respectively. The ferric reduction was 1.19 ± 0.40 and 1.69 ± 0.18 mg of ascorbic acid/1 g dry leaves in the aqueous and ethanol extracts, respectively. Hydroxyl scavenging was 65.0 ± 0.9 and 0.43 ± 0.50 mg of ascorbic acid/1 g dry leaves for the aqueous and ethanol extracts, respectively.
The present research suggests that the extract of *H. suaveleons* can be applied as a controlling antibacterial growth agent against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Streptococcus pneumoniae* and other bacterial pathogens. It is noteworthy that the ethanol extract was more effective than the aqueous one in terms of the antibacterial, phytochemical and antioxidant activities.

Keywords: Health care, inhibition, plant extract, antioxidant properties, *Hyptis suaveolens* L., phytochemicals, pathogenic bacteria

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INTRODUCTION

Medicinal plant-based remedies are used to prevent or treat disease and to maintain health. Some are also utilized as foods by many ethnic tribes of the world. Antibacterial agents derived from plants have been found effective against the growth and reproduction of bacteria. The growing resistance of bacteria to existing antibiotics has made it necessary to search for a new way of destroying or reducing them [1]. Due to this resistance, antibiotics become ineffective in treating disease. Therefore, microbiologists and other related natural and chemical scientists, such as biochemists, botanists, and biotechnologists, are deploying their knowledge and energy to discover ways to curb this trend [2].

Different tribes in the developing countries, such as Nigeria, Ghana, Kenya, Togo, and Niger, depend predominantly on herbal remedies to sustain most of the health challenges they encounter. Anand and Gokulakrishnan found that traditional healers in many regions of the world, including China and India, have used plants with medicinal properties to treat diseases, heal wounds, and prevent infections [3]. Gema Nieto noted that many of these plants are also consumed as food [4]. The effectiveness of herbal medicine in treating microbial infections is down to the bioactive compounds, such as flavonoids, phenols, phlobatannins, tannins, saponins, steroids, and glycosides, which have diverse physiological and pharmacological responses in the body [5].

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Phytochemicals, which are compounds found in plants, have been shown to have a protective effect against chronic diseases and to be effective in treating dangerous body conditions and neurodegenerative diseases. These bio-active compounds can be found by extracting molecules from plants and studying their medicinal properties [6]. These are non-nutritive chemicals that protect human beings from various diseases.

According to literature, extracts from the leaves of *Hyptis suaveolens* L. can heal disorders such as hemorrhoids, abscesses, and swellings. Indians, for instance, use this plant as a sudorific, laxative, stimulant, and galactagogue. The infusion of *H. suaveolens* is used for infections of the uterus and its leaf juice is taken for colic and stomach ache.

The extract from the roots of *H. suaveolens* is good for cleansing blood and also for women's diseases. In some parts of Asia, it is taken as a medicinal tea and in South America, as an essential oil and food. The flowering stems are edible and can also be used as a spice [7]. Therefore, we aimed to determine the phytochemicals and bacterial growth restriction potency of *H. suaveolens* extracts on some pathogenic bacteria.

STUDY OBJECTS AND METHODS

Plant sample collection. *Hyptis suaveolens* L. leaves were harvested from the plant's natural habitat in Uzairue, Edo State, Nigeria. The leaves were confirmed by Dr. Odoligie of the Department of Biological Science, Edo State University. The plant voucher No. LH 1116 was retained in the university herbarium.

Processing of leaf samples. The leaf samples of *H. suaveolens* were rinsed in distilled water and spread on concrete floor for three weeks to dry at room temperature of 27 ± 2°C. Then, it was pulverized into smooth powder using a mechanical grinder. In this process, 100 g of ground leaves were added to 250 mL of ethanol and 250 mL of water. The mixture was steeped for 24 h and then filtered to separate the liquid from the solid material. The filtrate from the ethanol extract was concentrated in vacuo, while the aqueous extract was evaporated in a water bath at 55°C. The semi-solid ethanol and aqueous extracts were kept in a sterile glass bottle for use.

Collection of test bacteria species. Five clinical bacteria isolates, namely *Streptococcus pneumoniae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, were obtained from the Microbiology Department of the Federal University of Technology, Akure. The American type cultures included *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, *K. pneumoniae* ATCC 49619, and *E. coli* ATCC 35218. The bacterial isolates were purified by sub-culturing on nutrient agar and incubated for 24 h at 37°C. The purified bacteria isolates were Gram-stained for cell morphology and identified as either Gram-positive or Gram-negative. The pure isolates were characterized and identified to species level by performing oxidase, catalase, hydrolysis of starch, indole, motility, triple sugar ions, coagulase, nitrate reduction, and Voges-Proskauer tests [8]. Before use, the isolates were grown in peptone water for 18 h. The turbid cultures were diluted with sterile distilled water to obtain the McFarland's standard of 10^7 column-forming units per milliliter (CFU/mL).

Extract inhibition test. The extracts inhibition test was performed on the test bacteria species by the agar well diffusion method. For this, 10^7 CFU/mL of the test bacteria was streaked on the surface of the Mueller-Hinton agar with a sterile swab stick and left for 2 h for the seeded bacteria to establish in the culture medium. Wells were created on the agar plates with a sterile cork borer with a diameter of 4 mm. Each of the wells was leveled up with 0.05 mL of the extract. The plates were left at room temperature for 2 h for the extract to diffuse and for the bacteria to establish in the culture medium before incubating for 24 h at 37°C. Bacterial inhibition was measured and the degree of sensitivity was recorded.

Minimum inhibitory concentration determination. The extract was evaluated by using the broth dilution technique. This involved adding 8 mL of Mueller-Hinton broth to test tubes, then adding 1 mL of the extract and 1 mL of a 24-h broth culture of the test bacteria. The tubes were then incubated at 37°C for 24 h and observed for bacterial growth. The minimum inhibitory concentration of the extract was determined as the lowest concentration at which no bacterial growth was observed.

Minimum bactericidal concentration determination. To determine the minimum bactericidal concentration of the extract, the tubes with the extract in the minimum inhibitory concentration without visible bacterial growth were shaken and 1 mL was obtained and pure-plated with the plate count agar. The plates were incubated for 24 h at 37°C. The plate without bacterial growth was taken as the minimum bactericidal concentration of the extract.

Qualitative phytochemical analysis of *H. suaveolens*. *Chemical methods.* The methods of Harbone and Williams and Trease and Evans were adopted to qualitatively determine the phytochemical components in the plant extracts, specifically tannins, alkaloids, flavonoids, cardiac glycosides (keller-killani test), saponins, phlobatannins, steroids, and terpenoids [9, 10].

Quantitative phytochemical analysis of *H. suaveolens*. *Total phenolic compounds determination.* A solution was prepared by adding 100 mg of the extract into 100 mL of distilled water. Then, 1 mL of this solution was taken with a pipette and added to a glass test tube. Next, 0.5 mL of 2 N Folin-Ciocalteu reagent and 1.5 mL of 20% Na_2CO_3 solutions were mixed with it and the volume was made up to 10 mL with distilled water. The mixture was left to stand for 2 h after being properly shaken, and the absorbance was read on a spectrophotometer at 765 nm. The obtained data were then used to estimate the phenol content by comparing it to the standard curves generated from different diluted concentrations of garlic acid.

Total flavonoids determination. The method was based on the formation of a flavonoids-aluminum complex with a maximum absorptivity at 415 nm. For this, 5 mL of the extract was mixed with 100 µL of 20% aluminum trichloride in methanol. After 40 min, the absorbance of the mixture was read at 415 nm. Blank samples were prepared from 100 mL of the plant extract and then mixed with a drop of acetic acid and 5 mL of methanol. The absorbance of 0.5 mg/mL of standard rutin in methanol was measured. The tests were performed in triplicate.

Total alkaloids determination. To determine total alkaloids, 5 g of the extract was mixed with 200 mL of 10% acetic acid prepared with ethanol in a 250-mL glass beaker. The mixture was covered and left to stand for 4 h. The solution was filtered and concentrated in a water bath to obtain one-quarter of the original volume. Then, the solution was mixed with concentrated ammonium hydroxide added drop-wise and left to settle for precipitate to form. The formed precipitate was harvested, washed with dilute ammonium hydroxide, and filtered. The residue, which is alkaloids, was dried and weighed.

Tannin determination. The extract concentrate was mixed with distilled water to a solution and boiled for an hour. The Folin-Denis reagent and a sodium carbonate solution were added to the boiled solution for color development. Using a spectrophotometer, absorbance was determined at 750 nm. The concentration of tannic acid was then calculated based on the tannic acid standard.

Saponins determination. In a reflux condenser containing pure acetone, saponins were extracted for 2 h. Then, they were exhaustively extracted for 2 h in a Soxhlet apparatus containing methanol. The methanol was evaporated and the extract was weighed. The remaining sample content was calculated as the percentage of saponins.

Steroids determination. One gram of the plant extracts was dissociated in a few drops of acetic acid. The mixture was warmed gently and cooled down under a running tap water. After that, it was mixed with a drop of concentrated sulphuric acid at the side of the test tube. Green color in that reaction indicated the presence of steroids [11].

Glycoside determination. The Keller-Killan test was used to determine cardiac glycosides. For this, 5 mL of the extract was mixed with 2 mL of glacial acetic acid incorporated with a drop of a ferric chloride solution. It was then underlaid with 1 mL of concentrated H_2SO_4. The appearance of a brown ring at the interface was an indication of deoxy-sugar, a characteristic of cardenolides. Below the brown ring might appear a violet ring and a green ring in the acetic acid layer in a graduate process throughout the thin layer.

In vitro* antioxidants determination.** ***Ferric reducing antioxidant potential of extracts. The ferric reducing antioxidant potential of the extracts was determined using the criterion described by Buricova and Reblova [12]. An extract of 0.1 g was added to water (20 mL), properly shaken to dissolve, and allowed settling. After filtering, 2.5 mL of the filtered extract was obtained and mixed with 2.5 mL of phosphate buffer (pH 6.6) and potassium ferrocyanide. It was incubated at 50°C and then mixed with 10% trichloroacetic acid, 5 mL of distilled water, and 1 mL of 0.1% ferric chloride. The absorbance of the samples and the standard was read using a spectrophotometer at 700 µm. All the measurements were performed in duplicate.

Free radical scavenging. An extract sample of 20 mL was mixed with 0.5 mL of 1 mM 1,1-diphenyl 1-2 picrylhydrazyl (0.05 mg/mL), dispersed in cuvettes, and kept for 20 min. Using a spectrophotometer, the absorbance of the mixed solution was measured at 520 µm. The absorbance was considered as mg of L-ascorbic acid/1 g dry weight of the plant substance. The calculation was used when the plant's extracts were replaced with a freshly prepared solution of ascorbic acid in deionized water (concentration from 0 to 1.6–100 mg/mL) [13]. The DPPH free radicals percentage was determined using the equation below. The experiment was performed in triplicate.

$$\text{DPPH scavenging effect} = \frac{A_0 - A_1}{A_0} \times 100 \qquad (1)$$

where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the extract or standard.

Hydroxyl radical scavenging. This test was based on the method of Halliwell *et al.* [14]. The hydroxyl radical scavenging assay of the plant's extracts was determined using iron-ascobate-EDTA-H_20 which reacted with deoxyribose to form thiobarbituic acid reactive substances. This reaction produced a pink chromagen at low pH when heated with trichloroacetic acid. The reaction mixture contained 4 mM deoxyribose, 0.3 mM ferric chloride, 0.2 mM EDTA, 0.2 mM ascorbic acid, 2 mM H_20, and extracts in different concentrations. After incubation at 37°C for 20 min, 0.4 mL of trichloroacetic acid (5%) and 0.4 mL of trichloroacetic acid (1%) were added to the mixture and boiled for 20 min. Using a spectrophotometer at 532 µm, the intensity of the pink chromogen was measured against a blank sample. The hydroxyl radical scavenging potency of the plant's extracts was determined as inhibitions of deoxyribose degeneration and calculated with the equation below. Ascorbic acid served as the positive control and tests were performed in triplicate.

$$\% \text{ inhibition} = \frac{A_0 - A_1}{A_0} \times 100 \qquad (2)$$

where A_0 was the absorbance of the control and A_1 was the positive control.

Determination of proximate and mineral contents. The method of the Association of Official Analytical Chemists was used to determine the proximate and mineral contents of the leaves samples [15].

Statistical analysis. The results were expressed as mean ± standard deviation (m ± SD). The obtained

data were subjected to a one-way analysis of variance (ANOVA). The least significant difference (LSD) was performed for the pairwise mean comparisons. To determine the considerable treatment amount at a 95% level of confidence, variance was considered statistically significant at ($p < 0.5$).

RESULTS AND DISCUSSION

The color, consistency, odor, and the time of extraction were observed and recorded. The physical characteristics of the extracts are shown in Table 1.

Antibacterial effects. The antibacterial potentials of the ethanol and water extracts of *Hyptis suaveolens* L. were tested on five clinical bacteria isolates (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Streptococcus pneumoniae*) and American type cultures (*P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, *K. pneumoniae* ATCC 49619, and *E. coli* ATCC 35218). The size of inhibition zones around the wells was used to calculate antimicrobial activity. The plant's extracts from both solvents had inhibitory effects on the tested bacteria (Table 2). The ethanol extract was most active against *E. coli* ATCC 35218 with an inhibition zone of 32 mm, followed by *K. pneumoniae* ATCC 49619 with an inhibition zone of 31 mm, and *P. aeruginosa* with an inhibition zone of 10 mm. The aqueous extract had the strongest inhibitory effect on *S. aureus* ATCC 25923 with an inhibition zone of 25 mm, followed by *E. coli* ATCC 35218 with an inhibition zone of 20 mm, and *P. aeruginosa* ATCC 27853 with an inhibition zone of 8 mm. *K. pneumoniae* and *P. aeruginosa* were not inhibited by the aqueous extract at the tested concentrations. Their resistance to the aqueous extract could be down to the low quantity of phytochemicals screened. However, these two species are among the Gram-negative bacteria commonly detected to form resistance to multiple drugs. This is explained by their built-in ability to find new ways of resistance due to a plasmid gene they possess. In addition, their outer membrane contains phospholipids that bound to the inner leaflet of the membrane and lipopolysaccharides that bound to the outer leaflet, which causes endo-toxic shock [16]. Yang *et al.* reported that any alteration in the outer membrane, like changes in hydrophobic properties or mutations in purines, can make them resistant, as well as other factors [17]. However, we found that the type cultures were more inhibited than the clinical isolates.

The minimum inhibitory concentrations of the ethanol extract on the tested bacteria species ranged from 50 to 200 mg/mL. The bacteria inhibited with small zones of inhibition were suppressed with high minimum inhibitory concentrations of 150–200 mg/mL, while those inhibited with larger zones were suppressed with minimum inhibitory concentrations of 50–100 mg/mL. Meanwhile, the minimum bactericidal concentrations of this extract were effective on the tested bacteria with concentrations of 50–100 mg/mL. The concentration of 50 mg/mL suppressed *S. aureus* ATCC 25923, *K. pneumoniae* ATCC 49619, *E. coli* ATCC 35218, and the clinical *S. aureus* species.

Our research suggests that the extract of *H. suaveolens* may be applied as a controlling antibacterial agent against the selected pathogens of *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, *E. coli*, *S. pneumoniae*, and several other bacterial pathogens that are threatening human health. Almost all of these species have formed resistance to the existing antibiotics known for their cure and prevention. Therefore, medicinal plants rich in biological compounds, such as *H. suaveolens*, can be used to suppress and reliably inhibit these bacteria. Edeoga *et al.* studied the antibacterial activity of *H. suaveolens* leaves against *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, *E. coli*, and *S. pneumoniae* [18]. We found

Table 1 Physical characteristics of *Hyptis suaveolens* L. extracts

Physical characteristics	Water extract	Ethanol extract
Time of extraction	24 h	24 h
Color	Dark green	Green
Consistency	Semi-viscous solid	Liquid
Odor	Characteristic	Pungent

Table 2 Antibacterial activity of water and ethanol extracts of *Hyptis suaveolens* L.

Organisms	Water extract, mm	Ethanol extract, mm	Water extract, mg/mL		Ethanol extract, mg/mL	
			MIC	MBC	MIC	MBC
Escherichia coli	13	20	150	100	50	50
Escherichia coli ATCC 35218	20	32	50	100	50	50
Streptococcus pneumonia	12	25	100	150	50	100
Klebsiella pneumoniae	0	29	100	150	150	100
Klebsiella pneumoniae ATCC 49619	10	31	50	100	50	50
Staphylococcus aureus	15	12	100	200	50	50
Staphylococcus aureus ATCC 25923	25	16	50	100	50	50
Pseudomonas aeruginosa	0	10	250	300	200	150
Pseudomonas aeruginosa ATCC 27853	8	18	200	250	150	100

MIC – Minimum inhibitory concentration

MBC – Minimum bactericidal concentration

some similarities and differences in the inhibition of these bacteria, which could be due to the age of the plants, climate change, environmental conditions, and the mode of extraction. Mishra *et al.* and Jin *et al.* reported that climate change and environmental stress could have an impact on gene expression and life cycle patterns in plants, as well as their phytochemical composition [6, 19]. Another factor could be the variability in secondary active metabolites produced in different plant parts, as well as their quality and quantity as they age.

Quantity and quality of phytochemicals. The qualitative phytochemical examination showed that both the water and the ethanol extracts yielded many secondary metabolites (Table 3). Alkaloids, phenol, flavonoids, glycosides, steroids, phlobatannins, saponins, and terpenoids were qualitatively and quantitatively present in the ethanol extract. They were also present in the water extract, except for saponins. These screened phytochemicals, though serving as protection for the plants under certain conditions, are essentially phytoconstituents of pharmacological potential useful in the health care system.

However, ethanol extracted these chemicals more than water. These chemicals were thought to be responsible for various degrees of inhibition observed on the tested bacteria species. The ethanol extract produced higher inhibition zones than the aqueous extract due to a higher content of phytochemicals. The presence of some of these chemicals in *H. suaveolens* has been qualitatively and quantitatively reported by Odusina and Oretuga [20]. Useful chemicals, such as *α*-phellandrene, *α*-copanene, 4-terpineol, *β*-pinene, *β*-elenene, *γ*-terpinene, and several others, have also been reported in *H. suaveolens* [21].

It has been shown that when solvents like ethanol and water are used for plant extraction, the extracted components are able to exhibit inhibitory effects on microorganisms [22]. In our study, the leaf ethanol extract of *H. suaveolens* showed a maximum degree of inhibition against the bacteria species with varied diameters.

***In vitro* antioxidants.** Table 4 represents the *in vitro* antioxidant assay of the ethanol and aqueous extracts of *H. suaveolens* leaves. The free radical scavenging activity in the aqueous and ethanol extracts was 1.44 ± 0.50 and 1.57 ± 1.40 mg of ascorbic acid/1 g dry leaves, respectively. The ferric reduction in the aqueous and ethanol extracts was 1.19 ± 0.40 and 1.69 ± 0.18 mg of ascorbic acid/1 g dry leaves, respectively. For hydroxyl scavenging, it was 65.00 ± 0.90 and 0.43 ± 0.50 mg of ascorbic acid/1 g dry leaves for the aqueous and ethanol extracts, respectively. The leaves possessed appreciable antioxidant properties. Since they contained large amounts of bioactive compounds, they could be screened for more medical-related indices to further justify their value in therapy. Although the body produces several of the antioxidants it uses, exogenous antioxidants found in diets are also very important for health maintenance. These free radical scavengers interact with free radicals and neutralize them to avert cellular injury [23].

The reducing power was stronger in the ethanol extract, which could be related to the hydrogen-denoting capacity of the free radical scavenged by the DPPH assay. The leaves of *H. suaveolens* could therefore be important in reducing the harmful effects caused by free radicals and in managing health issues such as Parkinson's disease, the aging process, dementia, and cancer [22]. The ferric-reducing activity of the ethanol leaf extract further strengthened the correlation of the

Table 3 Qualitative and quantitative phytochemicals in the extracts of *Hyptis suaveolens* L.

Phytochemicals	Water extract		Ethanol extract	
	Quality	Quantity	Quality	Quantity
Alkaloids	+	12.20 ± 2.60 mg/mL	+	26.20 ± 0.52 mg/g
Phenols	+	1.20 ± 0.54 mg/g	+	3.20 ± 0.20 mg/g
Tannins	+	168.00 ± 0.34 mg/100 g	+	318.00 ± 0.15 mg/100 g
Flavonoids	+	13.80 ± 0.24 mg/g	+	24.00 ± 0.02 mg/g
Glycosides	+	54.00 ± 0.18 mg/100 g	+	117.00 ± 0.05 mg/100 g
Saponins	–	10.00 ± 0.05 mg/100 g	+	13.60 ± 0.31 mg/g
Steroids	+	5.40 ± 0.63 mg/g	+	28.80 ± 0.42 mg/g
Phlobotannins	+	1.25 ± 0.14 mg/g	+	2.18 ± 0.12 mg/g
Terpenoids	+	1.16 ± 0.23 mg/g	+	2.31 ± 0.24 mg/g

"+" – present

"–" – absent

Table 4 *In-vitro* antioxidants of *Hyptis suaveolens* L.

FRAS DPPH, mg of ascorbic acid/1 g dry plant material		FRAP, mg of ascorbic acid/1 g dry plant material		Hydroxyl radical scavenging assay	
Water extract	Ethanol extract	Water extract	Ethanol extract	Water extract	Ethanol extract
1.44 ± 0.50	1.57 ± 1.40	1.19 ± 0.40	1.69 ± 0.18	65.00 ± 0.90	0.43 ± 0.50

Table 5 Proximate composition of *Hyptis suaveolens* L. leaves

Proximate parameters	Composition, %
Crude fiber	8.72 ± 1.40
Crude protein	13.40 ± 0.30
Ash	7.15 ± 1.20
Lipid	3.26 ± 0.70
Carbohydrates	70.18 ± 1.30

Table 6 Mineral composition of *Hyptis suaveolens* L. leaves, %

Na	Ca	N	P	K	Mg
0.50	1.08	3.02	1.03	1.78	0.73

observed free radical scavenging activity. However, in addition to the extract's suitability in managing certain diseases, as well as the correlation between the DPPH and FRAP values, it can also act as a free electron and engage with free radicals' conversion to militate a higher product that could break the radical chain reaction and possibly prevent cardiovascular diseases.

Nutritional contents. The proximate composition of the leaf extracts under study included reasonable crude fiber, crude protein, ash, lipid, carbohydrate, as well as chemical elements as sodium, calcium, nitrogen, phosphorus, potassium, and magnesium (Tables 5 and 6).

The leaves' valuable nutritional contents are useful for maintaining body cells and other health benefits. The essential mineral elements present in the leaves are highly important in human nutrition due to their role in health care. These minerals help repair physiological damages, which makes them vital elements as food supplements for a healthy immune system. For instance, Umedum *et al.* reported that copper is helpful in the elimination of free radicals and in many other physiological processes [24]. According to Rai *et al*., iron is used as an active site of many redox enzymes associated with cellular respiration, as well as oxidation and reduction in animals [25]. Edeoga *et al.* reported on fiber's physiological effect on the gastrointestinal ability to lower the rate of tracolonic pressure, which is useful in diverticular disease [26]. The authors also found that it has a biochemical effect on the absorption and re-absorption of bile acids, cholesterol, and dietary fats.

Despite the fact that several plants have been screened for their phytochemicals and antimicrobial activities, the majority of these plants are not edible. Those compounds which are biologically active and edible are important mostly in medicinal plants used in traditional medicine to avoid the danger of poisoning among local users of urban and rural areas. *H. suaveolens* will be used by many people since it is an edible plant with leaves rich in alkaloids with medical implications, as well as other health-benefiting metabolites [27].

CONCLUSION

The antibacterial activity of the *Hyptis suaveolens* L. extracts was due to the bioactive compounds present in the plant. This confirms and supports previous observations, suggesting that the extracts of *H. suaveolens* may be useful as a control agent against certain bacterial pathogens. The extracts also exhibited good phytochemical and antioxidant activities. It should be noted that the ethanol extract had better antibacterial, phytochemical and antioxidant properties compared to the water one. Further research is needed to identify, isolate, and characterize those compounds which could contribute to a new medicine that is readily available, affordable, and effective in treating infectious diseases.

CONTRIBUTION

Fred Coolborn Akharaiyi conceived the research idea, as well as designed and wrote the first draft. Chioma Bertha Ehis-Eriakha and Peter Taiwo Olagbemide reviewed the literature, while Faith Hukwu Igbudu analyzed the data. All the authors read and approved the final draft of the manuscript before submitting it for publication.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Fruit and vegetable purees as cryoprotectants for vacuum freeze-dried fermented milk products

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Abstract:
Fresh fermented milk products have a limited shelf life that can be extended by vacuum freeze-drying. Cryoprotectants are used to increase the survival of lactic acid microorganisms during freeze-drying. The most effective cryoprotectants are those of natural origin. Literature offers little information on the cryoprotective effects of fruit and vegetable purees. Therefore, we aimed to evaluate the effectiveness of fruit and vegetable purees in increasing the survival of lactic acid microorganisms during the freeze-drying and storage of fermented milk products.

We studied bioyogurt samples containing pumpkin, fig, and banana purees. Rational modes of freezing and freeze-drying were established on the basis of thermal analysis. The cryoscopic temperature was determined by differential scanning calorimetry. The proportion of frozen moisture was calculated using the Nagaoka formula. Standard methods were employed to evaluate the sensory characteristics of bioyogurts and determine their protein, fat, and non-fat milk solids contents, as well as titratable acidity and microbiological indicators.

The addition of pumpkin puree increased the cryoscopic temperature and reduced the freeze-drying stage and the total drying time by 13 h, depending on the amount of puree. However, the addition of sweet fig and banana purees decreased the cryoscopic temperature and increased the freeze-drying stage and the total drying time by 0.5–1.5 and 1.5–3 h, respectively. Based on the sensory evaluation of the freeze-dried bioyogurts, we selected the formulations with 15% of pumpkin and fig purees and 10% of banana puree. We found that the freeze-dried bioyogurts with puree had higher counts of lactic acid bacteria compared to the control. In the freeze-dried samples, the counts were higher at a storage temperature of 4 ± 2°C than at 20 ± 2°C.

Pumpkin puree provided the best survival of lactic acid microorganisms during freeze-drying and storage.

Keywords: Bioyogurt, vacuum freeze-drying, cryoprotectants, pumpkin puree, fig puree, banana puree, cryoscopic temperature, proportion of frozen moisture, lactic acid microorganisms, shelf life

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INTRODUCTION

Fermented milk products are widely used around the world due to their nutritional value and health benefits. These include yogurt, curdled milk, baked milk, kefir, cottage cheese, and other products. Their positive effect is associated with lactic acid and probiotic microorganisms. These microorganisms normalize the gastrointestinal tract and the lipid profile, enhance immunity, treat allergies, prevent intestinal cancer, maintain normal cholesterol levels, and have many other effects [1–5].

Lactic acid and probiotic bacteria cells in fresh fermented milk products gradually decrease in number, which has a negative effect on their quality. Vacuum freeze-drying is a promising way to preserve the quality of fermented milk products with a high content of viable cells of lactic acid microorganisms [6].

The freeze-drying technology expands the uses of fermented milk products. For example, freeze-dried yoghurt or curdled milk can be used as instant foods, drinks or sauces, in ready-made cereals or in the confectionery and bakery products. Their reduced weight and volume account for lower packaging,

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handling, and transportation costs. In addition, freeze-dried products can be stored at ambient temperature for a long time. This is convenient for their transportation to the northern regions or to the affected regions during natural disasters [7–9].

Freeze-drying involves removing frozen moisture by the ice-vapor phase transition, which reduces damage to cellular structures [10]. Previous studies have shown that some strains of probiotic lactic acid microorganisms tolerate freeze-drying better than others. This is associated with their size and composition of the cell wall and membrane [10]. During heat treatment and storage of freeze-dried yogurt, the viability of probiotic microorganisms can be affected by oxygen content, high temperature, low pH, water activity, and higher concentrations of solutes [11].

Using cryoprotectants is an efficient way to increase the survival of microorganisms during freezing or freeze-drying [12, 13]. Carbohydrates are known to have a protective effect on freezedried probiotics. They include trehalose, sucrose, lactose, fructooligosaccharides, galactooligosaccharides, and inulin [14–19]. Some proteins and antioxidants can also have a protective effect, including skim milk, soy protein, ascorbic acid, and L-cysteine [20]. Some salts, e.g., phosphates, can also act as cryoprotectants [21]. According to Shu *et al.*, the best effect can be achieved by using a combination of several cryoprotectants [20].

In this regard, it is important to study the influence of natural fruit and vegetable purees on the viability of microorganisms in fermented milk products during freeze-drying and further storage. Fruit and vegetable purees contain mono- and disaccharides, dietary fiber, mineral salts, antioxidants, and other substances with cryoprotective properties. We aimed to evaluate the effectiveness of some vegetable and fruit purees as cryoprotectants during the freezing and vacuum freeze-drying of bioyogurts. We also measured their effect on the viability of lactic acid, including probiotic, microorganisms during storage.

STUDY OBJECTS AND METHODS

We studied freshly prepared and freeze-dried bioyogurts produced with starter cultures based on the following new strains of lactic acid bacteria with technological and functional properties: *Streptococcus salivarius thermophilus* (strain HST-20), *Lactobacillus delbrueskii* subsp. *bulgaricus* (strain HLB-8), and *Lactobacillus acidophilus* (strain HLA-41). Purees from pumpkin (a source of pectins), as well as figs and banana (sources of inulin), were used as cryoprotectants. The puree samples were purchased from OptTorg Company, Russia. Their chemical composition is shown in Table 1.

Bioyogurt preparation. Bioyogurts were produced from reconstituted whole milk powder using the traditional yogurt technology. The test samples (with puree) were produced with the above-mentioned starter of new strains of lactic acid bacteria. The samples were obtained thermostatically by ripening at 37 ± 1°C. After reaching the required titratable acidity (88 ± 5°T), they were cooled to 4 ± 2°C. The puree amounts were based on literature analysis, preliminary experiments, and mass fractions of solids in the final product. Each type of puree was added to the bioyogurt in the amount of 10, 15, and 20% and mixed until a homogeneous consistency.

The control bioyogurt was produced with a typical starter culture without adding fruit or vegetable purees.

The control and test bioyogurts were then analyzed for their physicochemical, microbiological, and sensory parameters. Next, we determined the cryoscopic temperature and plotted the dependence of frozen moisture on temperature.

Thermal analysis. Thermal analysis was used to study the behavior of the bioyogurts during freezing and to determine optimal freezing and drying temperatures. The cryoscopic temperature of the bioyogurts was determined by differential scanning calorimetry (DSC) from +12 to –50°C on a Q20 unit (TA Instruments, USA) at an Ar flow of 50 mL/min.

The proportion of frozen water was calculated using Raoult's law to substantiate the Nagaoka formula, which describes the process of freezing water as:

$$\omega = 1 - \frac{t_{кр}}{t} = 1 - \frac{273 - T_{кр}}{273 - T}$$

where ω is the proportion of frozen water; $t_{кр}$ is the cryoscopic temperature, t is the current temperature, °C; $T_{кр}$ and T are the cryoscopic and current temperatures, K.

Based on the results, graphs were plotted showing the relation between frozen moisture and decreasing temperature. These data can be used to decide on the modes and terms of product storage.

Freezing. The bioyogurt samples were frozen in a freezer at –40°C with intensive air circulation at about 10 m/s to obtain a fine-grained structure. This mode is widely used in industrial freezers. Besides, 95% of moisture crystallizes at –40°C, which is sufficient for high-quality freeze-drying. Next, the trays with the frozen product were placed in an SVP-0.36 laboratory vacuum freeze-drying unit [22].

Table 1 Chemical composition of fruit purees under study

Indicator, %	Pumpkin puree	Banana puree	Fig puree
Moisture	82.9	76.2	83.2
Protein	1.7	1.1	0.6
Fat	6.2	0.5	0.2
Carbohydrates, including:	7.8	21.4	14.4
– mono- and disaccharides	3.8	17.3	11.1
– dietary fiber	1.5	2.4	2.7
– starch	2.5	1.7	0.6
Minerals	1.0	0.6	1.0
Antioxidants	0.004	0.003	0.005

Vacuum freeze-drying. Vacuum freeze-drying was carried out at the temperatures that ensured the removal of 85% of frozen moisture by the ice-vapor phase transition. This proportion of frozen moisture was based on our previous experiments and the recommendations of domestic and foreign researchers [23, 24]. The choice of the freeze-drying temperature was based on the thermal DSC analysis. The final drying temperature was 38–40°C. The total freeze-drying process lasted from 9 to 14.5 h, depending on the sample. The freeze-drying was considered to be completed when the final moisture in the freeze-dried samples was under 4%. The final moisture was determined by five repetitions.

Bioyogurt quality indicators. The quality indicators of the freeze-dried bioyogurt samples were determined by standard physicochemical, microbiological, and sensory analyses according to State Standard 3624-92 for titratable acidity, State Standard 3626-73 for moisture, and State Standard 30305.4-95 for solubility.

Storage of the samples. The freeze-dried bioyogurts were stored in vacuum light- and gas-tight packaging under two temperature conditions: at 4 ± 2°C in a refrigerator (control) and at 20 ± 2°C in a thermostat. The samples were stored for 12 months.

Sensory evaluation. Freshly prepared and freeze-dried bioyogurts were evaluated for color, taste, smell, consistency, texture, and general acceptance. Each indicator was evaluated on a five-point scale. The freeze-dried bioyogurts were preliminarily rehydrated with water at room temperature to the initial liquid state, with the required mass fraction of solids. The control was a rehydrated freeze-dried bioyogurt produced with a typical starter without fruit or vegetable purees.

The samples selected by the panelists were stored for further use.

Counting of lactic acid microorganisms. Counts of lactic acid microorganisms were determined in the bioyogurt samples before and after vacuum freeze-drying, as well as during storage after 3, 6, 9, and 12 months. This was done by their inoculation and cultivation in sterile skim milk, followed by the estimation of the most probable number of cells. The dishes were incubated anaerobically, while the cultures in sterile milk in the test tubes were incubated under aerobic conditions at 37°C for 5 days according to State Standard 33951.

RESULTS AND DISCUSSION

First, we performed thermal analysis to determine the cryoscopic temperature and the proportion of frozen moisture as the temperature decreased (Fig. 1).

As shown in Fig. 1, the addition of pumpkin puree increased the cryoscopic temperature, whereas the addition of fig or banana puree decreased this indicator. This was due to a higher content of mono- and disaccharides in figs and bananas compared to pumpkin. Their presence in the product, with other parameters being equal, is known to lower the freezing temperature [25].

Based on the differential scanning calorimetry (DSC), we plotted the dependences of frozen moisture on freezing temperature for each type of bioyogurt (Figs. 2–4).

As can be seen in Fig. 2, 85% of moisture in the control bioyogurt (without puree) turned into ice at –16°C. In the samples with 10, 15, and 20% of pumpkin puree, this proportion of frozen moisture formed at –14, –13, and –12°C, respectively.

As shown by Fig. 3, the addition of banana puree to bioyogurt decreased the proportion of frozen moisture at the same temperatures. Particularly, at –16°C, the control bioyogurt (without puree) had 85% of frozen moisture, while the sample with 10% of banana puree, 83%. The required proportion of 85% formed at a lower temperature of –18°C. The samples with 15 and 20% of banana puree had 85% of frozen moisture at –19 and –20 °C, respectively.

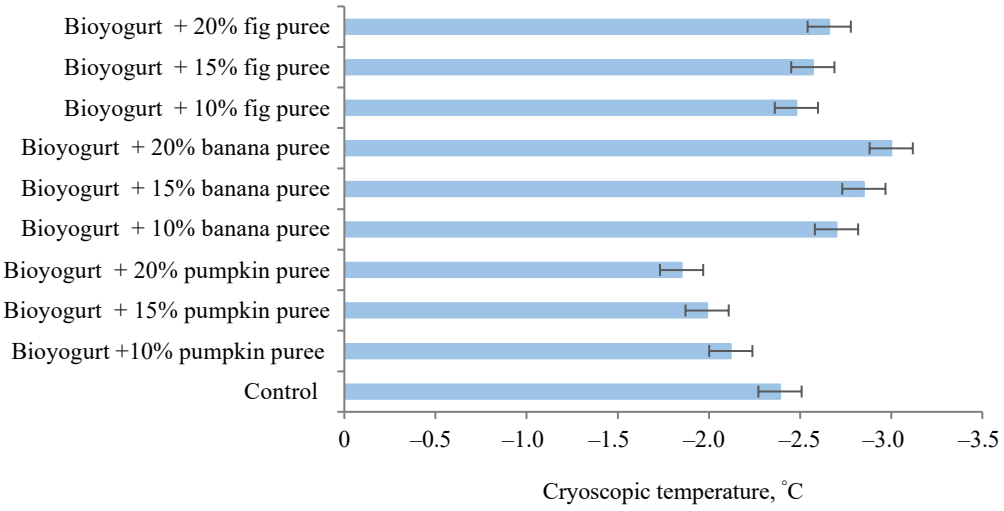


Figure 1 Cryoscopic temperature of the bioyogurts with fruit purees

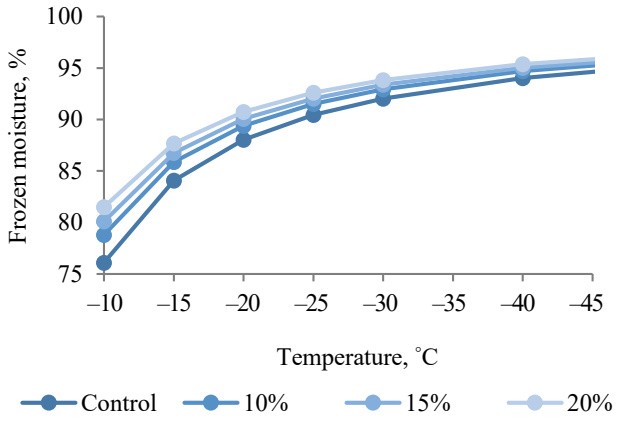


Figure 2 Frozen moisture in the bioyogurt with pumpkin puree in different amounts

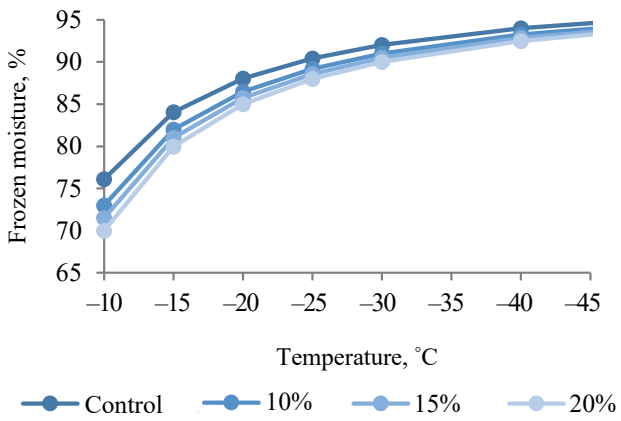


Figure 3 Frozen moisture in the bioyogurt with banana puree in different amounts

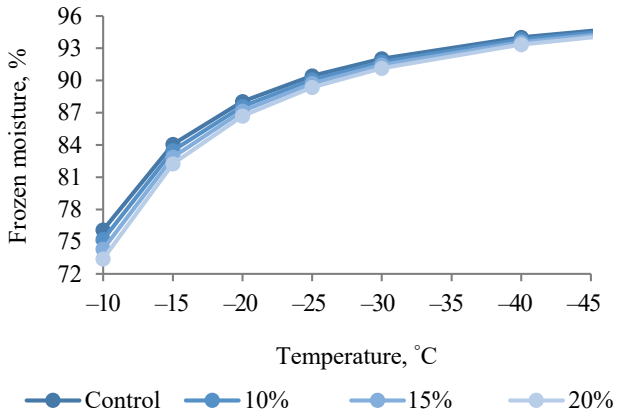


Figure 4 Frozen moisture in the bioyogurt with fig puree in different amounts

The bioyogurts with fig puree showed the same trend as the samples with banana puree (Fig. 4). The temperature at which 85% of moisture turned into ice decreased to –16.5 and –17.5°C in the samples with 10 and 20% of fig puree, respectively.

Based on the cryoscopic temperature and the proportion of frozen moisture, the bioyogurt samples were freeze-dried to 3.5–4.0% of moisture (Table 2).

As we can see, the addition of 10% of pumpkin puree only slightly changed the freeze-drying temperature of the bioyogurt. Yet, larger amounts of 15 and 20% increased the freeze-drying temperature by 3 and 4°C, respectively. We also found that the total freeze-drying time decreased from 12 h (control) to 11 and 9 h (bioyogurts with 10 and 20% of pumpkin puree, respectively).

Adding 10% of banana puree hardly changed the freeze-drying temperature, decreasing it by only 2–3°C. Yet, larger amounts of 15 and 20% lowered the temperature to –19.0 ± 0.5 and –20.0 ± 0.5°C, respectively, and therefore increased the total drying time by 1.5–3 h. Similar data were obtained for the bioyogurts with fig puree.

According to the results, introducing banana, fig, or pumpkin puree into the samples of bioyogurt did not lead to significant changes in the freeze-drying temperatures at which 85% of moisture was removed by the ice-vapor phase transition. This means that these products can be freeze-dried in the same machine with technical characteristics common for industrial machines. An increase in the freeze-drying time with a decrease in the freeze-drying temperature is a pattern that has been reported by other researchers as well [22, 26].

Next, we evaluated the quality indicators of rehydrated freeze-dried bioyogurts. Their physicochemical parameters are shown in Fig. 5.

According to the physicochemical parameters, the addition of purees led to some changes in the chemical composition of the bioyogurts. In particular, all the test samples had a lower content of protein compared to the control. On average, it amounted to 5–10% in the samples with the minimum amount of puree (10%) and 13–16% in the samples with the maximum amount of puree (20%).

The bioyogurts with banana and fig purees had a lower fat content of 2.6%, which is due to low fat in the purees. The opposite trend was observed in the samples with pumpkin puree. Since pumpkin puree contains more fat than the other purees, the samples with 10 and 20% of pumpkin puree had a fat content of 3.5 and 3.8%, respectively.

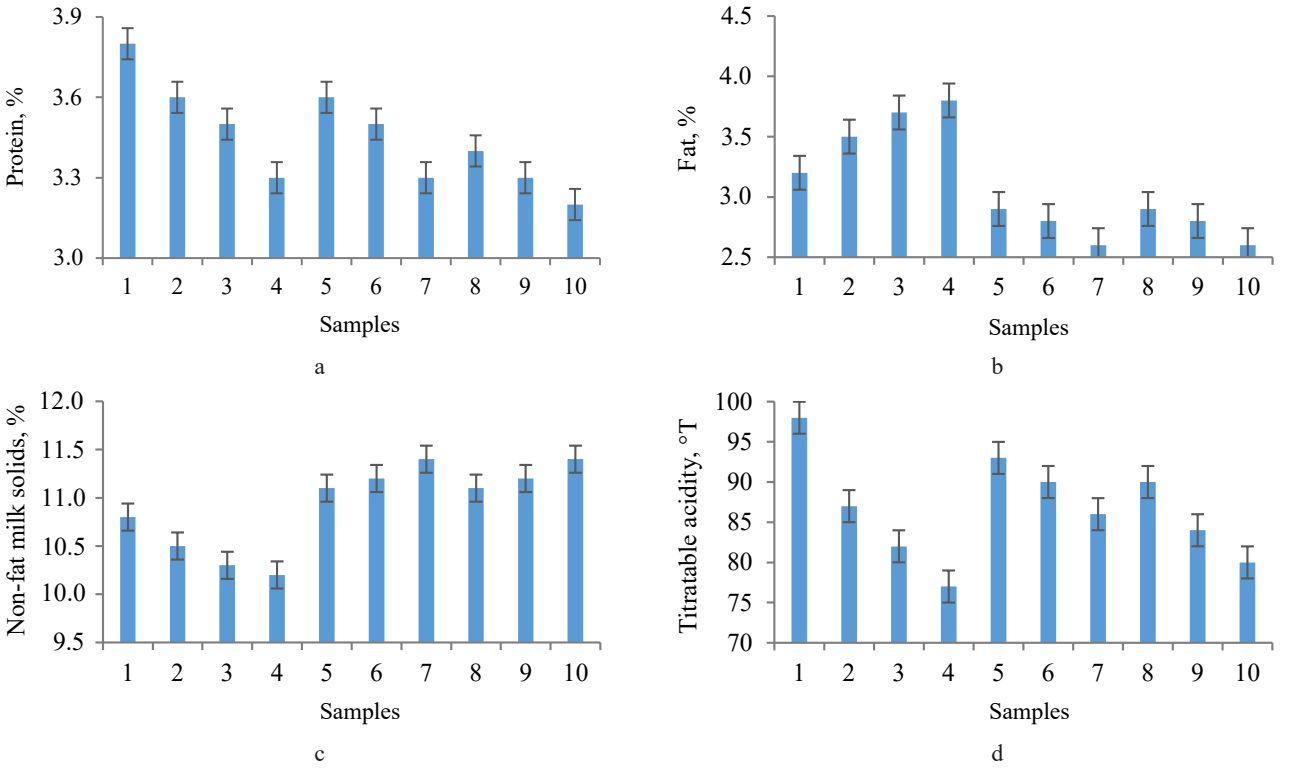
The mass fraction of non-fat milk solids was within the normal range in all the samples. In particular, it was 10.2–10.5% in the samples with pumpkin puree and 11.1–11.4% in the samples with banana and fig purees.

All the test samples of freeze-dried bioyogurts had decreasing values of titratable acidity, which is due to the presence of various acids in the purees.

The physicochemical and microbiological parameters of all the samples were in line with the regulatory requirements for this type of product. For this reason, we proceeded with only sensory evaluation.

Table 2 Modes of vacuum freeze-drying of bioyogurts with fruit purees

Bioyogurt	Freeze-drying temperature, °C	Final drying temperature, °C	Average total freeze-drying time, h
Control (without puree)	–16.0 ± 0.5	39.0 ± 0.5	12.0 ± 0.5
Pumpkin puree bioyogurt			
10%	–14.0 ± 0.5	39.0 ± 0.5	11.0 ± 0.5
15%	–13.0 ± 0.5	39.0 ± 0.5	10.0 ± 0.5
20%	–12.0 ± 0.5	39.0 ± 0.5	9.0 ± 0.5
Banana puree bioyogurt			
10%	–18.0 ± 0.5	39.0 ± 0.5	13.5 ± 1.0
15%	–19.0 ± 0.5	39.0 ± 0.5	14.5 ± 0.5
20%	–20.0 ± 0.5	39.0 ± 0.5	15.0 ± 0.5
Fig puree bioyogurt			
10%	–16.5 ± 0.5	39.0 ± 0.5	12.5 ± 0.5
15%	–17.0 ± 0.5	39.0 ± 0.5	13.0 ± 1.0
20%	–17.5 ± 0.5	39.0 ± 0.5	13.5 ± 1.0



1 – control (bioyogurt without puree), 2 – bioyogurt with 10% of pumpkin puree, 3 – bioyogurt with 15% of pumpkin puree, 4 – bioyogurt with 20% of pumpkin puree, 5 – bioyogurt with 10% of banana puree, 6 – bioyogurt with 15% of banana puree, 7 – bioyogurt with 20% of banana puree, 8 – bioyogurt with 10% of fig puree, 9 – bioyogurt with 15% of fig puree, 10 – bioyogurt with 20% of fig puree

Figure 5 Physicochemical parameters of rehydrated yogurts

The results of the sensory evaluation of the rehydrated freeze-dried bioyogurts are presented in Figs. 6–8.

The sensory evaluation of the bioyogurts showed that adding 10% of pumpkin or fig purees led to slight changes in sensory indicators. In particular, the panelists noted light color shades and weak aromatic notes characteristic of the added purees. Yet, the bioyogurts with 10% of banana puree acquired a pronounced banana taste and aroma.

Consistency was not affected by adding 10% of any of the studied purees.

Adding 15% of puree caused significant changes in the sensory characteristics of the bioyogurts. The samples had a more pronounced smell, taste, and color characteristic of the added purees. The panelists also noted a thicker consistency of these samples.

The bioyogurts with 20% of puree had almost no smell or taste of yogurt, acquiring the smell and taste of the added puree. These samples had the thickest consistency compared to the other bioyogurts.

Based on the sensory evaluation, we selected the bioyogurts with 15% of pumpkin and fig purees,

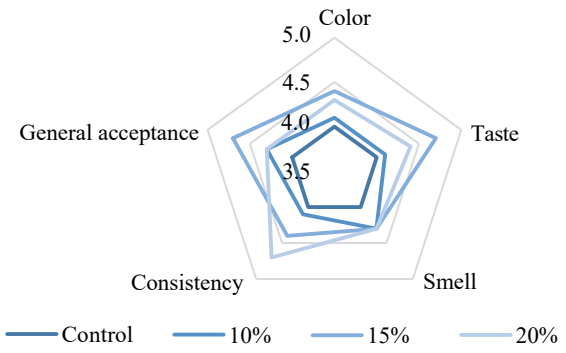


Figure 6 Sensory indicators of rehydrated freeze-dried bioyogurts with pumpkin puree in different amounts

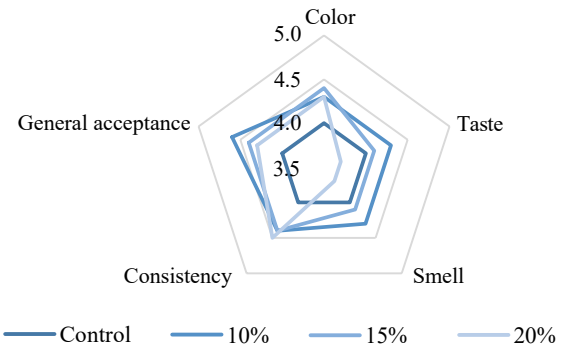


Figure 7 Sensory indicators of rehydrated freeze-dried bioyogurts with banana puree in different amounts

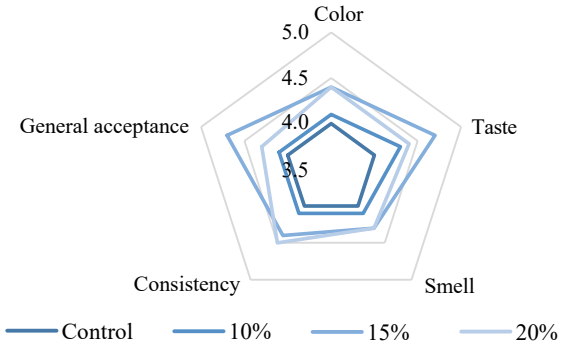


Figure 8 Sensory indicators of rehydrated freeze-dried bioyogurts with fig puree in different amounts

as well as the bioyogurt with 10% of banana puree, for further research.

The count of lactic acid microorganisms is one of the regulated microbiological indicators of fermented milk products. The counts for the freshly prepared and rehydrated freeze-dried bioyogurts are presented in Table 3.

We found that all the rehydrated freeze-dried bioyogurts had slightly lower counts of lactic acid microorganisms than the freshly prepared samples. However, their counts remained above the required level of at least 1.0×10^7 CFU in 1 g/cm^3. Notably, the rehydrated bioyogurts with puree had a greater count of lactic acid microorganisms. This may be due to the presence of mono- and disaccharides, as well as soluble dietary fiber, in the puree.

Physically, purees contain substances that penetrate the cells and those that do not. There is a displacement hypothesis that explains the protective effect of penetrating cryoprotectants. According to this hypothesis, when ice crystals form in the object of freezing, cryoprotectants displace concentrations of inorganic ions (salt effect) from the hydration shell of proteins and from the membranes without directly interacting with them. There is also a replacement hypothesis that claims that some penetrating cryoprotectants are able to replace water molecules associated with the polar part of membrane phospholipids, thus preventing the salt effect. Non-penetrating cryoprotectants (e.g., sucrose, fructose, dextran, etc.) "colligatively" displace salts from near-membrane water layers. As a result, during ice formation, salts are concentrated outside the cells, which reduces their damaging effect. Also, non-penetrating cryoprotectants enhance the effect of penetrating cryoprotectants [13].

Our data were consistent with the results of other studies on the freeze-drying of yoghurts. For example, Venir *et al.* studied the freezing and freeze-drying of low-fat yogurt and yogurt with 10% of sucrose and 10% of blueberries [27]. After freezing, the counts of lactobacilli and streptococci in these yoghurts decreased by 0.7 and 1.7 log CFU/g, respectively, while after freeze-drying, by 1.5 and 2.3 log CFU/g, respectively. The authors suggested that the yogurt with sucrose and blueberries retained more viable microorganisms due to the cryoprotective effect of sucrose.

The selected test and control samples of freeze-dried bioyogurts were put into storage for 12 months at 4 ± 2 and 20 ± 2°C. The results of studies during storage are presented in Figs. 9 and 10.

Table 3 Counts of lactic acid microorganisms in bioyogurts before and after freeze-drying

Samples	Freshly prepared bio-yogurt before freeze-drying, CFU/g	Rehydrated bioyogurt after freeze-drying, CFU/g
Control (bioyogurt)	2.5×10^8	6.0×10^7
Bioyogurt with 15% of pumpkin puree	6.0×10^8	5.0×10^8
Bioyogurt with 10% of banana puree	2.5×10^8	2.0×10^8
Bioyogurt with 15% of fig puree	5.0×10^8	3.0×10^8

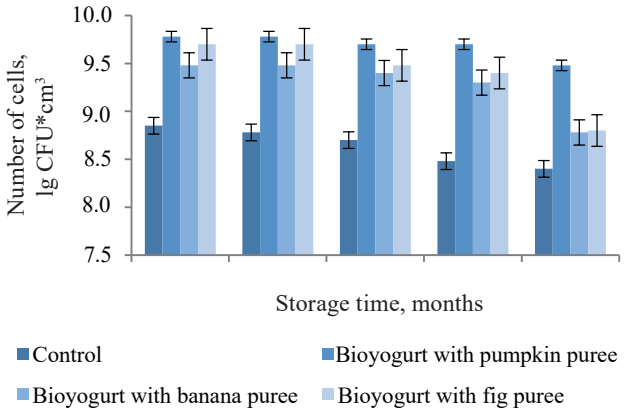


Figure 9 The number of cells of lactic acid microorganisms in freeze-dried bioyogurts by stage of storage at 4 ± 2°C

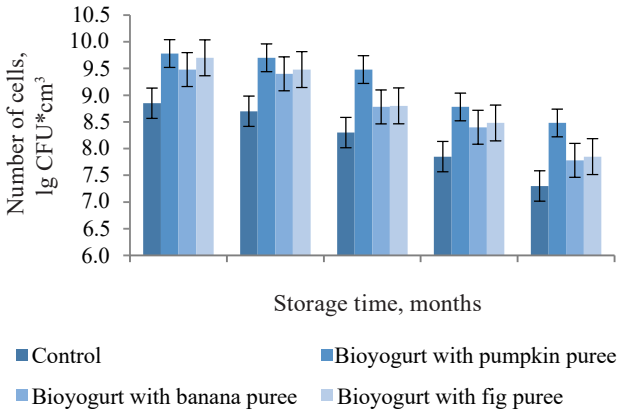


Figure 10 The number of cells of lactic acid microorganisms in freeze-dried bioyogurts by stage of storage at 20 ± 2°C

The numbers of cells of lactic acid microorganisms during storage indicate their high viability in freeze-dried bioyogurts based on new starter cultures.

Their safety was found to be affected by the storage temperature and the type of puree used.

After 12 months of storage at 4 ± 2 and 20 ± 2°C, the smallest count of lactic acid microorganisms was detected in the control sample. Throughout this period, the bioyogurts with puree had higher counts at both storage temperatures than the control. In addition, they met all the requirements established for bioyogurts.

All the samples of freeze-dried bioyogurts had a higher count of lactic acid microorganisms at a storage temperature of 4 ± 2°C. We found that pumpkin puree contributed to a better survival of lactic acid microorganisms during freeze-drying and storage at both temperatures compared to the fruit purees.

Our data were consistent with the results of studies on freeze-drying and storage of fermented milk products supervised by Professor I.A. Radaeva at the All-Russian Research Institute of the Dairy Industry (VNIMI) in the 1970–1980s. In those studies, after 15 months of storage at 4°C, the number of surviving lactic acid bacteria in sweet and fruit yogurt decreased 10 times compared to freshly prepared yogurts. The shelf life of fruit yoghurts stored at 20°C was 12 months and at 1–4°C, 18 months. Such a long shelf life made these products suitable for astronauts [28]. Some researchers noted slight changes in the number of viable *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in dried yogurt stored under vacuum at 4°C or under nitrogen for 9 months [29].

Saarela *et al.* reported that freeze-dried microorganisms *Lactobacillus* and *Bifidobacterium* survived better at low temperatures than at room temperature [30].

Araújo *et al.* studied the protective effect of tropical fruit by-products on *Lactobacillus paracasei* L-10, *Lactobacillus casei* L-26, and *Lactobacillus acidophilus* LA-05 during freeze-drying and storage [31]. They found that the by-products of acerola, cashew, and guava had a protective effect and increased the stability of probiotic lactobacilli during freeze-drying and during 90 days of storage at 4.0 ± 0.5 and 25.0 ± 0.5°C. The authors attributed the protective effect to the contents of monosaccharides, phenolic compounds, and dietary fiber in the by-products.

Our study showed that pumpkin, fig, and banana purees have cryoprotective effects during freezing and freeze-drying of fermented milk products due to the presence of mono- and disaccharides, proteins, antioxidants, and dietary fiber in them.

CONCLUSION

Our study showed the effectiveness of introducing pumpkin, fig, and banana purees into bioyogurt as cryoprotectants during freeze-drying. These purees can also enrich bioyogurts with prebiotics (mono- and disaccharides, dietary fiber, and antioxidants) and increase the viability of lactic acid microorganisms. Mono- and disaccharides, minerals, and antioxidants contained in puree reduce the level of cryodamage of lactic acid microorganisms during freezing, contributing to their better survival and preservation after freezedrying.

The addition of puree to bioyogurts affects their thermophysical characteristics. We found that adding 15% of pumpkin puree allows for raising the freeze-drying temperature from –16 to –13°C, with all the other parameters being equal. This can decrease specific energy consumption during freeze-drying and reduce its time. All the studied bioyogurts with puree can be freeze-dried under vacuum using domestic industrial freeze-drying units.

Adding puree to bioyogurts can also significantly improve their sensory profile and expand the range of freeze-dried fermented milk products of this type.

CONTRIBUTION

I.S. Krasnova developed a general idea of using plant-based puree as a cryoprotectant during freezing and freeze-drying based on literature analysis, devised a plan of research, produced bioyogurt samples,

evaluated their quality, analyzed the results, and formulated conclusions. V.I. Ganina developed research methodology, determined the counts of lactic acid microorganisms, analyzed the results, and formulated conclusions. G.V. Semenov performed thermal analysis and summarized the data on thermal analysis, freezing, and vacuum freeze-drying, analyzed the results, and formulated conclusions.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Thymoquinone-related knowledge (1915–2022): A comprehensive bibliometric analysis

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Abstract:
Nigella sativa L. and its active component, thymoquinone, attract a lot of scientific attention. However, very few review articles on this issue have been published so far, and no review relied on the method of bibliometrics, which is currently the most prominent technique for citation mapping.
This review involves a total of 2072 studies on thymoquinone reported in the Scopus database between 1915 and September 15, 2022. The data obtained were processed using the VOSviewer software, MS Excel, and Scopus Analytic. The review introduces some prospective research areas based on theme mapping, knowledge trending, bibliographic coupling, and keyword co-occurrence networks. The authors, documents, journals, institutions, and countries were ranked based on the knowledge impact and the number of publications.
The 2072 selected publications belonged to 7605 scholars, with 3.67 authors per document on average. The average number of citations per document was 68.84. The Phytotherapy Research Journal scored as the top source. M.N. Nagi proved to be the top-cited author with 2076 citations, while Saudi Arabia appeared to be the most productive and cited country. The best-studied areas were represented by such topics as anti-inflammatory properties of thymoquinone, cytokine network, and arthritic disease model. Molecular-based approaches in conjunction with ethno-knowledge may be of assistance in comprehending the cellular mechanisms of thymoquinone and establishing its efficacy against a variety of diseases.

Keywords: Bibliometric analysis, thymoquinone, *Nigella sativa* L., performance analysis, Scopus database

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INTRODUCTION

Nigella sativa L. (*Ranunculaceae* family) is also called black caraway, black cumin, nigella, kalonji, or siyahdaneh. Its seeds are commonly known as black seeds in English and *Alhabbah Al-saudaa* in Arabic. Black seeds are a popular traditional medicine against a vast range of ailments in many regions of the world, especially in the Middle East and the Far East. *N. sativa* is cultivated in the Mediterranean region, western Asia, the Middle East, southern Europe, and northern Africa.

For centuries, *N. sativa* seeds have been used to treat fever, asthma, infection, inflammation, chest congestion, eczema, cough, bronchitis, flatulence, obesity, chronic headache, dysmenorrhea, diabetes, and diarrhea [1–4]. *N. sativa* contains some active ingredients that showed antioxidant, analgesic, anti-inflammatory, anti-asthmatic, antipyretic, antibacterial, antihypertensive, and antineoplastic properties [5]. *N. sativa* is a substantial and prospective source of several bioactive compounds, including 4-terpineol, p-cymene, thymoquinone (Fig. 1), dithymoquinone, thymohydroquinone, and t-anethol. In addition, *N. sativa* contains vitamins, fatty acids, proteins, mineral elements, and vital amino acids. *N. sativa* seeds also contain nigellimine, nigellidine, saponine, nigellcine, and water-soluble triterpenes.

Thymoquinone (2-isopropyl-5-methyl-1, 4-benzoquinone) exhibits antioxidant, antihistaminic, anti-Alzheimer, anticancer, analgesic, hepatoprotective, and anti-

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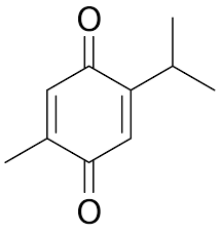


Figure 1 Thymoquinone: chemical structure

ischemic properties, besides being an excellent neuroprotector, insecticide, histone protein modulator, and renoprotector [6, 7].

Thymoquinone inhibits cancer at the stages of proliferation, migration, and invasion. It also functions as an anticancer agent against various human malignancies, including breast, pancreatic, blood, oral, prostate, bone, head and neck, cervical, lung, and liver cancers [8, 9]. Thymoquinone causes apoptosis and controls pro- and anti-apoptotic gene expression [8]. It effectively facilitates *miR-34a* up-regulation, increases *miR-34a* levels *via p53*, and suppresses *Rac1* expression [10]. In addition, thymoquinone diminishes the phosphorylation of *NF-B* and *IKK*, as well as the metastasis and the activity of *ERK1/2* and *PI3K*. Thymoquinone suppresses the spread of cancer cells by activating *JNK* and *p38* [11]. It owes its anticancer impact to its antioxidative potential and ability to reduce oxidative stress. Other scientists believe that thymoquinone induces apoptosis in cancer cells by inflicting oxidative damage [11, 12].

Thymoquinone can function as an antioxidant and a pro-oxidant in a dose-dependent way; it acts as an antioxidant at low concentrations and as a pro-oxidant at high ones. Thymoquinone showed a good potential against doxorubicin, a drug used to cause cardiotoxicity and free radicals that cause oxidative damage [13]. Thymoquinone also speeds up cell growth in rats, relieves oxidative stress caused by Fe-NTA, and works well as a chemoprotective phytochemical [14]. In recent research, thymoquinone proved to be an effective antidiabetic drug in healthy male volunteers: it lowered cholesterol and triglyceride levels while increasing high-density lipoprotein, glucose-induced insulin secretion, and insulin sensitivity [15].

As a natural substance, thymoquinone has a considerable pharmaceutical potential. For instance, it has become subject of in-depth research that determined its ability to stop the proliferation, migration, and invasion stages of human malignancies. However, its cancer-activity still remains understudied. Moreover, thymoquinone medication development requires adequate clinical studies. The most urgent task is to define the relationship between its structure and antitumor activity. Only then will scientists be able to address such issues as purification or commercial production of *N. sativa*-derived substances.

In general, thymoquinone appears in hundreds of studies that focus on a wide range of aspects. This enormous amount of bibliographic data needs an effective and comprehensive review that would combine this intellectual variety from a new perspective. This goal can be achieved using the interdisciplinary method of bibliometric techniques that include mathematics, statistics, data mining, mapping, and visualization [15]. Bibliometric methods involve performance analysis, keyword co-occurrence, co-authorship mapping, co-citation, and bibliographic coupling. All these techniques combined can reveal the knowledge structure, hotspots, international collaboration, trending, and road-mapping for future research [16, 17]. The current study was designed to analyze and visualize thymoquinone-related publications using the VOSviewer software, where the abbreviation stands for *visualizing scientific landscapes.*

STUDY OBJECTS AND METHODS

Selecting the database and keywords. Bibliometric analyses reflect the coverage of their underlying databases in the sense that the coverage essentially specifies what is included in the analysis. The bibliometric assessment contextualizes these publications against the database, which is likewise reliant on the coverage. Scopus was founded in 2004 by Elsevier, which claims it to be the most complete overview of the world's research outputs, monitored by a team of subject matter experts. Scopus's goal is to create the largest possible database of high-quality research publications. Scopus differs from Web of Science (WoS) in that WoS promotes quantity above quality, while Scopus attempts to balance between the two. Both Elsevier and Clarivate Analytics provide subscription-based databases, but we chose to use the Scopus database for our research objective.

Selecting the keywords is one of the fundamentals of bibliometric research. More data can be gathered and evaluated to yield findings that can be expanded upon in understanding performance, knowledge structure, hot spots, and other indicators of the bibliometric analysis. The bifurcation that accompanies all scientific research related to thymoquinone means that databases require several keywords since the research conducted on this natural compound included agriculture, chemistry, traditional and prophetic medicine, pharmacology, and computational biology. In this study, we chose the word *thymoquinone* as the keyword to search in the Scopus database. Thus, the search results are comprehensive for all types of related studies.

Sample size. The method of bibliometric analysis is applied when the number of bibliometric data is considerable, and the literature review is too broad for a manual examination. As a rule, bibliometric analysis is recommended in cases when the number of references exceeds 200 [18]. The average category of normalized citation impacts of bibliometric studies with smaller

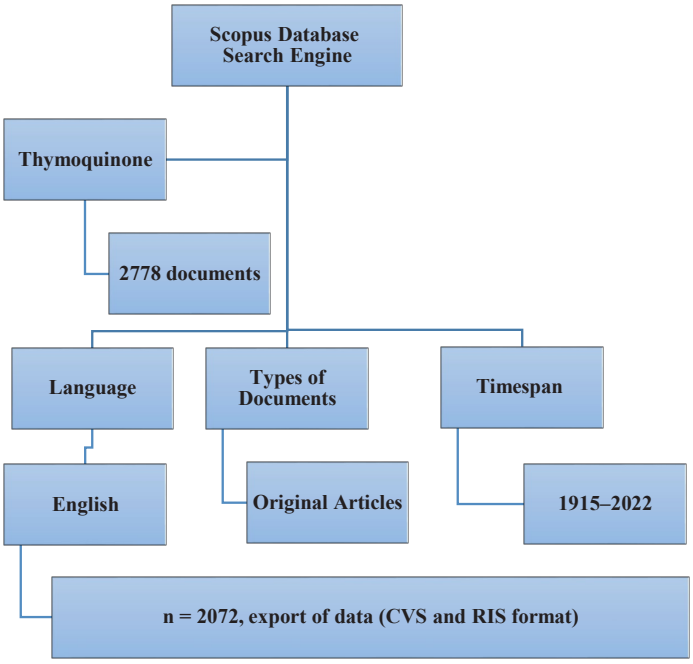


Figure 2 Search strategy, sampling criteria, and exportation of bibliographic data: ar – article; cp – conference paper

sample sizes (< 200 documents) varies greatly, making this method unreliable. The present research included trial-and-error approaches and keyword filtering, and the sample size was enough to apply the method of bibliometric analysis.

Search results. We checked some article titles in the Scopus database for the keywords to confirm that the bibliographic data were relevant to the study subject. The preliminary findings (n = 2778) included articles, reviews, letters, notes, editorials, errata, conference papers, short surveys, book chapters, conference reviews, data papers, and retractions (1915–2022). In addition, we collected bibliographic data for original research published in English, i.e., journal articles and conference papers (Fig. 2). This step made it possible to refine the conclusions. Finally, we downloaded the data on 2072 articles published in 1915–2022 into a CSV spreadsheet using Microsoft Excel.

Performance analysis and bibliographic mapping. The integrated Scopus analyzer helped us determine the volume of annual research and their citations, as well as the performance of the thymoquinone-related research. We used the method of regression analysis to define the incremental annual increase. Bibliographic mapping was performed using the VOSviewer platform. VOSviewer is a program for creating and displaying bibliometric networks. These networks may be built via citation, bibliographic coupling, co-citation, or co-authorship relationships and can comprise journals, researchers, or individual articles. VOSviewer also has text-mining tools for creating and visualizing co-occurrence networks of key phrases collected from the scientific literature.

RESULTS AND DISCUSSION

Overview. We identified 7605 scholars for 2072 sources with 3.67 as the average number of authors per document. The average number of citations per document was 68.84, which means that thymoquinone research is well-regarded in the academic community. The interdisciplinary character of the subject indicated a collaboration among specialists from many fields. Table 1 covers the general information regarding the documents utilized for this study. The most productive

Table 1 Main bibliographic data*

Description	Results
Timespan	1915–2022
Sources (journals, books, etc.)	949
Documents	2072
Average years from publication	2.18
Average citations per documents	68.84
Average citations per year per doc	1333.1
References	2072
Article	1117
Conference paper	21
Keywords	
All keywords	17 136
Author's keywords	4531
Index keywords	14 402
Authors	
Authors	7605
Documents per author	3.67

* The average number of citations per year was calculated by dividing the total number of citations by the number of years for all the documents

source, institution, and country proved to be *The Biomedical Sciences Instrumentation*, King Saud University (Saudi Arabia), and Saudi Arabia, respectively. The highest number of published articles belonged to such subject areas as *Biochemistry* and *Genetics and Molecular Biology*. H. Benghuzzi, affiliated with Global Training Institute, Flowood, United States, appeared to be the most productive author (n = 43). H. Benghuzzi's research focused on the effect of thymoquinone on cardiomyocyte, SiHa, and SH-SY5Y cell lines [19–21].

Performance analysis. Over the last eleven decades (1915–2022), the number of publications increased at an average yearly rate of 19.38%. The last ten years saw 75.2% of the total research production. According to the Scopus database, the first article on thymoquinone belonged to McPherson and Stratton [22]. They described the action of *α*-benzoyl-p-tolylhydrazine upon thymoquinone. They referred to thymoquinone as a previously identified chemical [22]. This indicates that thymoquinone had been discovered before 1915; however, the Scopus database has no information on the discovery.

Figure 3 shows the annual escalation of publications. The upward trend was fitted using a polynomial trendline (order 6; R-squared = 0.9676). When data vary, a polynomial trendline is represented as a curved line. The number of data fluctuations or bends in the curve establish the polynomial order. After the commencement, the number of publications began to climb drastically in 2000 and accounted for 15 scientific articles before reaching 259 in the year of 2021. This was before the number of publications reached 259 in 2021. The obtained results mean that thymoquinone attracts the attention of the global scientific community, and such a significant number of studies indicates its significance [5].

Research impact: citation networking. Scientific impact can be described using different scales, ranging from high to low for researchers and papers to national and institutional dimensions. Numerous studies concentrate on scientific achievement, scholarly network analysis, and scientific impact metrics. We are currently witnessing a dramatic rise in publications that feature the issue of scientific impact and how it has changed through time in the *science of science*. However, many of these studies focus on a certain historical period. Citation networks are a common tool for measuring scientific impact, although heterogeneous scholarly networks have lately drawn more attention. The structural measure, citation analysis, and behavioral complexity are all important factors in quantifying scientific effects in the diverse scholarly network [23, 24].

This section of our research highlights the most important and relevant sources on thymoquinone. According to the Scopus dataset, the 2072 papers we selected came from 949 sources. Table 2 shows the distribution of the top ten most pertinent sources. According to the number of publications, *Molecules* (25), *Phytotherapy Research* (22), and *PLoS ONE* (18) proved to be the top-publishing sources. The *Phytotherapy* research journal scored as the top source based on all the bibliometric metrics, e.g., the number of publications, total citations, average citation, *h*-index, and CiteScore (Table 2). As for the *h*-index, all the articles published in *The Journal of Ethnopharmacology*, *Planta Medica*, and *Anticancer Research* have been cited.

Analyzing bibliometric citations is a useful method for judging authors' output in scientific literature and knowledge generation [27]. Figure 4 shows the top scholars who have engaged in thymoquinone research throughout the years. These top prolific 40 authors were mapped using VOSviewer's density visualization out of 7605. Density visualizations provide a quick overview of the main authors in a bibliometric citation. For each of the 40 authors, we calculated the total strength of the citation links with other authors and selected the authors with the greatest total link strength. Nagi proved to be the top-cited author with 2076 citations, followed by Badary (n = 1793), Al-Shabanah (n = 1247), and Al-Bekairi (n = 1050). Nagi (Saudi Arabia) has worked on the antioxidant mechanisms of this natural compound on chemically induced disease models of liver, heart, lung, and kidney. The team used thymoquinone to eliminate experimental carcinogenicity in the liver [28–33]. Nagi's citation effect reflects extensive work on the

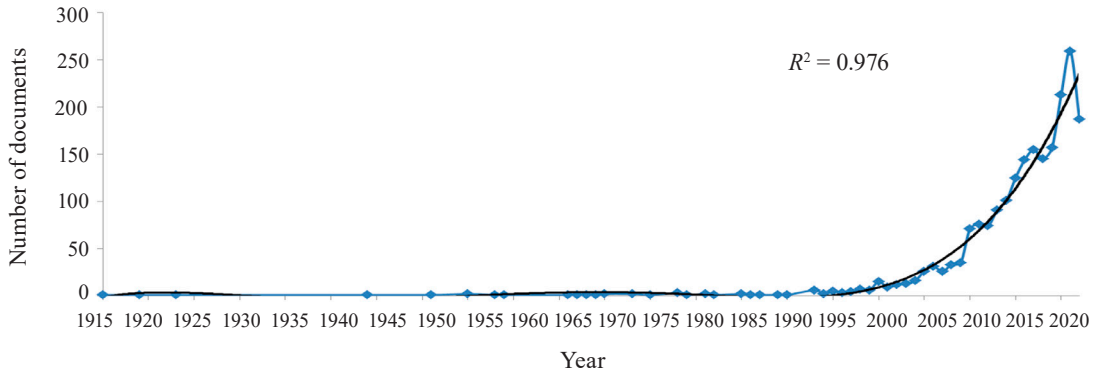


Figure 3 Annual escalation of documents. The incremental trend was fitted using a polynomial trendline (order 6; R^2 = 0.9676)

Table 2 Top-cited sources

Source	Documents	Citations	CA	*h*-index	CS 2021	Total link strength
Phytotherapy research	22	2315	105.22	13	9.3	214
Life Sciences	16	1109	69.31	12	8.0	109
Planta Medica	8	1088	136.00	8	6.2	141
Plos One	18	959	53.27	16	5.6	75
International Immunopharmacology	11	749	68.09	11	7.0	150
Food Chemistry	10	636	63.60	9	10.0	47
Anticancer Research	5	602	120.40	5	4.1	87
Journal of Ethnopharmacology	7	592	84.57	7	6.9	91
Chemico-Biological Interactions	15	575	38.33	11	9.0	113
Molecules	25	437	17.48	15	5.9	132

CA – citation average; *h*-index – the maximal value of *h* means that the given author/journal has published at least *h* papers that have each been cited at least *h* times [25]; CS – CiteScore, a metric that reflects the average annual number of citations to recent papers published in that journal [26]

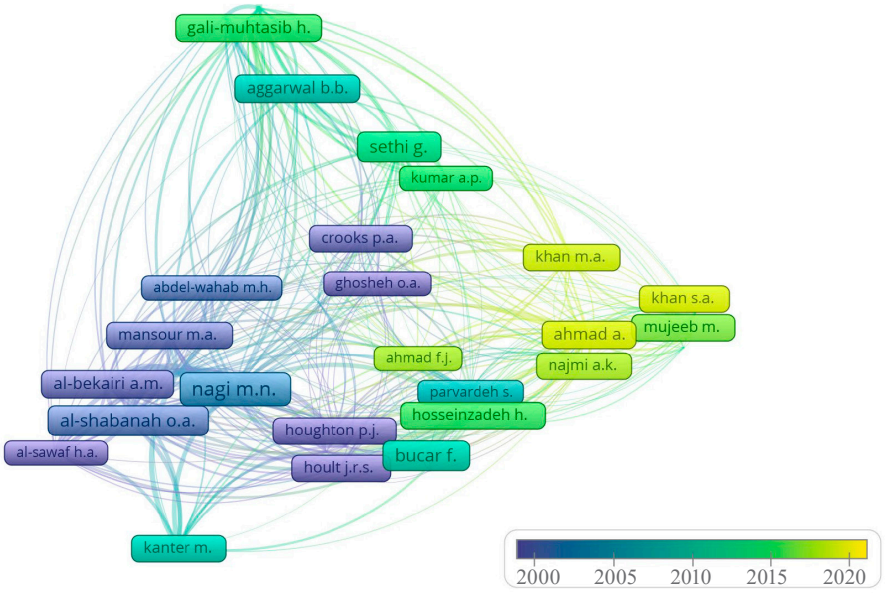


Figure 4 Overlay visualization of the top-cited authors and their distribution over time (2000–2022). This range of years was automatically selected by the software

antioxidant properties of thymoquinone. Badary (Egypt) has worked on the anticlastogenic and superoxide anion scavenging activities of thymoquinone. They also demonstrated the protective properties of thymoquinone on benzo(a)pyrene-induced forestomach carcinogenesis, doxorubicin-triggered hyperlipidemic nephropathy, galactose- and aluminum chloride-induced neurotoxicity in rats, ifosfamide-induced Fanconi syndrome, 20-methylcholanthrene-induced fibrosarcoma tumorigenesis, etc. Figure 4 visualizes the research dynamics. The multicolored frames indicate the dynamics of the knowledge structure. The authors in the yellow frames started their research after 2018.

Citation counts assess the scientific effect of the particular publication on the assumption that a higher number of citations indicates a more widely endorsed publication. Document analysis identifies the intellectual structure of a topic of knowledge by determining the quantity and authority of cited literature. Table 3 displays the top ten most-cited papers according to the Scopus citation data, with worldwide citations ranging from 184 to 1411. In particular, Burits and Bucar, Houghton *et al.*, Badary *et al.*, and Worthen received 1411, 692, 330, and 327 citations, respectively, and were ranked as the top four most-cited publications [34–37]. Burits and Bucar investigated the antioxidant properties of thymoquinone using two thin-payer chromatography screening methods, a diphenylpicrylhydracyl assay, a non-enzymatic lipid peroxidation in liposomes, and a deoxyribose degradation assay. They proved that thymoquinone is an effective ·OH radical scavenging agent. Houghton *et al.* studied the inhibition of eicosanoid generation in leukocytes and membrane lipid peroxidation by the pre-administration of fixed oil of *N. sativa* and thymoquinone [35]. They demonstrated that thymoquinone was very potent against

Table 3 Top-cited documents

Document	DOI or Pubmed link	Citation	Reference
Burits and Bucar	10.1002/1099-1573(200008)14:5<323::AID-PTR621>3.0.CO;2-Q	1411	[34]
Houghton *et al.*	10.1055/s-2006-957994	692	[35]
Badary *et al.*	10.1081/DCT-120020404	330	[36]
Worthen	https://pubmed.ncbi.nlm.nih.gov/9673365	327	[37]
Yi *et al.*	10.1158/1535-7163.MCT-08-0124	276	[39]
Gali-Muhtasib *et al.*	https://pubmed.ncbi.nlm.nih.gov/15375533	276	[40]
Sethi *et al.*	10.1158/1541-7786.MCR-07-2088	264	[41]
Ghosheh *et al.*	10.1016/S0731-7085(98)00300-8.	264	[42]
Nagi *et al.*	10.1006/phrs.1999.0585	258	[33]
Mansour *et al.*	10.1002/cbf.968	249	[28]

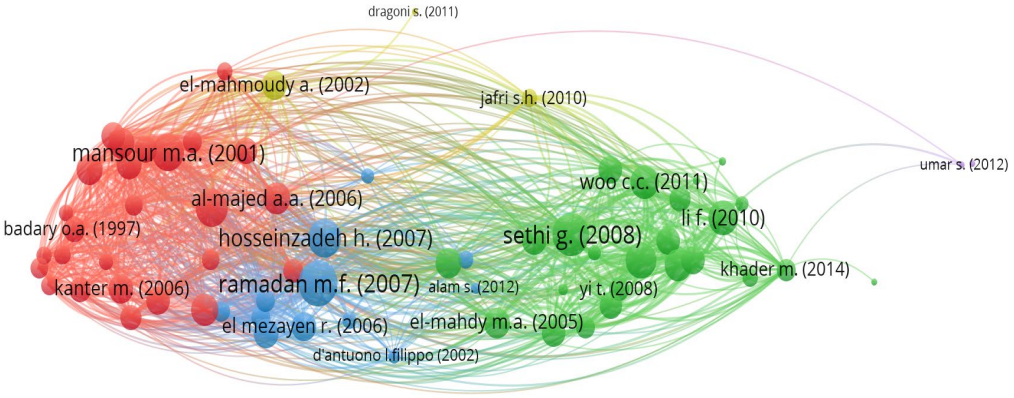
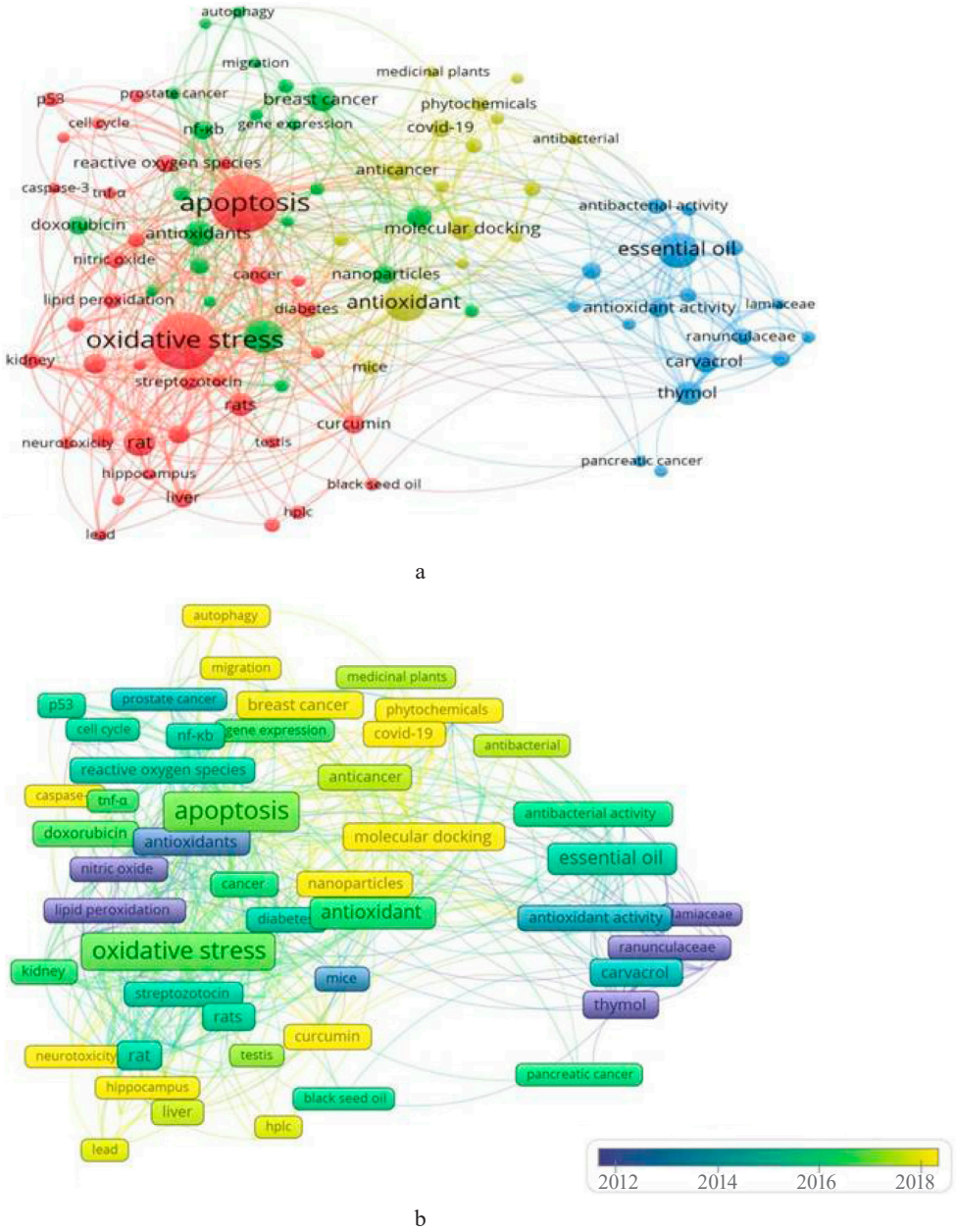


Figure 5 Density visualization of the most bibliographically coupled documents. Circles represent the total link strength for each document. Of the 2072 documents, 82 meet the threshold of citation (120). For each of the 82 documents, we calculated the total strength of the bibliographic coupling links with other documents

5-lipoxygenase and cyclo-oxygenase. Their pharmacological results on oil provided credence to the long-standing usage of *N. sativa* and its products as a remedy for rheumatism and other similar inflammatory conditions. Badary *et al.* also confirmed the superoxide anion scavenging properties on an iron-dependent microsomal lipid peroxidation assay [36]. The antioxirodant properties of thymoquinone are still under investigation and have not yet been determined as a result of its distinctive chemical composition and the multiplicity of mechanisms for antioxidants. Many theories have been proposed to explain this property. Some scientists believe that it is the mitochondrial respiratory chain that triggers thymoquinone antioxidant activities in the cell because it converts the supplied thymoquinone to its hydroquinone form [38].

Bibliometric coupling. Bibliometric coupling evaluates previous articles on a particular topic, identifies major researchers, and elucidates the nature of scholarly talks on the subject. Of the 2072 documents, 82 meet the threshold of citation, which was 120. For each of the 82 documents, we calculated the total strength of the bibliographic coupling links with other documents and selected the documents with the greatest total link strength.

Figure 5 illustrates a scientific mapping, which uses the full counting method to reveal the most bibliographically coupled essential documents and how they are related. The number of local citations was used to assess the scientific impact of each document. Based on the total link strength and subject effect, we found five clusters, each in a distinct hue of red, purple, green, yellow, and blue. These five clusters had the total link strength and links of 2746 and 202, respectively. The red cluster was anchored by Burits with citation, total link strength, and links of 1406, 48, and 31, respectively [34]. It was followed by Badary and Worthen [36, 37]. They examined the role of the antioxidant capacity of thymoquinone in suppressing chemically induced oxidative injuries. The green cluster was headed by El-Mahdy *et al.*, Sethi *et al.*, Khan *et al.*, and Woo *et al.* [41, 44, 43, 45]. This cluster's documents focused on the anticancer activity of thymoquinone in various *in vivo* and *in vitro* models. These authors proposed possible involvement of the PPAR-*γ* pathway and nuclear factor-κB activation and inhibition of antiapoptotic gene products as mechanisms [43–45]. Ramadan and Hossienzadeh headed the blue cluster: their research was on the neutraceutical applications of thymoquinone [46, 47]. The yellow cluster contained three publications that supported the research publi-



a

b

Figure 6 Density (a) and overlay (b) visualization for the most frequent keywords. Circles represent the number of occurrences. Different colors of the frames define the trending knowledge in each cluster

shed by El-Mahmoudy in 2022. It demonstrated that thymoquinone inhibited the expression of inducible nitric oxide synthase in rat macrophages [48]. We obtained only two documents in the fifth cluster. This violet cluster documents the effect of thymoquinone on inflammation, cytokine network, and arthritic disease model [49, 50].

Lexical analysis: keyword co-occurrence. Thematic evolution is a crucial bibliometric method: it is a primary technique that gives a historical perspective on research and offers a science-based model concentrating on future research directions. It highlights the most important research topics and demonstrates how they have developed over time, offering insights into the future direction that research should follow [51, 52]. The authors used 4531 keywords in thymoquinone-related research. We selected only 248 keywords. For every keyword, we calculated the total strength of the co-occurrence links with other keywords using VOSviewer and clustered them into four categories marked as red, green, blue, and yellow. Figure 6a shows the keywords with the greatest total link strength. Cluster one (red) included 37 keywords. Its most frequent keywords were *apoptosis* and *oxidative stress*. Reactive oxygen species (ROS) and the oxidative stress they cause play a significant part in the process of apoptosis. Apoptosis may be prevented or delayed by antioxidants and thiol reductants [53]. The green cluster (n = 70) was led by *inflammation*, *antioxidant*, *cytotoxicity*, *doxorubicin*, and *breast cancer*. Extensive

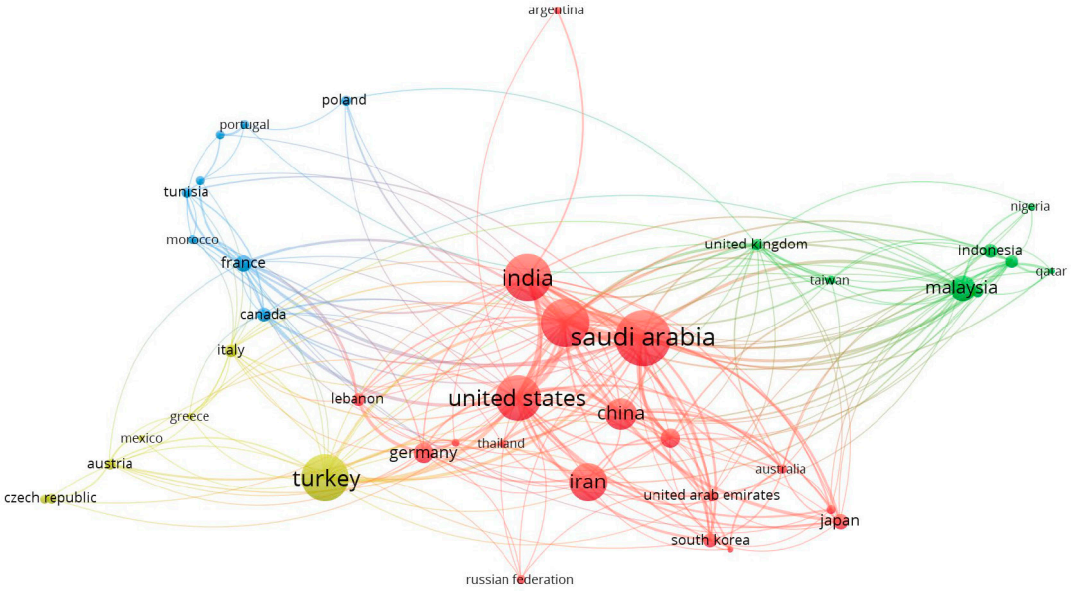


Figure 7 Mapping of co-authorship

Table 4 International collaboration as measured by co-authorship

Country	Documents	Citations	Total link strength
Saudi Arabia	342	9766	297
Egypt	275	8131	227
India	267	6119	124
Turkey	267	3886	53
United States	248	9346	166
Iran	183	3724	24
China	141	3270	58
Malaysia	92	1615	61
Germany	66	2799	38
Pakistan	62	953	42
France	45	1292	49
Japan	43	1260	35
South Korea	39	1598	31
Indonesia	37	110	7
Canada	36	1024	28
Jordan	33	635	31
Lebanon	32	1591	28
Italy	31	1082	19
United Kingdom	28	1182	32
Iraq	25	409	19

research conducted over the last two decades has uncovered the mechanism by which persistent oxidative stress may rise to chronic inflammation, which may, in turn, mediate most chronic illnesses, including cancer [54]. Keywords related to the chemical composition, antibacterial, and antimicrobial properties of the *N. sativa* essential oil dominated the third cluster (blue, n = 77). Cluster four (yellow) with 66 words involved *antioxidant*, *molecular docking*, and COVID-19. VOSviewer generated an automatically-determined time limit for the overlay visualization (Fig. 6b). The time frame was from 2012 to 2018. This knowledge structure aligns with the scientific productivity that began in the past two decades. The yellow frames display the most popular terms, which in turn reflect the most cutting-edge research and knowledge.

Geographical and authorship mapping. Understanding the trajectory of research across a wide variety of study fields requires a solid grasp of the author cooperation network. This partnership frequently results in establishing academic hubs that enhance the growth and future expansion in that study area. Figure 7 presents a co-author network that depicts the intellectual relationships between scholars on a country-by-country basis. Based on co-authorship, Fig. 7 identifies the nation that has contributed the most publications. Of 100 different countries, 42 countries with four clusters, 254 links, and a total link strength of 811 met the cut-off point, with the minimal number of papers being ten and its minimal number of citations being five. The diameter of the circle represents the total number of publications per country. Line thickness and circle spacing are used to gauge the level of collaboration. The total strength of a country's relations is determined by the number of documents published by authors from two or more different nations. Four main networks emerged: Saudi Arabia (red), Malaysia (green), France (blue), and Turkey (yellow). However, Saudi Arabia, Egypt, India, Turkey, and United States proved to be the leading countries (Table 4).

CONCLUSION

This research results have a considerable theoretical and practical significance. The publication introduces a comprehensive historical overview of scientific literature during the past eleven decades. First, it highlights the most influential and productive authors,

publications, and nations. Second, scientists that work with thymoquinone can use this article to determine prospective co-authors and journals where to publish their findings. Third, our article gives academics a chance to concentrate their attention on the most relevant, prominent, and recent papers. Fourth, the results of this study may be used by researchers who employ neural networks and data scientists to choose the research topics we identified as gaps. For instance, more study is required to evaluate the precise mechanism of thymoquinone in the mitochondrial oxidation system. Thymoquinone is a well-known antioxidant supplement that promotes health. However, it can potentially be harmful to cells and organs due to its propensity to encourage the oxidation of biomolecules under certain circumstances. Furthermore, the reduced metabolite thymohydroquinone may be more effective than thymoquinone since hydroquinones have been discovered to display higher antioxidant and prooxidant activity than their parent quinones.

However, this study relied entirely on the Scopus database for pertinent papers. We had to leave few documents out because they lacked the necessary details. Future research projects may employ other databases, such as Web of Science (WoS) and Google Scholar, to use a variety of review approaches and give a more detailed quantitative and qualitative summary. The shortcomings of the methodologies in terms of in-depth study balance out their benefits in terms of a wider scope. We failed to investigate the approaches and models to perform a more critical analysis of the research issue, but this is a fundamental flaw in all bibliometric approaches since they frequently focus more on the outputs than on the actual subject matter.

CONTRIBUTION

S.I. Abdelwahab and M.M.E. Taha conceived and designed the manuscript, contributed data and analysis tools, and performed the analysis. S.I. Abdelwahab and A.A. Mariod collected the data and wrote the paper.

CONFLICT OF INTEREST

Authors declare no conflict of interests regarding the publication of this article.

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Plant extracts and essential oils in the dairy industry: A review

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Abstract:
Plants have been used as food additives worldwide to enhance the sensory qualities of foods and extend their shelf life by reducing or eliminating foodborne pathogens. They also serve as therapeutic agents due to their beneficial effects on human health through their anti-cancerous, anti-inflammatory, antioxidant, and immune-modulatory properties.
Plants can be added to food as a dry powder, grated material, paste, juice, or as an extract that can be produced by a variety of methods. Plant extracts and essential oils are concentrated sources of bioactive phytochemicals that can be added to food in small amounts in a variety of forms. These forms include liquid, semi-solid, or dry powder for easy and uniform diffusion. Encapsulation can protect bioactive compounds from temperature, moisture, oxidation, and light, as well as allow for controlling the release of the encapsulated ingredients. Nanoemulsions can enhance the bioactivity of active components.
This review explains how plant extracts and essential oils are used in the dairy industry as antimicrobial materials, analyzing their impact on starter bacteria; as natural antioxidants to prevent the development of off-flavors and increase shelf life; and as technological auxiliaries, like milk-clotting enzymes, stabilizers, and flavoring agents. Therefore, plant extracts and essential oils are a better choice for the dairy industry than plants or their parts due to a wide range of applications, homogeneous dispersion, and ability to control the concentration of the bioactive ingredients and enhance their efficiency.

Keywords: Plant extracts, essential oils, dairy products, natural antimicrobials, milk-clotting enzyme, natural antioxidants

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INTRODUCTION

Plants have been utilized as medicine and preservatives, as well as food flavorings, since ancient times. They are used fresh or dry and have many different forms of use, including plant parts such as leaves, roots, flowers, seeds, crusts, tubers, or herbs. They are also applied in crushed or ground forms, or as extracts prepared in different ways [1]. Plants rich in secondary components (alkaloids, organosulfur compounds, glycosides, flavonoids, tannins, phenols, coloring agents, and resins) are classified as medical and aromatic plants [2]. Medical and aromatic plants are used for aromatic, coloring, preservative, and antioxidant purposes as spices and food additives. Phenolics are free radical eliminators and metal chelators. They can inhibit the lipid peroxidation and exhibit various physiological activities as antioxidants. Many foodborne pathogenic and spoilage bacteria, as well as molds and yeasts, can be inhibited by phenols and extracts rich in such substances [1]. In addition, they have potential in the prevention and treatment of some chronic diseases, including cancer, diabetes, and cardiovascular disease [3]. Essential oils (terpenes, esters, alcohols, ketones, aldehydes, and phenols) are of great interest to the food and cosmetic industries, as well as medicine, due to their wide range of biological properties (antimicrobial, antifungal, antioxidant, anti-inflammatory, insecticide, analgesic, anticancer, cytotoxic, etc.) [4].

Milk and dairy products are the most common foods in the diets of all age groups. Their popularity can be attributed to milk's unique components and properties, as well as the fact that a wide variety of foods can be prepared using this ingredient. The market for value-added functional foods has expanded manifold due to the consumers' increased awareness of, and interest in, following healthy dietary strategies to achieve health benefits from foods beyond their basic nutrition [5]. With the introduction of fortified foods, there has been a surge in using plants and their extracts as valuable additives in dairy products because they contain

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numerous bioactive components that perform a variety of functions. Additionally, plants have been used for centuries in milk and dairy products as technological auxiliaries (e.g. milk-clotting enzyme preparations, cheese wrappers) or as natural preservatives due to their antimicrobial and/or antioxidant properties [6].

Plant extracts are concentrates of bioactive phytochemicals obtained through extraction technologies including steam distillation, cold pressing, or solvent extraction, with or without pretreatment. In addition to classic approaches, new extraction methods have also been investigated, such as ultrasonic, microwave, and supercritical fluid extraction. Essential oils can also be extracted from various parts of plants, depending on the species and variety [7]. The purpose of extraction is to obtain the largest quantity of bioactive components from plants. With the help of solvents, soluble plant metabolites are separated from the insoluble cellular marc. After the solvent is removed, the products can be used in liquid, semi-solid, dry-powdered, or encapsulated form. Nowadays, plant extracts are increasingly becoming important additives in the dairy industry due to their high content of bioactive compounds that have antimicrobial and antioxidant activity [8]. The aforementioned compounds also delay the development of off-flavors and improve the shelf life and color stability of food products. Due to their natural origin, they are excellent candidates to replace synthetic compounds, which are generally considered to have toxicological and carcinogenic effects [9]. Additionally, plant extracts can be added to food in low concentrations with efficient distribution and uniformity. Encapsulation protects them from temperature, moisture, oxidation, and light, while allowing scientists to control the release of encapsulated ingredients, as well as to mask off-flavors and unpleasant odors [10]. Therefore, we mainly aimed to review the possible uses of plant extracts as antioxidants, antibacterials, and antifungals in the dairy industry, as well as to study their effects on the starter bacteria. Furthermore, we analyzed the use of plant extracts instead of renin enzyme to coagulate milk and determined their effect on the yield of the resulting cheese, as well as its physiochemical and sensory properties.

RESULTS AND DISCUSSION

Plant extracts as natural antimicrobials. ***Mode of action.*** Antimicrobials are compounds used for food preservation by controlling the growth of spoilage-causing and pathogenic microorganisms. There are a range of natural compounds with antimicrobial activity that have been identified from various sources (plants, animals, or microbes). However, due to the structural differences between Gram-negative and Gram-positive bacteria, the efficacy of antimicrobial agents mayvary [11]. In plants, natural compounds exerting antimicrobial activity are phenolic compounds, alkaloids, sulfur-containing compounds, and terpenoids, as well as essential oils and their constituents [12–15]. Generally, natural compounds with different chemical groups can permeate or disrupt the cytoplasmic membrane, allowing the passage or release of non-specific compounds. Increased cell membrane permeability leads to the release of intracellular compounds, especially potassium, calcium, and sodium ions, causing irreversible damage [14, 16]. Natural compounds may also inhibit the ATPase enzyme responsible for the energy generation of the cell, which leads to cell death [8, 17].

In a study by Gonelimali *et al.*, the plant extracts significantly affected the cell membranes of Gram-positive and Gram-negative bacteria, as demonstrated by the decline in pH and the hyperpolarization of the cell membrane [18]. Some fatty acids, in particular, have the ability to interfere with the structure of the cell membrane, displacing phospholipids and increasing their permeability. Five of them are acetylenic: 6-hexadecinoic, 6-heptadecinoic, 6-octadecinoic, 6-nonadecinoic, and 6-eicosinoic acids, whereas the other three are saturated: palmitic, heptadecanoic, and stearic acids [13]. The activity of polyphenols depends on the number and position of hydroxyl groups. Polyphenols may inhibit the enzymes of microorganisms, possibly through interactions with sulfhydryl groups or through less specific interactions with proteins [19]. Miklasińska-Majdanik *et al.* reported that phenolic compounds partially damaged the bacterial membrane, inhibited virulence factors such as enzymes and toxins, and suppressed bacterial biofilm formation [20]. In addition, some natural polyphenols, aside from their direct antibacterial activity, exert a synergistic effect when combined with common chemotherapeutics.

Essential oils also have antibacterial, antioxidant, and antimutagenic properties, as well as possible health benefits. These natural compounds, which are generally recognized as safe (GRAS), reduce lipid oxidation in foods and hence hold potential as natural food additives [21]. There are many types of compounds in essential oils which have proven antimicrobial properties. They include phenolic monoterpenes (thymol and carvacrol), phenylpropanoids (eugenol), alcoholic monocyclic monoterpenes (*α*-terpineol and terpinen-4-ol), as well as bicyclic monoterpene hydrocarbons (*α*-pinene) and ketones (camphor). The acidic nature of the hydroxyl group of phenols facilitates a hydrogen bond with the enzyme active center, which is responsible for their high activity [22]. Terpenoids can rupture the cell membrane due to their lipophilic nature.

Challenges of using plant extracts as antimicrobials in the dairy industry. *In vitro*, several studies have demonstrated that plant extracts and essential oils of aromatic and medicinal plants have antimicrobial activity against pathogens and spoilage microorganisms associated with food contamination [23, 24]. However, these results cannot be strictly valid due to the complexity of the food matrix. Moreover, many factors interfere with the activity of these compounds, such as proteins, lipids, packaging, storage temperature, type

of microorganism, and compound stability [17, 25]. Proteins and lipids, for example, can wrap around the surface of the microorganism, forming a physical barrier that prevents the bioactive compound from coming into contact with the microorganism, thus reducing its efficacy [17]. In a similar report, the antimicrobial activities of cinnamon and clove essential oils were lower in the high-fat milk samples than in the skim milk samples [26]. Dairy products are foods with a high content of proteins, lipids, minerals, and vitamins. So, when adding extracts and essential oils of aromatic plants to dairy products as antimicrobials, we must take into account the following factors:

1. Natural compounds added as antimicrobials should be in greater concentrations than those tested *in vitro*. According to Gammariello *et al*., the concentration of active compounds used to inhibit the growth of pathogenic microorganisms in Fior di Latte cheese was significantly higher than the level tested *in vitro* [27]. The minimum inhibiting concentration of pomegranate essential oil against *Listeria monocytogenes* and *Staphylococcus aureus* (105 CFU/mL each) was higher than 2.5 mg/mL in a culture medium, while its concentration of 40 mg/mL in Cheddar cheese failed to inhibit the same population of those microorganisms [28]. Hassanien *et al.* also mentioned that the 0.1% concentration of black cumin essential oil reduced the growth of *L. mononocytogenes*, *S. aureus*, *Escherichia coli*, and *Salmonella enteritidis* in a culture medium, while in cheese, such concentrations were not effective against *S. aureus* and *L. monocytogenes* [23];
2. In some cases, mixing some plant components at low concentrations has a higher antimicrobial effect than adding them separately, which proves their synergistic effect [17];
3. Dairy products contain all the nutrients necessary for the microbial growth of cultured cells, allowing for a faster recovery of cells damaged by natural antimicrobials [29];
4. The contents of natural compounds can decrease during processing and storage. Libran *et al.* reported a decrease in the content of compounds from basil and tansy essential oils added during cheese production [30]. In another study, rosemary essential oil added to sheep milk during cheese manufacturing had a loss of 37.49% [24]; and
5. Microencapsulation can improve the stability of natural substances throughout processing and storage [31]. Nanoemulsions of plant extracts can decrease the quantity of a required effective dose and enhance the material's bioactivity against bacteria by allowing them to penetrate the cell membrane and thus destabilize its lipid bilayers [32]. For example, a nanoemulsion of anise extract performed better than bulk extract as an antimicrobial agent against some foodborne pathogenic bacteria [33].

Plant extracts as antibacterials in dairy products. Several plants in various forms (powder, essential oils, extracts, etc.) have been successfully used in dairy foods. Plant extracts of cinnamon, garlic, lemongrass, cress, rosemary, sage, and oregano individually inhibited the population of *L. monocytogenes* in processed cheeses [34]. According to Shan *et al*., all the extracts of cinnamon stick, pomegranate peel, grape seed, oregano, and clove inhibited the growth of *S. aureus*, *L. monocytogenes*, and *S. enterica* in cheese [28]. As a result, these extracts, especially clove, have the potential to be employed as natural food preservatives. Cayenne and green pepper extracts also reduced the *S. aureus* population in Egyptian Kareish cheese [35]. Mahajan *et al.* reported that the aqueous extracts of pine needles improved the microbiological properties of low-fat Kalari, an Indian hard cheese, due to their antioxidant and antimicrobial properties [36]. Sulfur-containing compounds are credited with antimicrobial activity in plant-based compounds, particularly diallyl sulphides in *Allium* species, terpenoids (carvone and limonene) in spearmint essential oil, eugenol in clove oil, and thymol in thyme oil. Ginger's antimicrobial activity is attributed to several compounds, including gingerols, gingerdiols, and shogaols [37–39]. Table 1 lists bioactive components and their functional qualities in plants used to manufacture functional dairy products.

In addition, the essential oils of aromatic plants also showed anti-bacterial activity in food preservation, even with Gram-negative bacteria. Gram-negative bacteria have an effective permeability barrier consisting of a thin lipopolysaccharide exterior membrane, which could restrict the penetration by the extruding plant extracts. Gram-positive bacteria have a mesh-like peptidoglycan layer which is more accessible to permeation by plant extracts [40]. In Feta cheese and Iranian white cheese, oregano (0.1%) and thyme (0.1%), salvia (0.1%), basil (1%), and black cumin essential oils had antimicrobial activity against *L. monocytogenes* [41, 42]. In Iranian white cheese inoculated with *E. coli* O157:H7 and treated with black cumin essential oil, the pathogen growth was significantly lower compared to the control during storage [43]. Adding clove essential oil to Paneer cheese increased its shelf life to 10 days in the treated cheese compared to 5 days in the control sample. Furthermore, the control samples had a higher microbial count compared to the treated cheese. Clove essential oil added at concentrations of 0.5 and 1% dramatically reduced the growth rate of *L. monocytogenes* in cheese at 30 and 7°C. However, high concentrations of clove oil may adversely affect the sensory properties of food. Thus, small concentrations may be enough to ensure low bacterial load and, therefore, food safety [44].

The addition of aqueous licorice and cinnamon extracts to yoghurt exhibited the strongest inhibitory effect on *Helicobacter pylori* development when compared to the control yoghurt [45]. According to Mahgoub *et al*., adding 0.2% *Nigella sativa* essential oil to the cheese improved its physicochemical and sensory qualities. In addition, it provided the most effective antibacterial capability against *S. aureus*, *S. enteritidis*, and *E. coli* [46]. In goat milk-based

yoghurt containing *Lactobacillus acidophilus* and roselle extract, higher antimicrobial activities were observed against *Bacillus cereus*, *E. coli*, *S. aureus*, and *Salmonella typhi*. This could be attributed to the production of higher antimicrobial compounds such as antimicrobial peptides and organic acids [47].

Plant extracts as antifungals in dairy products. Fungi are spoilage microorganisms that grow in foodstuffs during storage, reducing their nutritional value and sometimes producing mycotoxins. As a result, foods become unfit for consumption [8]. The growth of fungi on the cheese surface can be inhibited by using some plant-based compounds. For example, cinnamon leaf and bark essential oils (≤ 10% (v/v) showed the highest antifungal activity during the ripening of Appenzeller cheese [48]. Also, incorporating cinnamon oil with 5% cinnamaldehyde into a film coating of spreadable cheese delayed the growth of *Aspergillus niger* and *Penicillium expansum* [49]. Molds and yeasts were not detected in UF-soft cheese fortified with ginger and garlic extracts until the end of storage, 42 and 90 days, respectively [37, 50]. Sağdıc *et al.* found that garlic and thyme extracts inhibited most molds and yeasts in soft cheese [51]. Plant extracts can also help to delay or prevent the formation of mycotoxins. Vazquez *et al.* found that eugenol (200 µL/mL) added to Arza Ulloa cheese reduced the synthesis of citrinin, a toxin generated by *Penicillium citrinum* [37]. Sindhu *et al.* also found that the essential oil isolated from curcuma leaves (1.5%) inhibited aflatoxin formation [52]. At concentrations of 0.50–1.5%, the oil of *Satureja hortensis* L. exhibited antibacterial activity, while its alcoholic extract had no effect on *S. aureus* mycelia growth. Similarly, the *S. hortensis* essential oil implanted in fresh cow's cheese prevented *S. aureus* growth, but its ethanol extract did not appear to be effective [53]. Labneh, a concentrated yoghurt with 0.2 ppm essential oils of thyme, marjoram, and sage, had a 21-day shelf life compared to the control, with yeast and mold observed in the control from the 14th day onwards [54]. Labneh containing 0.3% cinnamon oil, on the other hand, had a longer shelf life (8 days) when stored at 6°C compared to the control product [55].

Effect of plant extracts on starter culture activity. Starter cultures are responsible for fermentation and provide the desired sensory qualities to the finished product. Plant extracts and essential oils, which are intended to suppress pathogenic bacteria, prevent spoilage, or improve sensory characteristics, have been demonstrated in numerous studies to have no effect on the activity of starting cultures. Lactic acid bacteria are the most resistant bacteria to antimicrobial agents in plant extracts and essential oils at concentrations that limit the growth of pathogenic microorganisms [17]. In particular, the count of lactic acid bacteria in sheep's cheese was not reduced when rosemary essential oil was added to inhibit the growth of *Clostridium tyrobutyricum* [25]. In a similar report, Gammariello *et al.* found that 13 extracts and essential oils of orange species, grapefruit, spring lemon, parsley, and lemon Boyajian did not affect the survival of lactic acid bacteria in Fior di Latte cheese, while decreasing the population of pathogenic bacteria [27]. Furthermore, treating Argentinean cheese with 200 mg/kg of oregano oil had no influence on *Lactococcus lactis*, *Lactobacillus bulgaricus*, or *Streptococcus thermophilus* growth or acidifying activity, compared to the control [56].

The addition of 0.03% *Mentha longifolia* oil to Feta cheese resulted in the highest viability of *Lacticaseibacillus casei* at low pH, compared with the other treatments containing < 0.03%. Electron microscopy showed that essential oils caused no harm to *L. casei* [57]. Other studies indicated that some plant extracts may improve starter activity. The total count of starter cultures (*L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*) was higher in the ginger-fortified UF-soft cheese and Egyptian white cheese pickled in a brine solution containing fresh ginger extract, compared to control cheese [38, 58]. Inversely, some studies indicate that some extracts have an adverse effect on the growth of starter cultures. For example, adding 2.5 µg/mL of thyme oil to Coalho cheese reduced the viable cell count of *L. monocytogenes* and the counts of starter cultures composed of *L. lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis* [59].

Adding *Cinnamom umverum* and *Allium sativum* aqueous extracts to goat, cow, and camel milk had no significant effect on the acidification through fermentation [60]. The aqueous extracts of rose flower, spearmint, dill, and green tea, as well as chamomile essence increased *Bifidobacterium bifidum* and *L. acidophilus* growth in probiotic milk and yoghurt and kept the bacteria alive until the end of storage, with no need for additional nutrients [61–63].

In another study, adding *A. sativum* or *Cinnamomum verum* water extracts to both cow and camel milk yoghurts boosted *B. bifidum* viability for 21 days of storage, compared to the control yoghurts. This was correlated to the presence of vitamins, minerals, amino acids, and polyphenolics in *A. sativum* and *C. verum*, among other factors. Camel milk has more free amino groups and a higher buffering capacity than cow milk, which leads to increased *B. bifidum* viability [64]. Haddadin found that increasing concentrations of ethanol olive leaf extract accelerated bacteria growth and allowed them to attain optimal acidity in less time [65]. The samples containing 0.6% of the extract had the highest bacteria count and the bacteria were viable until the end of fermentation. Polyphenols (oleuropein and other secoiridoids), flavonoids (rutin, flavonol), and luteolin-7-glucoside are stimulatory components in ethanol olive leaf extract.

In a different study, adding *Diospyros kaki* L. leaf extract to yoghurt increased the rate of acidification and decreased the time required to complete fermentation, contributing to the viability of the starter culture. In particular, the increase in the counts of *S. thermophilus*

and *Lactobacillus delbrueckii* ssp. *bulgaricus* was the highest (2.95 and 1.14 log CFU/mL, respectively) in *D. kaki* yoghurt [66]. The water cinnamon extract had no effect on the probiotic population, although *Lactobacillus* species and *S. thermophilus* counts in yoghurt increased for up to 7 days during storage [45].

Ziarno *et al.* showed that the herbal extracts from valerian, sage, chamomile, cistus, linden blossom, ribwort plantain, and marshmallow did not inhibit the growth of lactic acid bacteria in fermented milk, such as yoghurt [67]. However, they can gradually inhibit fermentation at concentrations above 2% (w/w) and hence can be used to prevent post-acidification of fermented milk. In general, the plant extracts significantly increased the growth and acidification rates of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. Plant extract components, including monosaccharides, formic acid, and hydroxycinnamic acid, as well as neochlorogenic, chlorogenic, and caffeic acids, play a stimulatory role and cause a beneficial effect on the growth of yoghurt culture bacteria through fermentation [68].

The viability of probiotic bacteria in fermented milk is also affected by the addition of essential oils. The yoghurts with mint, bee balm, and ziziphora essential oils (0.001%) exhibited higher viability *of L. acidophilus* LA-14 and *Bifidobacterium animalis* ssp. *lactis* BB-12 than the control samples. However, the yoghurt with eucalyptus essential oil had lower viability [69]. The survivability of probiotic bacteria in yoghurt and cheese were unaffected by the essential oils of *M. longifolia*, *Teucrium polium*, *Cuminum cyminum*, *Allium ascalonicum*, and *Pimpinella anisum* [57]. The viability of *L. casei* in a bio-yogurt containing various amounts of *T. polium* essential oil was significantly reduced after 28 days of storage. A probiotic yoghurt without essential oils and a bio-yogurt containing 60 ppm of *T. polium* oil had the highest overall viable count of *L. casei* (6.47 log CFU/mL). However, higher concentrations of the oil resulted in decreased bacterial counts [70]. Sarabi-Jamab & Niazmand reported that the population of *L. acidophilus* in bio-yogurt with varied concentrations (25, 40, 70, 100, and 130 g/L) of *Mentha piperita* and *Ziziphora clinopodioides* essential oils considerably decreased after 7 days of storage [70]. Yet, their viability did not change significantly, compared to the control. In ice cream, the probiotic bacteria *L. acidophilus* La-5 and *B. bifidum* Bb-12 were more stable during storage at –20°C for 90 days when made with tiger-nut extract, compared to the control [71].

Plant extracts as natural antioxidants. ***Mode of action.*** Antioxidants are essential for lowering oxidative reactions in food systems and the human body. In food systems, they retard lipid peroxidation and the formation of secondary lipid peroxidation products. Antioxidants also help to reduce protein oxidation, as the interaction of lipid-derived carbonyls with proteins alters their function [72]. Dairy products contain lipids rich in polyunsaturated fatty acids and their esters are easily oxidized by molecular oxygen over time. This oxidation may occur during the manufacture, storage, or distribution of final products. Light, oxygen, and transition metal ions are important factors leading to oxidative changes. Deleterious changes in dairy products caused by lipid oxidation include not only off-flavors but also the loss of color and nutrients, and the accumulation of compounds that may be detrimental to consumers' health [73]. Objectionable odors and flavors in oxidized products are caused by product subcomponents forming compounds such as hydrocarbons, aldehydes, and ketones [74].

Synthetic antioxidants are commonly used to increase the shelf life of food products, including TBHQ, BHA, and BHT. They reduce the rate of lipid oxidation and hydrolysis, as well as stabilize free radicals. According to the International Dairy Foods Association, these antioxidants are not allowed to be added to milk. Therefore, dairy products are fortified with natural antioxidants that delay lipid oxidation and hydrolysis, reduce nutritional losses, prevent free radical damage, and provide a variety of health benefits [75].

Antioxidants can be divided into primary and secondary based on how they work to reduce lipid oxidation. Primary antioxidants act as H donors to the lipid-free radicals formed during lipid oxidation and rearrangement into a stable form. Secondary antioxidants act as chelators of metal ions, decompose hydroperoxide into non-radical species, deactivate singlet oxygen, and absorb ultraviolet radiation. They can also act as oxygen scavengers to slow down the rate of radical formation [76].

Plant antioxidants can be divided into three categories: phenolic compounds, vitamins, and carotenoids [77]. Phenolic compounds have a large diversity of structures: from simple molecules (e.g., ferulic, gallic, and caffeic acids) to polyphenols (tannins and flavonoids) [78]. Vitamins E and C are the most important plant antioxidants. Vitamin E is a lipid-soluble vitamin made up of four tocopherols and four tocotrienols, each having four isomers (α, β, α, and δ), but only α-tocopherol can be absorbed by the human body. Vitamin C is a water-soluble vitamin found in a variety of fruits and vegetables [79]. Therefore, these compounds have been considered promising candidates as potential protectors against lipid oxidation. The presence of an antioxidant is one of the fastest ways to reduce fat oxidation [80, 81].

Plant extracts as antioxidants in dairy products. Plant extracts containing high amounts of phenolic compounds act as H donors, radical scavengers, or metal chelators. Scientists have studied the antioxidant properties of sedge, marjoram, wild marjoram, caraway, basil extract, ginger, plum concentrates, aloe vera, mustard, tea catechins, rosemary extracts, and other plant extracts [82–84]. The antioxidant activity of milk increased significantly when plant extracts were introduced before bacterial fermentation [85].

Santos *et al.* used rosemary extract to prevent fat oxidation in cow milk fortified with fish oil. When

tested at 60 and 110°C, an ethanol extract of rosemary added to 400 mg/kg of butter increased the butter's oxidation stability [86]. Plant extracts enhanced the antioxidant activity and overall phenolic content in the fermented milk. For example, marjoram extract added to yoghurt had a significant antioxidant effect on both the first and the last days of storage (28 days) [85]. *Cudrania tricuspidata* L. and *Morus alba* L. leaf extracts improved yoghurt's antioxidant activity and total phenolic content. *C. tricuspidata* leaf extract exhibited the highest antioxidant activity [68]. Srivastava *et al.* found that the goat milk yoghurt fortified with 2% beet root or 2% ginger extracts had the highest antioxidant activity evaluated by the DPPH assay, followed by the cow milk yoghurt with 2% ginger extract [87]. Furthermore, fortifying milk and yoghurt with 2% red ginseng extract increased its oxygen radical absorption capacity and radical-scavenging activity (DPPH), but reduced the DNA damage caused by H_2O_2, compared to the control yoghurt [88]. The cheese fortified with clove extract was shown to have the strongest antioxidant and antibacterial qualities, compared to the cheeses fortified with other extracts (cinnamon stick, oregano, pomegranate peel, and grape seed) [28].

In recent years, herbal ghee has primarily been sold as medical ghee on the global market [89]. This product has a typical flavor, a bitter or pungent aftertaste, and a dark color. The antioxidant activities of vidarikand (*Pueraria tuberosa* L.), shatavari (*Asparagus racemosus* L.), and ashwagandha (*Withania somnifera* L.) extracts were evaluated against the synthetic antioxidant BHA. Compared to the aqueous extracts, the herbs' ethanol extracts were more effective in avoiding the formation of peroxide value and conjugated dienes in ghee. Puerarin, daidzein, genistein, and daidzin are active ingredients in vidarikand [90].

Table 1 Plants and their bioactive compounds with antimicrobial and antioxidant activities in some dairy products

Plants	Scientific name	Bioactive components	Applications	References
Thyme	*Thymus vulgaris* L.	Thymol (phenolic monoterpenes)	Ricotta cheese, Coalho cheese, Mimicking models, Fior di Latte cheese, Feta cheese, Labneh, Butter	[22, 27, 42, 51, 54, 59]
Basil	*Ocimum basilicum* L.	Carvacrol (phenylpropanoids)	Ricotta cheese, Serra da Estrela cheese, Ice cream	[30, 42, 82–84]
Cloves	*Eugenia caryophyllata* L.	Eugenol (*α*-terpineol and terpinen-4-ol)	Yoghurt, Paneer cheese, ArzaUlloa cheese	[28, 37, 44]
Cinnamon	*Cinnamomum zeylanicum*	Cinnamaldehyde	Cheddar-based media, Spreadable cheese, Appenzeller cheese, processed cheeses	[24, 25, 28, 48, 50, 55, 60]
Ginger	*Zingiber officinale* L.	Gingerols, Gingerdiols, Shogaols	Fortified cheese, UF-soft cheese	[38, 39, 58, 83, 87]
Oregano	*Origanum vulgare* L.	Carvacrol, Thymol, *γ*-terpinene, *p*-cymene, Carvacrol methyl ethers	Cheddar-based media, Feta cheese	[24, 25, 42, 56]
Black cumin	*Nigella sativa* L.	Thymoquinone, Thymol, *α*-hederin	Iranian white cheese, Feta cheese, Domiati cheese	[23, 41–43]
Roselle	*Hibiscus Sabdariffa* L.	Calyx, Chlorhexidine, Amoxicillin-clavulanic acid, Tetracycline, Metronidazole	Yoghurt	[47]
Garlic	*Allium sativum* L.	Oil-soluble organosulfur compounds: include Allicin, Ajoenes, Allyl sulfides, Actericidal, Antibiofilm, Antitoxin, and Anti-quorum	UF soft cheese, soft cheese, processed cheeses	[24, 38, 49, 51, 60]
Sage	*Salvia officinalis* L.	Geraniol, Pinene, Limonene, Carnosol, Saponin, Catechins, Apigenin, Luteolin, Rosmarinic, Carnosine, Vanillic, Caffeic acids	Sour cream, Fior di Latte cheese, Cheddar cheese, Yoghurt, Ghee, Butter oil	[24, 67, 82–84, 92]
Rosemary	*Rosmarinus officinalis* L.	1,8-cineole, Borneol, Camphor, Caffeic acid, Rosmarinic acid, Luteolin-7-O glucoside, Carnosic acid, Ursolic acid, Carnosol, di- and triterpenes	Ghee, Butter oil, Sour cream, Yoghurt, Sheep's cheese, Cheddar-based media, Cottage cheese, Herbed cottage cheese, Flavored yoghurt	[24, 86, 92]
Lemon grass	*Cymbopogon* L.	Myrcene, limonene, citral, geraniol, citronellol, geranyl acetate, neral, and nerol	Indian soft cheese, Yoghurt, Coalho cheese, processed cheeses	[24]
Dill	*Anethum graveolens* L.	Quercetin, Kaempferol, Myricetin, Catechins, Isorhamnetin, Carvone, Limonene	Milk	[62, 63]

Parmar *et al.* discovered that a 7% ethanol extract of *Terminalia arjuna* L. bark was particularly effective in preventing auto-oxidation of both cow and buffalo ghee during storage. The extract had a substantial ability to increase the antioxidant potential of ghee, with the efficacy being greater in cow ghee than in buffalo ghee. The Arjuna-fortified ghee had a shelf life of 8 days at 80 ± 1°C, compared to only 2 days for the control ghee sample [91].

Sage and rosemary extracts have been the most widely used herbs to prolong the shelf life of ghee and butter oil [92]. These extracts have many times the antioxidant activity of synthetic antioxidants like BHA and BHT [93]. Butter oil supplemented with dihydroquercetin (DHQ) as a natural antioxidant showed the strongest oxidative stability in the accelerated test. The addition of 50, 100, 150, and 200 ppm of DHQ increased the shelf life of butter oil by 1.9, 2.8, 2.99, and 3.53 times, respectively [94]. Similarly, adding 80 mg of olive mill waste water or pomace to 1 kg of butter provided oxidative stress resistance during storage both under ambient thermal conditions (25°C) and the oven conditions (60°C) for three months [95]. During storage, the butter made from sour cream supplemented with 2% sage or rosemary had higher oxidative stability and lower secondary oxidative products, including malonaldehyde and ketones, than the control butter. However, rosemary herb was found to be more efficient than sage in slowing lipolysis in butter [96].

Similarly, Merai *et al.* found that the ghee made from butter and 0.6% Tulsi (*Ocimum sanctum* L.) leaves extract was as stable as the ghee containing 0.02% of BHA after 8 days of high temperature storage (80 ± 2°C) [97]. The phenolics found in Tulsi leaves appeared to be the primary contributors to ghee's increased oxidative stability. Furthermore, Farag *et al.* reported that adding thyme and cumin essential oils to butter prevented it from spoiling at room temperature and was more efficient than butylated hydroxy toluene [98].

Plant extracts as natural milk-clotting enzymes. The global increase in cheese production, along with a decreased supply and higher prices of calf rennet, has led to the search for alternative milk-clotting enzymes as suitable rennet substitutes. Plant clotting enzymes, also known as plant proteases, have become a subject of growing interest in the cheese industry due to their availability, simple purification processes, and low cost, as well as stability [99, 100]. The selection of a suitable plant coagulant depends on the optimum conditions for enzyme activity (pH, temperature, salt, solvents, etc.), milk-clotting activity/proteolytic activity (MCA/PA ratio), and the rheological and sensory properties of final products [101].

Plant proteases. In general, the main classes of milk-clotting proteases are aspartic, serine, and cysteine proteases. The number and type of enzymes vary from one species to another and depend on the plant parts [101]. As chymosin, some plant proteases can cleave a few sites at αs1- and β-caseins, which may occur in maintaining the micelle stability. These regions of αs1- and β-caseins are sometimes near the micelle surface and contribute to electrostatic repulsion between casein micelles. The removal of these parts could greatly assist the gelling process. First, the initial instability of micelles is increased and coagulant access to β-casein is improved. Second, the removal of these parts increases the flexibility and/or susceptibility of caseins to rearrangements in gel [102].

Plant proteases also play a significant role in the early stages of cheese ripening. The hydrolysis of caseins in cheese by residual coagulants produces essential substrates for some bacterial microflora, whose breakdown allows for flavor development during maturation. The strength of these impacts on the cheese quality depends on the type of plant coagulant used, its amount, and its enzymatic activities [103]. Some plant milk-clotting proteases are presented in Table 2.

Several studies have reported that most plant proteases with milk-clotting activity (MCA) are stable in various pH ranges (4.5–10) and temperatures (20–80°C), with the optimum pH around 6.5 and maximum activity around 60°C. The protease isolated from pumpkin seed extract curdled milk at a pH range of 4.5 to 8.5 and a temperature range of 20 to 80°C. It was resistant to solvents, salts, and surfactants, and was more effective on κ-casein than β-casein [100]. The best coagulation conditions for the pineapple, kiwi, and ginger extracts were pH 5, 6.6, and 6.6, respectively, and temperatures 45, 40, and 45°C, respectively [104]. A novel cysteine protease extracted from *Ficus johannis* L. by cation exchange chromatography was stable in a variety of pH ranges (3.0–10.5), with the optimum at 6.5, and showed maximal activity at 60°C. The purified protease had significant activity against κ-casein when compared to α- and β-casein. In the presence of high salt concentrations, the enzyme was virtually totally active [105]. Ben Amira *et al.* showed that when the pH was dropped to 3, the MCA/PA ratio rose, surpassing that of chymosin. The lowest ratio attributed to the extract at pH 6 was mainly related to its high proteolytic activity, as well as to its low MCA. Melon extracts also showed high milk-clotting activity over a wide range of temperatures (45–75°C), while kiwi and ginger extracts showed high activity over a lower temperature range, with a maximum of 40 and 63°C, respectively [101].

Effect of plant proteases on yield and physiochemical properties of cheese. The rheological properties of milk gels and sensory characteristics of cheeses produced by plant proteases vary according to the type of coagulant, its enzymatic activities, and its concentrations. Most plant enzymes are not suitable for industrial-scale cheese production, where a large portion of protein is lost due to excessive hydrolytic activities [106]. For example, the curd yield produced using kiwi (17.8%), melon (15.1%), and ginger (15.4%) extracts was lower than that produced using commercial rennet (20.2%). Kiwi extracts had textural properties comparable with those obtained using calf rennet, thus

Table 2 Some plant milk-clotting proteases

Plant	Scientific name	Type of protease	Names	Numbers
Cardoon	*Cynara cardunculus* L.	Aspartic	Cardosin	8 (A to B)
Artichoke	*Cynara scolymus* L.	Aspartic	Cynarase	3 (A , B, C)
Wild thistle	*Cynara humilis* L.	Aspartic	Cardosin	1 (A)
Asian rice	*Oryza sativa* L.	Aspartic	Oryzasin	1
Milk thistle	*Silybum marianum* L.	Aspartic	Enzymatic extract	–
Cotton thistle	*Onopordum acanthium* L.	Aspartic	Onopordosin	1
spear thistle	*Cirisum vulgare* L.	Aspartic	Cirsin	1
Red star-thistle	*Centaurea calcitrapa* L.	Aspartic	Enzymatic extract	–
Afghan fig	*Ficus johannis* L.	Cysteine	Ficin	1
Lebbeck	*Albizia lebbeck* L.	Cysteine	Enzymatic extract	–
Fig	*Ficus carica sylvestris* L.	Cysteine	Ficin	2
Golden kiwifruit	*Actinidia chinensis* L.	Cysteine	Actinidin	1
Crown flower	*Calotropis gigantea* L.	Cysteine	Calotropain	4 (FI, FII, DI, DII)
Ginger	*Zingiber officinale* L.	Aspartic & cysteine	Ginger	3 (GPA, GPB, GPC)
Chaguar	*Bromelia hieronymi* Mez	Serine	Hieronymain	3
Solanum coagulans	*Solanum dubium* Fresen	Serine	Dubiumin	1
Lettuce	*Lactuca sativa* L.	Serine	Cucumisin	1
Egyptian balsam	*Balanites aegyptiaca* L.	Aspartic & serine	–	2

Source: Ben Amira *et al.* [101]

holding the best potential as a milk coagulant in cheese production. Melon extracts, however, produced a fragile gel and a low curd yield [107]. The cheese made with the purified enzyme obtained from *F. johannis* had similar textural properties and chemical compositions to the cheese produced using commercial calf rennet [105]. All rheological parameters indicated a strong milk gel formed using pumpkin seed extracts. The peptidase sequence was homologous with that of cucumicin-like peptidase [99, 105].

Abebe & Emire used *Calotropis procera* L. enzyme leaf extract as an alternate milk coagulant to produce fresh cheese [108]. The highest cheese yield and the fastest clotting time were 17.89 kg of cheese/100 kg of milk and 14:50 min, acquired with 10 g of *C. procera* powder at 60°C extraction, respectively. There was no difference in coagulation time among the milk samples with varied milk fat structures. However, whey was extracted from homogeneous cream curds more quickly than from non-homogenized cream curds [109]. Camel milk is difficult to convert to cheese using regular rennet, so pineapple, kiwi, and ginger enzyme extracts are used to help make cheese from camel milk. Kiwi extract showed the highest curd yield (20.71%) when compared to pineapple (19.74%) and ginger (11.50%) extracts [106]. Mazorra-Manzano *et al.* reported that kiwi had a higher output than melon and ginger but was lower than chymosin in cow milk [107]. When compared to the camel cheese with pineapple and ginger extracts, the camel cheese with kiwi extract had higher amounts of water-soluble vitamins, primarily B_7 (3.75034 mg/g), B_{12}, and B_5, as well as higher mineral contents, primarily Na (605.2 ppm) and Ca (63.11 ppm). The water content was lowest in the camel milk cheese made with ginger extract, whereas the protein content was higher in the cheese made with pineapple extract than kiwi or ginger extracts [104]. Gad & Abd El-Salam mentioned that higher concentrations of rosemary extract increased the rennet coagulation time of skim milk. The antioxidant activity of the skim milk/rosemary extract blends was improved by heat treatment, calcium chloride addition, and pasteurization [110]. Furthermore, by suppressing oxidation, rosemary extract as a natural antioxidant could extend the shelf life of Cheddar cheese or cheese powder [111].

Plant extracts and sensory properties of dairy products. Flavor, an important sensory component, is one of the variables influencing customer preferences. The flavor of food is determined by the presence of volatile aromatic compounds, which take different forms in different foods [112]. Flavoring compounds can be roughly classified into plant-based, artificial, and biotechnologically formed flavors. Plant-based flavors are separated from plant-based sources rich in aromatic compounds, spices like vanilla, or herbs. Modupalli *et al.* reported that plant-based food flavoring agents are naturally occurring polyphenolic compounds, organic esters, acids, alkaloids, and carotenoids [113]. Dry aromatic plants, as well as their essential oils and extracts, are used in dairy products in order to give them a distinctive and attractive taste and smell [2]. In cheese, the ethanol cinnamon extract improved the flavor and overall quality of flavored processed cheese, whereas lemon grass and cress extracts improved its odor and color, respectively [35]. The UF-soft cheese produced with the ethanol ginger extract became more pleasant compared to the control cheese, especially during storage [38]. The major compounds responsible for the unique ginger flavor are gingerols and other volatile oils. Over storage, gingerols are converted

into a series of homologous compounds known as shogaols, which are more pungent than gingerols [114]. Mahajan *et al.* improved the flavor, texture, and acceptability of low-fat Kalari cheese by using aqueous pine needle extracts [36]. Similarly, the aqueous extracts of *Inula britannica* L. increased the odor and flavor of a Cheddar-type cheese [115]. The cottage cheese with 8 and 9% aqueous green tea extract acquired a pleasant, moderately expressed green tea flavor and taste, whereas the cottage cheese with a high level of tea extract (> 9%) had a bitter and disagreeable tea flavor [116, 117].

Essential oils improve the flavor and smell of dairy products and also extend their shelf life. The highest overall acceptability during storage was achieved by Iranian white cheese containing 0.75% basil oil, followed by the sample containing 0.5% salvia oil. However, adding 0.75 and 1% salvia oil impaired the samples' odor and taste [43]. Iranian white cheese and Domiati cheese were also the most preferable and had the highest flavor scores when supplemented with 1 and 0.2% black cumin essential oil [38, 41]. Inversely, the goat cheeses treated with essential oils had a bitter flavor, whereas Feta cheese had a strong off-taste due to large quantities of clove and tea tree oils required for antibacterial activity [118]. Furthermore, Foda *et al.* observed that high concentrations of spearmint oil can generate concerns about changes in white cheese's sensory properties, to the point that the panel test revealed the highest acceptability at lower oil concentrations [119].

In fermented milk, adding the extracts of *D. kaki* leaf and *Nelumbo nucifera* L. leaf to yoghurt increased its viscosity, water-holding capacity, bitterness, and texture smoothness. The *D. kaki* – fortified yoghurt contained a high content of flavoring components such as acetaldehyde, acetoin, and diacetyl, which gave it the best taste in the sensory evaluation. The *N. nucifera*-fortified yoghurt showed the largest amount of acetic acid in the volatile complex analysis and the highest pH value [66]. According to Zaky *et al.*, adding 2 μL/100 mL of dill and caraway essential oils to Labneh made from buffalo's milk increased the total volatile fatty acids of Labneh during storage [120]. In addition, it improved its antioxidant activity and sensory qualities, compared to the control. Ghalem & Zouaoui added *Rosmarinus officinalis* L. oil to yoghurt at concentrations of 0.14, 0.21, 0.29, and 0.36 g/L and stored it for up to 21 days [121]. The herbal yoghurt enhanced with 0.14 g/L of the oil received the highest score for taste, flavor, and texture from the panel. Trivedi *et al.* tested basil in various forms (juice and dried powder) as an ice cream flavoring agent. Compared to the control, adding basil juice (up to 2%) lowered protein, fat, total solids, ash, and total carbohydrate contents, as well as melting resistance and pH [122]. Coffee extract was also used to produce a distinct probiotic coffee ice cream with the desired coffee flavor that improves the consumer's emotional state and aids in calorie burning [123]. Table 3 represents some dairy products fortified with flavoring plant extracts and their bioactive components.

Encapsulated plant extracts in dairy products. Encapsulation is a process of entrapping one substance into another to improve the bioavailability of high-value compounds [129]. It can be used to mask undesirable

Table 3 Plants and their bioactive components used as flavoring agents in some dairy products

Plants	Scientific name	Bioactive components	Applications	References
Cinnamon	*Cinnamomum zeylanicum*	Cinnamaldehyde	Soft cheese, Processed cheese, Yoghurt	[124, 125]
Lemon grass	*Cymbopogon* L.	Eral, Citronellal, Linalool, Geranial, Limonene, 6-Methyl Hept-5-En-3-One, Caryophyllene, *β*-Myrcene	Indian soft cheese, Yoghurt, Coalho cheese, Processed cheese	[35, 125, 126]
Ginger	*Zingiber officinale* L.	Gingerols, Shogaols, other volatile oils	UF-soft cheese	[38, 114, 127]
Pine needle	–	–	Low-fat Kalari cheese	[36]
Green tea	*Camellia sinensis* L.	Theanine, caffeine, chlorophyll, and various types of catechins	Cottage cheese	[116]
Basil	*Ocimum basilicum* L.	Eugenol, *β*-caryophyllene	Iranian white cheese, Yoghurt, Ice cream	[43, 122, 128]
Black cumin	*Nigella sativa* L.	Thymoquinone, P-Cymene, T-Anethole, Sequiterpene Longifolene, Nigellicimine, Nigellicimine N-Oxide, Pinene, Thymol	Iranian white cheese, Domiati cheese	[38, 41]
Spearmint	*Mentha spicata* L.	Menthol, Epoxyocimene, Linalool, Menthone, Eucalyptol, *Neo*-menthol	White cheese	[119, 125]
Dill	*Anethum graveolens* L.	Quercetin, Kaempferol, Myricetin, Catechins, Isorhamnetin, Carvone, Limonene	Milk	[120]
Caraway	*Carum carvi* L.	Carvone and limonene	Labneh	[120]
Rosemary	*Rosmarinus officinalis* L.	Camphene, Pinene, Limonene, Myrcene, Camphor, Thujone, Verbenone, Cuminic Aldehyde	Yoghurt	[121]

flavors and odors, protect biologically active compounds from adverse interactions with other substances, and improve stability under a variety of environmental conditions, including temperature, moisture, oxidation, and light [10]. Salama *et al.* summarized numerous forms for encapsulating bioactive compounds, including nano-capsules, micro-capsules, nano-emulsions, micro-emulsions, solid lipid nanoparticles, liposomes, and others. The nano- or micro-capsules are excellent systems that deliver bioactive compounds for direct absorption. Dairy products fortified with encapsulated plant extracts have better nutrition and health benefits. There is a variety of materials used for encapsulation, including gum Arabic, modified starches, maltodextrins, alginates, pectin, carrageenan, hydrogenated vegetable oils, bees wax, soy proteins, gelatins, whey proteins, sodium caseinates, and others. Below are some examples of recent and most common applications of encapsulation technology in dairy products [130].

The encapsulation of sage extract in liposome improved its antimicrobial activity against pathogenic bacteria and affected the physicochemical properties of the resulting yoghurt. Particularly, acidity increased, while diacetyl and acetaldehyde decreased when the extract was added at rates of 5, 10, 15, and 20%. The yoghurt's viscosity first increased but then decreased during storage [131]. The low antioxidant activity in dairy products was corrected by fortifying them with doum fruit extract in a liposome form. The encapsulated doum extract has a significant impact on yoghurt's chemical analysis, especially at higher concentrations [132]. El-Messery *et al.* investigated the encapsulation of mango peel phenolic extract and its use in milk beverages [133]. They found that these flavored drinks were well-liked by consumers. They were high in antioxidants, phenolic compounds, and many other bioactive components. In addition, the encapsulated extract did not alter the chemical or rheological aspects of the beverage. In another study, chitosan microcapsules containing beetroot or ginger aqueous extracts were added to fermented camel milk in the presence of probiotic bacteria. The 10% concentration of the beetroot aqueous extract microcapsules increased the survivability of probiotic bacteria, whereas the amount of ginger was only 1%. The best effects were achieved by the chitosan beads with the beetroot aqueous extract [134]. Jaboticaba-loaded nanoemulsion (up to 15%) was added to cow milk to make it rich in phenolics and exhibit high antioxidant activity [135]. Adding the fig leaf extract microencapsulated with alginate and/or skim milk improved the cheese sauce's properties, microbiological quality, and shelf life [136]. The control cheese and the functional cheese supplemented with 2% liposomal encapsulated saffron extract showed the greatest difference in terms of chemical composition and color. The cheeses containing the encapsulated saffron extract were significantly harder and chewier, compared to the control. However there was no significant difference in adhesiveness, cohesiveness, and gumminess among the cheese samples. Based on the findings, liposomal encapsulation was considered an efficient method for the delivery of saffron extract to ricotta cheese as a novel functional food [137].

In another study, the encapsulated Arjuna herb extract improved the vanilla chocolate dairy drink in all the parameters tested by the response surface methodology [138]. Barretto *et al.* reported that the encapsulated anthocyanin provided yoghurt with high stability and large amounts of antioxidant and phenolic compounds. Encapsulation gave anthocyanin more efficiency and biological activity [139]. Sawale *et al.* studied the effect of heat treatment on the stability of the *T. arjuna* extract and its content of phenols and antioxidants when it was used in the free and encapsulated forms to fortify the vanilla chocolate-flavored milk drinks [140]. They found that sterilization had a negative effect on the extract in the free form and its content of antioxidants and phenols. The encapsulated extract, however, was stable enough to protect the bioactive compounds. Lourenço *et al.* studied the encapsulation of pineapple peel hydroalcoholic extract rich in phenolic compounds. To preserve these ingredients, maltodextrin, inulin, and Arabic gum were used as encapsulation materials by spray drying. The resulting powder had a good flow ability and suitable handling properties. Also, this encapsulation method ensured high antioxidant activity, while the non-encapsulated extract had low activity in the same conditions [141].

CONCLUSION

As compared to plants or their parts, the use of plant extracts and essential oils in the dairy industry has received a lot of positive feedback from both dairy producers and consumers. Plant extracts can be used to enhance the flavors, antioxidant and antibacterial properties of fortified dairy foods, as well as their visual appeal. Additionally, plant extracts can be added to dairy products in a variety of forms, free or encapsulated, to protect active ingredients from the external environment and control their release. They can also be incorporated as a nanoemulsion to enhance the compounds' bioactivity. Plant extracts and their essential oils have different effects on microorganisms, depending on the type of microbe, the type and concentration of the plant extract, and the interaction of antimicrobial chemicals with the food matrix. Most studies indicate that plant extracts and essential oils included in various dairy products increase the survival of probiotics and starter bacteria while acting as antimicrobials against pathogenic and spoilage-causing microorganisms. With the growing demand for cheese in the world, plant-based coagulants can be a good substitute for calf rennet, which is currently in low supply. Different plant-derived proteases can be used in milk coagulation and cheese-making. However, to produce cheeses that are comparable to those made with calf rennet, we need to select the right type of

plant proteases (specific or non-specific action) and their enzymatic activity (milk-clotting activity/proteolytic activity ratio), depending on the type of cheese to be produced (ripened or non-ripened). In the dairy industry, plant extracts can also be used to either increase desirable ingredients (vitamins, fiber, and minerals) or to partially replace undesirable ingredients (salt and sugar).

CONTRIBUTION

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

CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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Use of animal origin protein concentrates in bread baking

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Abstract:
Protein deficiency in the human diet is a widespread problem that affects all body systems. Nutrition adjustment appears to be one of the most effective ways to prevent this problem. This study was aimed at investigating the possibilities of using animal origin protein concentrates in bread baking industry.
Study objects included five breads: one control and four samples containing protein concentrates. The test wheat bread samples contained 7, 9, and 11% of milk protein concentrate and 7% of whey protein concentrate to the mass of flour in the dough. Rheological parameters of the dough were obtained using an alveograph and a farinograph. The specific volume and sensory characteristics of the baked products as well as crumb deformation were evaluated. The nutritional value of two samples – control and with 9% of milk protein concentrate addition – was determined by calculation.
Whey protein concentrate had a negative influence on the dough rheology. Low water binding capacity and specific volume as well as hard crumb make the usage of this product in bread baking unacceptable. The palatability test showed that bread supplemented with 9% of milk protein concentrate had the best sensory characteristics, compared to control and other test samples (with 7 and 11% of this concentrate). The predicted protein content was equal to 11.6 g/100 g that is 35% higher than in the control sample. Consequently, milk protein concentrate has a potential to improve biological value of the bread that can be further helpful to prevent the protein deficiency.
Milk protein concentrate can be successfully used in the bread making process as an ingredient to correct the nutritional and biological value of baked products.

Keywords: Fortified bread, whey protein, milk protein, rheology, protein concentrate

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INTRODUCTION

Protein is one of the most important nutrients in the human diet, which functions are enzymatic, construction, protective, transport, and others [1]. Protein is a source of essential amino acids, which determine its biological value. The quality of the protein is evaluated with amino acid score – the ratio of essential amino acids content in the investigated protein to the similar essential amino acids in the reference one. Amino acid score equal to 100% indicates the optimal amino acid compositio nof the product. If the content of one or more essential amino acids is less than 100%, then these acids are considered as limiting, and the protein is named imperfect. An unbalanced diet leads to the disruption of the normal human body functioning and occurs, among other things, because of mono-diets, fasting, and vegetarianism. A diet based on the protein with one or a few limiting essential amino acids should beadjusted.

Enrichment of the food products with protein components is one of the current trends in the developing of the products aimed at preventing protein deficiency in the human diet. Such a mass-consumption product as bread, which is included in the daily ration of the majority of Russians, is promising for enrichment. In 2020, the consumption of bread products in the Russian Federation amounted to 116 kg per capita.

Wheat flour is one of the main ingredients in the bread formulation. Top-grade flour which is milled from soft wheat is mainly used for bread baking. The wheat grain itself contains 11.8 g of protein and all essential amino acids. As a result of milling wheat, various types of flour are obtained, namely, top, first, and second grade, as well as whole-wheat flour. At the same time, the content of protein, vitamins, and amino acids

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depends on the yield of the flour – the more the yield, the lower the content of these nutrients [2]. The top-grade wheat flour contains the least amount of these nutrients. The protein of this flour contains limiting essential amino acids, the most deficient of which are lysine and methionine (Table 1). The biological value of the whole-wheat flour is also imperfect due to the lack of lysine.

It is possible to provide a full-fledged amino acid composition of in bakery products by introducing high-protein components of plant or animal origin into the bread formulation. Pulses such as pea, soy, and chick-pea are widely used for bread production fortification. For example, high-protein flour was obtained from pinto beans by grinding and extrusion. The 5% introduction of this flour instead of wheat flour allowed increasing the content of the essential amino acid lysine in the finished product by 48%. The dough resistance to kneading, which is an important parameter of the dough making process, has also increased [4]. The addition of soy protein in concentrations from 0 to 30% led to the decrease in the specific volume of bread from 2.61 to 1.31 cm^3/g [5]. The decrease in the bread specific volume was caused by the addition of pea protein powder. With the maximum dosage of the additive equal to 25%, the volume of bread decreased by 116 cm^3 relative to the control sample [6]. Consequently, the use of soy and pea proteins in baking leads to the lower visual volume and, as a result, the unsatisfactory sensory characteristics of the product.

The alternative flours of different origin, namely, lint, lupine, and others are a great alternative to the pulses [6–9]. The addition of 10% pea protein powder instead of flour to bread made from buckwheat and flaxseed flour had no negative affect on the physical and chemical quality indicators of the dough. In the baked product, the index of essential amino acids increased from 34 to 40, and the protein content accounted for 17.1% [6]. The bread baked from a mixture consisting of 88.8% wheat flour, 8.2% low-fat soy flour and 3.0% whey protein concentrate resulted in bread with a high protein concentration with an increased content of available lysine [10]. Lupine seed protein is distinguished by a high protein content (for more information, see Table 2). The flour obtained from lupine seeds has found application in the production of short crackers, buns, and hard dough cookies [11]. In addition, cricket flour, *Oncorhynchus tschawytscha* L. fish powder, and others are potential raw materials for the development of high-protein bread [12, 13].

The use of the protein additives discussed above allowed increasing the total protein and lysine content in the finished product. Nevertheless, the specific volume, that determines softness, appearance, and sensory evaluation of the product, decreased. At the same time, plant origin proteins do not contain all essential amino acids and its digestive value is only 62–80%. Amino acid composition of animal origin proteins, as a rule, is full-fledged, and its digestibility is 93–96%. Thus, these albumens may act as a source of the lacking essential amino acids, which inclusion in the diet is necessary to maintain normal human body functioning.

Highly concentrated forms of milk protein – concentrates and isolates – contain up to 80–95% protein and are also perspective for products with functional properties [14, 15]. Milk protein concentrate is obtained from skimmed milk by ultrafiltration and subsequent spray drying. Whey protein concentrate is

Table 1 Amino acid composition and amino acid score of the top-grade and whole-wheat wheat flour [3]

Essential amino acid	Reference protein FAO/WHO, 2011	Top-grade wheat flour	Whole-wheat flour	Top-grade wheat flour	Whole-wheat flour
	Content, g/100 g			Amino acid score, %	
Isoleucine	3.20	4.17	4.96	130.30	146.60
Leucine	6.60	7.83	6.96	118.60	105.50
Lysine	5.70	2.43	3.12	42.60	54.70
Methionine + Cysteine	2.70	3.43	3.69	127.00	136.70
Phenylalanine + Tyrosine	5.20	7.28	7.77	140.00	149.40
Threonine	3.10	3.02	3.12	97.40	100.60
Tryptophan	0.85	0.97	1.12	114.10	131.80
Valine	4.30	4.57	4.40	106.30	102.30

Table 2 Protein content in the studied raw materials

Raw material	Production	Protein content, g/100 g
Protein fraction of pinto bean flour	Kelly Bean Co, USA	39.2
Soy protein	Yihai Kerry Groups, China	86.6
Pea protein powder	Bio Planet, Poland	78.4
Lupine	–	≤ 40.0
Cricket flour	Kreca Ento-Food BV, Netherlands	66.1
Whey protein concentrate	Fonterra Unifood, New Zealand	80.0
Milk protein concentrate	Fonterra Unifood, Australia	85.0

obtained from cheese whey using the similar technology. The products have a neutral creamy milk taste and are able to bind flavor components. Therefore, its use in baking can have a positive effect on the flavor of the finished product. Both concentrates also contain lactose which is a reducing sugar capable to react with amino acids due to which bakery products have an attractive color. Lactose is not fermented by the yeast *Saccharomyces cerevisiae*, that is why it is not changed during the fermentation process and remains in the same amount. Consequently, the color of the crust will be even more intense.

Milk and whey protein concentrates produced by Fonterra are distinguished by a high biological value. The protein content of these products is 85 and 80 g/100 g, respectively. At the same time, soy protein concentrate contains 71 (N×6.25) g of protein and all essential amino acids. The content of each essential amino acids in 100 g of protein, except for phenylalanine, is less than in whey protein [16]. Comparative amino acid composition of protein concentrates of animal and plant origin is presented in Table 3. According to the given data, whey protein concentrate has higher essential amino acids content, therefore, its use as a fortifier is more effective.

The aim of this research is to substantiate the possibility of using the animal protein products, namely, whey and milk protein concentrates as food additives improving the biological and techno-functional properties of bakery products.

STUDY OBJECTS AND METHODS

Wheat bread with 7, 9 and 11% of milk protein concentrate and 7% of whey protein concentrate as experimental samples as well as a control bread were made by single-phase method and then analyzed.

Materials. Wheat flour (Petersburg Mill Plant, St. Petersburg, Russia), fresh yeast (SAF-NEVA, Russia), salt, and tap water were used in the bread making process. Protein ingredients such as milk protein concentrate (MPC 485) and whey protein concentrate (WPC 450) were supplied by Unifood company.

Nutrient composition of materials. The nutrient composition of whey and milk protein concentrates was studied in the Fonterra laboratory using the following methods: moisture – express method (moisture meter MOC63u, Japan) according to State Standard 29246-91; protein (N×6.38) – State Standard 34454-2018; fat – State Standard ISO 1736-2014; lactose – State Standard 34304-2017; calcium and sodium – inductively coupled plasma optical emission spectrometry (ICP-OES). The amount of protein in dry matter was defined by calculation.

The moisture content of the wheat flour was determined by the express method by drying in a moisture meter at 160°C, the nutrient composition was specified by the manufacturer.

Bread dough preparation process. The introduction of raw materials in the form of protein concentrates changes the content of dry matter in the formulation mixture. Therefore, it is necessary to adjust the percentage of water required for kneading. The control sample was kneaded according to the formulation illustrated in Table 4. The dry ingredients were mixed for 1 min at speed 1 (Bear Varimixer, Denmark). Then water was gradually added, and kneading continued for 3 and 7 min at speeds 1 and 2, respectively.

After this, the formulation was adjusted based on the dry matter amount which indicated in Table 5. Table 6 shows the content of dry matter in the formulation mixture per 100 kg of the flour. The percentage of other ingredients is demonstrated in Table 4. The theoretical

Table 3 Amino acid composition of whey and soy protein concentrates [13, 16]

Essential amino acid	Whey protein concentrate	Soy protein concentrate
	Content, g/100 g	
Isoleucine	7.0	4.8
Leucine	11.4	7.9
Lysine	9.4	6.4
Methionine + Cysteine	2.6	1.4
Phenylalanine + Tyrosine	3.5	5.2
Threonine	7.4	4.5
Tryptophan	2.1	1.6
Valine	6.4	5.0

Table 4 Formulation of control sample

Ingredient	Dough, g	Dough, % to wheat flour
Top-grade wheat flour	500.0	100.0
Water (22°C)	290.0	58.0
Food salt	7.5	1.5
Fresh yeast	10.0	2.0
Total dough	807.5	161.5

Table 5 Weight fraction of dry matter in raw materials

Ingredient	Dry matter content, %
Top-grade wheat flour	84.8
Salt	96.5
Fresh yeast	25.0
Milk protein concentrate	91.6
Whey protein concentrate	92.6

Table 6 Dry matter content in raw materials, per 100 kg of wheat flour

Ingredient	Dry matter content, kg				
	Control	Experimental samples			
		Milk protein concentrate, 7%	Milk protein concentrate, 9%	Milk protein concentrate, 11%	Whey protein concentrate, 7%
Top-grade wheat flour	84.8	84.8	84.8	84.8	84.8
Food salt	1.4	1.4	1.4	1.4	1.4
Fresh yeast	0.5	0.5	0.5	0.5	0.5
Milk protein concentrate	–	6.4	8.2	10.1	–
Whey protein concentrate	–	–	–	–	6.5
Total dry matter	86.7	93.1	94.9	96.8	93.2
Hydration, %	58.0	62.3	63.5	64.8	62.3

Table 7 Formulation of breads

Ingredient	Ingredient quantity, % to wheat flour			
	Milk protein concentrate, 7%	Milk protein concentrate, 9%	Milk protein concentrate, 11%	Whey protein concentrate, 7%
Top-grade wheat flour	100.0	100.0	100.0	100.0
Water (22°C)	64.0	67.0	70.0	52.0
Salt	1.5	1.5	1.5	1.5
Fresh yeast	2.0	2.0	2.0	2.0
Milk protein concentrate	7.0	9.0	11.0	–
Whey protein concentrate	–	–	–	7.0
Total dough	174.5	179.5	184.5	162.5

hydration of the test samples was calculated based on the given hydration of the control sample equal to 58%. According to the data in Table 6, the addition of only 7% of both milk protein concentrate and whey protein concentrate allowed increasing the calculated water absorption capacity of the dough by 4.3%. The actual quantity of water added was determined using a farinograph. The final formulations of all samples are shown in Table 7.

Effect of protein concentrates on the dough quality. The influence of milk and whey protein concentrates on rheological properties of the dough was investigated. Protein ingredients and wheat flour were premixed according to the formulation (Table 1) and then analyzed. Bread baked with no addition of protein was chosen as a control sample.

Resistance to extension and extensibility. Dough extensibility (L) and resistance to extension (P) as well as the ratio of these values (P/L), that describe the viscoelastic properties of the dough, were measured using a Chopin alveograph (Chopin Technologies, Paris, France). Analysis was performed according to State Standard R 51415-99. The amount of water was calculated based on the moisture content of raw materials.

Water absorption and dough development during mixing. The moisture content of test samples was adjusted using a Brabender farinograph (Brabender, Duisburg, Germany) according to State Standard R 51404-99. The dough consistency of 500 FU guarantees the best possible processing properties. After adding the required amount of water, we measured water absorption of the dough, dough development time, stability (S1), and degree of softening 12 min after the curve maximum (DS ICC; fall in viscosity). The optimal amount of water was used in dough preparation process.

Baking process. After fermentation (30 min, 24°C) dough was separated into 250 g pieces, shaped and transferred into a bread pan. Then bread was placed in the proofing cabin (MIWE klima, Germany) for 70–80 min at t = 36°C and W = 75%. The bread was baked in a rotary oven (Revent, Sweden) for 22 min at t = 220°C (landing temperature 250°C). The samples were cooled for sixteen hours before analysis.

Effect of protein concentrates on techno-functional properties of the breads. Specific volume was measured by the displaced grain method according to State Standard 27669-88. Crumb texture was evaluated using a texture analyzer Structurometer ST-2 (Quality laboratory LLC, Russia). The method is based on measuring the crumb deformation under constant force.

Sensory evaluation of the breads. Palatability test was performed according to State Standard 5667-65 by the following criteria: overall appearance, crust color, crumb structure, flavor, and taste.

Impact of protein concentrates on the nutritional value of the breads. The nutritional value of the best and the control samples was calculated considering baking loss equal to 11%.

RESULTS AND DISCUSSION

Nutrient composition of materials. The nutrient composition of the used raw materials determines the nutritional value of the final product. The composition

Table 8 Nutrient composition of top-grade wheat flour and protein concentrates

Ingredient	Content per 100 g					
	Moisture, %	Protein (N×6.38), g	Fat, g	Lactose, g	Calcium, mg	Sodium, mg
Top-grade wheat flour	15.2	10.3	1.1	0.0	18.0	3.0
Milk protein concentrate	8.4	81.1	1.5	5.0	2100.0	70.0
Whey protein concentrate	7.4	78.2	5.1	3.7	415.0	226.0

of the top-grade wheat flour and protein concentrates is illustrated in Table 8. The moisture content of the milk and whey protein concentrates at the moment of production was equal to 5.7 and 4.2%, respectively. Such low moisture content is conditioned by spray drying technology, during which the product is dehydrated to a moisture content of 4–9%. At the time of the study, the moisture content of the products was 8.4 and 7.4%. Proteins were in non-hermetic conditions, therefore, the moisture content during storage increased. The milk protein concentrate and whey protein concentrate had a similar protein content – 81.1 and 78.2 g/100 g, respectively, which was 787.4 and 759.2% higher than in wheat flour.

The ultrafiltration process is the separation of milk into components of different sizes by passing it through a membrane under pressure. This process results in products with different levels of water, protein, lactose, and minerals. Milk protein concentrate has a lower fat content. This is because of product origin: milk protein concentrate is made from skimmed milk. The content of lactose and calcium in milk protein concentrate, on the contrary, is higher. Since both dairy products are obtained using the same technology – ultrafiltration with spray drying, this difference is due to the products' nature. Cheese whey contains more sodium, while skimmed milk is rich in calcium [3].

Effect of protein concentrates on the dough quality. During alcoholic fermentation, carbon dioxide is released, due to which the dough expands, forming pores. With an increase of the dough extensibility, its gas-holding capacity and the volume of the test semi-finished product increase. Gas-holding capacity is a quality indicator that depends on the visco-elastic dough properties. At the same time, the more high-quality gluten is in the flour, the higher its gas-holding capacity. The value of this indicator determines the volume and crumb structure of baked products.

Resistance to extension and elasticity were determined using an alveograph. The maximum value of elasticity reflects the force that must be applied to start the gluten network deformation process. Therefore, the smaller the value of resistance to extension, the easier it is to stretch the dough and the greater increase in volume during fermentation is expected.

The dough prepared with the introduction of the minimal whey protein concentrate amount (7%) had an unsatisfactory consistency. As a result, the dough became very sticky and inconvenient in the further work process. It was not possible to perform the analysis with an alveograph due to the strong liquefaction. This effect may be caused by the weakening effect of whey concentrates on the flour gluten [17]. The destruction of disulfide bonds leads to an increase in the fluidity of the dough and a decrease in its gas-holding capacity. Thus, it was decided to exclude a dough sample with whey protein concentrate from the further research.

Milk protein concentrate, on the contrary, strengthened the dough. The maximum volume of the bubble decreased by 47% relative to the control, the dough resistance to extension during inflating into a bubble increased by 81%. Coarsely ground amaranth flour, added to the dough instead of 5% wheat flour, had a similar effect on extensibility – the L value decreased by 57% [18].

A farinograph was used to control the water absorption capacity of the semi-finished product. The added amount of water is necessary to achieve a consistency of 500 FU. The water absorbed by the flour during the kneading process contributes to the formation of gluten. The amount of water is important for forming a dough with optimal rheological characteristics. Too much or, vice-versa, too little water addition leads to a sticky or strong dough, respectively. The water absorption capacity of flour determines the ability of the dough to retain carbon dioxide and, as a result, the volume of the bread. Water absorption capacity depends on the content in wheat of such components as water-soluble proteins, pentosans, and damaged starch.

Beside water absorption capacity determination, the dough development time – the time taken to achieve the maximum consistency of 500 FU; stability (S1) – unchanged dough structure without a fall in viscosity; softening (DS) – decrease in consistency; and degree of softening twelve minutes after reaching the maximum curve (DS ICC) can be read on the farinograph curve. The width of the curve reflects the elasticity of the dough.

Flour, called weak, quickly reaches the desired consistency. At the same time, stability is maintained for a short period of time, and the consistency curve sharply decreases. Strong flours, on the other hand, take longer to develop up to 500 FU, remain stable for some time, and have a slight fall in consistency. The results of the analyzes carried out both instruments are demonstrated in Table 9. Figure 1 shows farinograph curves of two dough samples: control and with 7% of milk protein concentrate addition.

The 7% of milk protein concentrate addition increased water absorption of the test sample by 6% compared to the control one, which is 1.7% higher than

Table 9 The results of dough samples analysis

Quality indicator	Control sample	Milk protein concentrate, 7%
Water absorption, %	58.0	64.0
Dough development time, min:s	01:58	01:24
Kneading stability, min:s	09:36	07:19
Softening, FU	47	57
Resistance to extension, mm WG	113	205
Extensibility, mm	95	45
Resistance to extension/ extensibility	1.19	4.56

theoretically stated. This advantage may be explained by the ability of milk protein concentrate to bind water due to the special protein structure of the product [17]. The sample containing milk protein concentrate had sharper fall in consistency and faster fall in stability due to the higher water absorption – the test sample kept 500 FU line 2:17 min less than the control one. Thus, the protein concentrate had a negative effect on the strength of the flour, which subsequently led to obtaining the product with a smaller volume. Wheat flour demanded much time to reach the desired consistency, which fell more smoothly. Therefore, the strength of the studied flour may be considered as normal.

7% of whey protein concentrate addition led to a decrease in water absorption capacity by 6% relative to the control. This value is 10.3% less than the theoretical one. Therefore, dry matter in the form of whey protein concentrate is not able to bind additional moisture. This decrease in dough hydration may also be due to the weakening of the gluten. As a result, the ability of gluten-forming proteins to bind moisture is reduced.

In [19], a farinograph was also used to determine the effect of roasted yellow split pea added as a substitute for 10% of wheat flour on the baking characteristics of the formulation mixture. The introduction of the protein additive allowed increasing the water absorption capacity to 61.5%, which is 3% higher than one of the control sample. At the same time, the stability of the test sample also increased and was maintained 1:54 min longer than in the control sample.

Effect of protein concentrates on techno-functional properties of the baked products. To evaluate the bread quality, the specific volume and deformation of the crumb were determined. The measurement results are shown in Table 10.

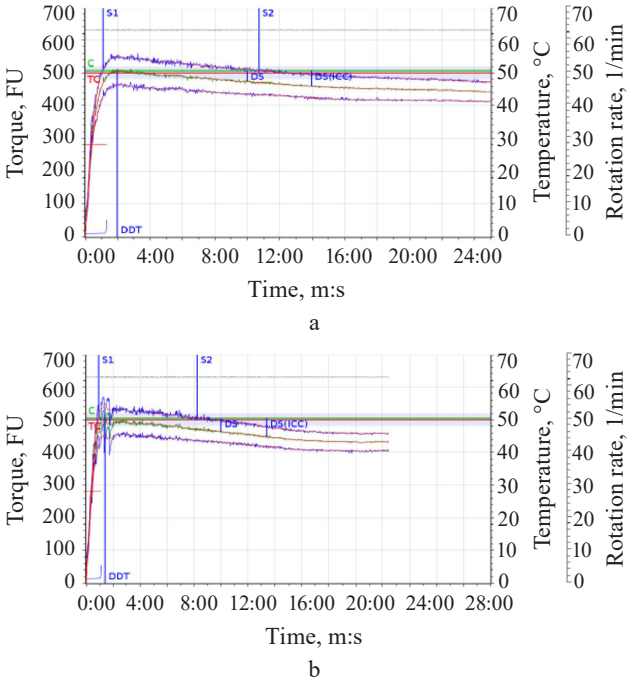

Figure 1 Farinograph curves of the dough samples: (a) control sample; (b) sample with milk protein concentrate, 7%

The specific volume is the ratio of the product volume to its mass. This indicator reflects the softness of the product – the smaller the specific volume, the worse the appearance, less the porosity, and more the density of the bread crumb. All test breads with milk protein concentrate had a smaller specific volume. The addition of protein in the amount of 7, 9 and 11% led to a decrease in the index by 0.5, 0.43 and 0.34 cm^3/g relative to the control sample, respectively (Fig. 2). Whey protein concentrate had a greater negative impact on this indicator – the product with a minimum content of whey protein had 0.72 cm^3/g less specific volume than the control sample. The introduction of another protein additive – ground chia seeds – to replace 5% of wheat flour, performed in [20], allowed obtaining the bread with a comparable value of specific volume – 2.57 cm^3/g.

The decrease in volume of test samples may be due to the worse gluten network development. This was caused by the addition of extra dry protein ingredients that are not able to form gluten. Moreover, lactose, a disaccharide contained in dairy products, is not fermen-

Table 10 Techno-functional properties of breads

Quality indicator	Control sample	Milk protein concentrate, 7%	Milk protein concentrate, 9%	Milk protein concentrate, 11%	Whey protein concentrate, 7%
Specific volume, cm^3/g	2.93	2.43	2.50	2.49	2.21
Total deformation, mm	8.69	6.56	5.57	5.25	3.85
Plastic deformation, mm	3.41	2.22	1.74	1.68	1.09
Elastic deformation, mm	5.28	4.34	3.83	3.57	2.76

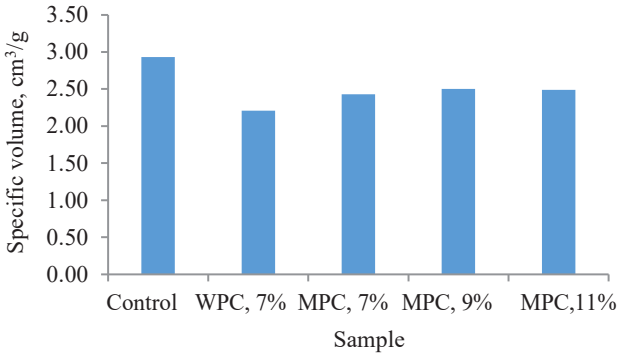

Figure 2 Specific volume of the control bread and breads with and milk protein concentrate (MPC) in different amounts and whey protein concentrate (WPC)

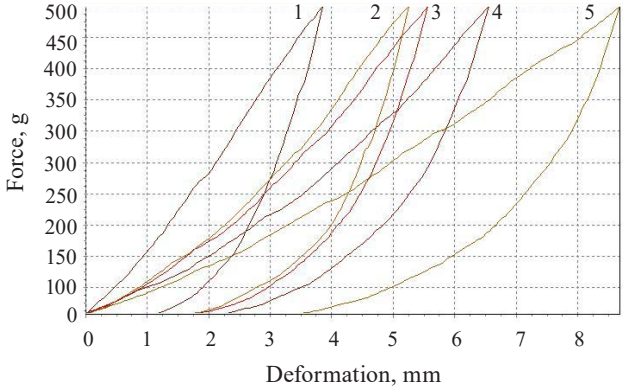

Figure 3 Effect of force on crumb deformation of breads studied: 1 – whey protein concentrate, 7%; 2 – milk protein concentrate, 11%; 3 – milk protein concentrate, 9%; 4 – milk protein concentrate, 7%; 5 – control sample

ted by baking yeast [21]. Consequently, gas formation in the dough decreased, and the bread appeared to be less fluffy.

The crumb texture was evaluated by measuring its elastic and plastic deformation under the constant force. The analysis of the deformation characteristics of the samples' crumb after sixteen hours of baking is illustrated in Fig. 3. The total deformation (H_t) is an indicator describing the degree of product softness. The higher the value of the H_t is, the softer the bread. The highest value of the initial softness was noted in the control sample. Milk protein concentrate addition had a negative effect on the product softness. With minimal (7%) and maximum (11%) amounts of the concentrate, the softness decreased by 24.5 and 39.6%, respectively. The hardness of the crumb is directly related to the specific volume and depends on the degree of gluten network development during kneading and on the content of fermentable sugars in the dough.

The sample containing whey protein concentrate, which crumb appeared to be twice as hard as the control sample's one, was the least deformable. This difference may be due to the low water absorption capacity of whey protein relative to milk protein.

Sensory evaluation of the baked products. Bread quality depends not only on its rheological characteristics, but on the sensory ones. The crust should be evenly brown, but not too dark. The crumb of the wheat bread should be light-coloured, not too tough, and have fine porosity. Sensory evaluation was performed by a group of ten people considering the listed requirements.

Bread baked with addition of whey protein concentrate had too low specific volume and dense crumb. These disadvantages are illustrated in Fig. 4 and can be explained by low gas-holding capacity of the flour. This, in turn, is caused by the destroyed disulfide bones and, therefore, weak gluten. If dough holds little carbon dioxide released during the proofing, the specific volume of the baked product is low, and the porosity is coarse. Due to the overall negative influence of this protein concentrate on the rheological and sensory parameters of the bread, baked sample was excluded from the further sensory evaluation. However, the positive influence of whey protein on the crust color should be stated. That can be explained by the high lactose content in the whey protein concentrate; as a result, sugars and amino acids are actively undergo the Maillard reaction [16].

Profilogram that illustrated in Fig. 5 reveals the most acceptable consumers' characteristics of the sample 3 with 9% of milk protein concentrate addition. The tasters also selected sample 4 with 11% of protein concentrate addition.

Figure 4 Appearance and crumb structure of breads: 1 – control sample; 2 – whey protein concentrate, 7%

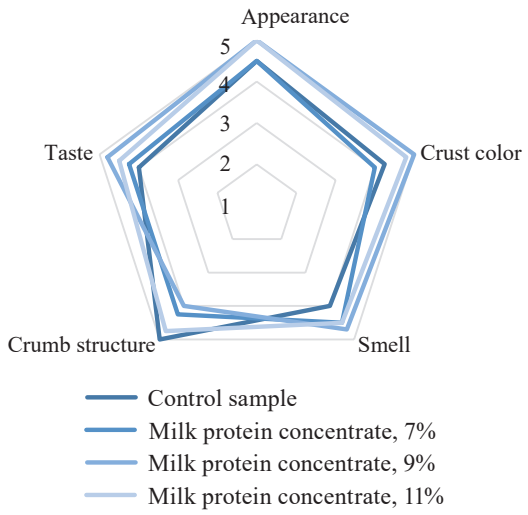

Figure 5 Profilogram of sensory evaluation of breads with milk protein concentrates

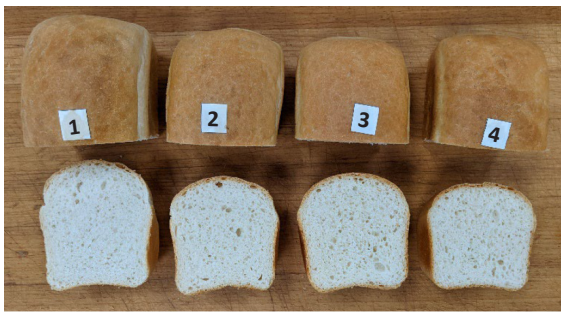

Figure 7 Crumb structure of bread samples: 1 – control sample; 2 – milk protein concentrate, 7%; 3 – milk protein concentrate, 9%: 4 – milk protein concentrate, 11%

The highlighted samples had more attractive flavor characteristics relative to the control one. Some consumers described the smell and the taste as creamy and milky, while the control sample was assumed as too insipid. This advantage is due to the ability of proteins to bind flavor compounds. The sample 3 was distinguished by a uniformly colored golden crust (Fig. 6).

With the increase of the added concentrate dosage, the texture of the products became harsher and less uniform (Fig. 7), that had no negative effect on the overall sensory evaluation of the products.

Impact of protein concentrates on the nutritional value of breads. The nutritional values of the optimal sample, kneaded with the 9% of milk protein concentrate, and the control one are presented in Table 11. It was calculated without considering the amount of protein destroyed during the baking.

According to the data presented in Table 11, the predicted protein content in the chosen sample is equal to 11.6 g/100 g that is 35% higher than in the control sample. Therefore, the food ingredient in the form of milk protein concentrate has the potential to be used to create a product with functional properties.

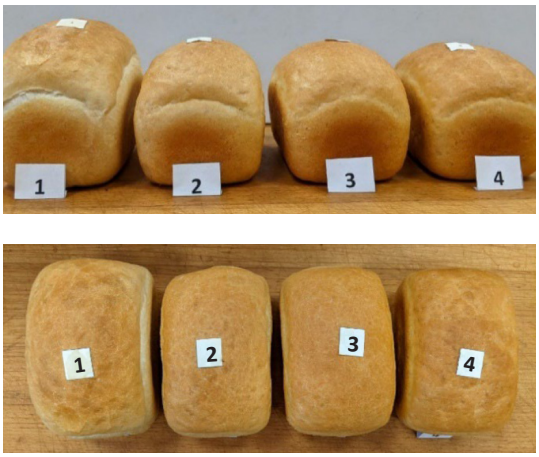

Figure 6 Appearance of breads: 1 – control sample; 2 – milk protein concentrate, 7%; 3 – milk protein concentrate, 9%; 4 – milk protein concentrate, 11%

Table 11 The nutritional value of the breads under study

Sample	Content per 100 g of finished product			
	Protein, g	Fat, g	Carbohydrates, g	Caloric value, kcal
Control	7.5	0.82	43.4	240.0
Milk protein concentrate, 9%	11.6	0.73	38.1	230.6

CONCLUSION

Whey protein concentrate is not recommended to be used in the bread baking due to its negative impact on the rheological and sensory quality indicators of the dough and baked product. Low water absorption capacity has led to the lower volume yield of the bread which is technologically and economically unprofitable.

As a result of the analysis carried, the sample baked with 9% of milk protein concentrate added was established as the best. Thus, milk protein concentrate can be considered as a promising raw material for bread production. This product addition resulted in bread with attractive consumers' characteristics and had a positive effect on the volume yield of breads. The predicted nutritional value allows concluding that milk protein concentrate has the potential to increase the biological value of the product. The amino acid composition of finished products is recommended to be analyzed in order to substantiate the possibility of using milk protein as a functional food product.

CONTRIBUTION

All the authors have an equal contribution to the article and are equally responsible for any potential plagiarism.

CONFLICT OF INTEREST

The authors declared no conflict of interests regarding the publication of this article.

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Microbiological safety criteria for products from unconventional raw materials: raw bear fat

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Abstract:
Fat of game animals is a prospective raw material for bioactive additives. Before such a product enters the market, food science has to make sure it is safe for consumption.

This research featured subcutaneous adipose tissue of brown bears tested with standard methods for microbiological safety indicators. The microbial properties were studied on liquid and solid nutrient media. Staphylococcus was profiled using a VITEK 2 Compact biochemical automatic analyzer and Gram-positive cards (Bio-Mérieux, France). The analysis followed the Technical Regulations of the Customs Union TR TS 021/2011 On Food Safety (December 09, 2011).

The microbial count for mesophilic aerobic and facultative anaerobic microorganisms was 1.5×10^3 CFU/g. The fat samples revealed no molds, yeasts, or *Escherichia coli* bacteria. Liquid and solid nutrient media made it possible to describe the qualitative profile and cultural properties of the bear fat microflora against pork fat, which served as control. The automatic system identified Gram-positive, coagulase-negative, and oxidase-positive *Staphylococcus lentus* and *Staphylococcus sciuri*. In line with the modern classification, they belong to the new genus of *Mammaliicoccus*, namely *Mammaliicoccus sciuri*.

Subcutaneous adipose tissue of brown bears needs to undergo a microbiological safety test before consumption. Bear fat requires additional research in order to become a safe raw material for food products and bioactive additives.

Keywords: Brown bear fat, subcutaneous adipose tissue, microbiological indicators, *Mammaliicoccus lentus*, *Mammaliicoccus sciuri*

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INTRODUCTION

The Strategy for Improving the Quality of Food Products in the Russian Federation through 2030 introduces several ways to develop an independent national food quality management system. This document stresses that the technical regulations for certain food products need a system of food quality indicators. New quality indicators will make it possible to test new foods with unconventional plant and animal raw materials, as well as to develop new biologically active additives and functional foods [1–5].

Adipose tissue of game animals is a potential source of bioactive additives [6]. In traditional medicine, bear (*Ursus arctos* L.) fat is an excellent anti-burn ointment. It also treats various diseases of the upper respiratory tract and heals skin damage of various severity. Bear fat is applied to relieve back and joints pain symptoms. The Mongols consume it raw; the Yakuts use twice-melted bear fat to treat tuberculosis [7]. Bear fat also is a popular folk remedy against gastrointestinal diseases and atherosclerosis [8, 9]. Customers buy it mostly online, and no technical regulations have been developed for it so far. As a result, the market is full of bear fat products of unknown safety status.

Raw fat is most often obtained from hibernating game animals, e.g., bears, badgers, marmots, etc. It is

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divided into subcutaneous and visceral. Most animal fat deposits are subcutaneous and are located right under the skin. Visceral adipose tissue is scarce and envelops the internal organs of well-fed adult specimen.

Meat and fat of farm and game animals can transmit zoonotic infections to people. Table 1 classifies them according to the carrier.

Game animals, being asynanthropic, are potential carriers of zoonotic diseases that can be transmitted to humans via infected meat and offal or via infected domestic and synanthropic animals. Table 2 describes the most common zoonoses of asynanthropic game animals.

Science knows more than a thousand pathogens of infectious diseases, 60% of which are of a zoonotic nature, i.e., people get them from animals. About 70% of such cases are connected with wild animals [10].

People usually get infected by inhaling contaminated aerosol secretions of rodents, e.g., feces, saliva, and urine. Meat and offal obtained from infected animals are also contagious. To be used as food, game meat and offal are to undergo a veterinary and sanitary examination, according to the Law on Veterinary Medicine of the Russian Federation (N 4979-1, May 14, 1993).

Meat and meat products contain saprophytic, sanitary-indicative, opportunistic, and pathogenic microorganisms. Microbial contamination occurs in endogenous and exogenous ways. Endogenous contamination takes place when the animal is still alive. Posthumous endogenous infection is usually associated with damaged intestines or ungutted carcasses. Exogenous contamination happens when microorganisms penetrate from the environment during butchering, storage, transportation, and processing. Retrocession from the gastrointestinal tract, i.e., endogenous infection of deep tissues in a living animal, occurs as a result of starvation, physical strain, diseases, and injuries. Microbial contamination *in vivo* is connected not only with the digestive system, but also with those systems that contact with the external environment, i.e., genitourinary, respiratory, and integumentary. Microbiotic studies of game animals are important precisely because the microflora of slaughter products depends on the composition of microorganisms the animal had when it was alive.

Bear microbiota is a popular subject of foreign studies. Bear intestinal microbiomes differ from those of other omnivores because bears have no caecum. The caecum restricts the rate at which nutrients pass through the intestinal tract. Apparently, it serves as a reservoir for microbial populations that replenish the microbiome diversity depending on the diet and health. Therefore, bears intestinal microbiomes are vulnerable to systemic changes in diet, health, or other factors. Gillman *et al.* believe that fecal samples provide insight into the intestinal microbiota of black bears, as well as other carnivores and omnivores with simple intestinal morphology [11].

Table 1 Zoonotic infections

Animals	Carrier	Infection
Synanthropic	Rats, pigeons, etc.	Anthrax, brucellosis, leptospirosis, ornithosis, etc.
Domesticated	Cattle, pigs, chicken, etc.	
Asynanthropic	Hares, bears, badgers, etc.	

Table 2 Zoonotic diseases transmitted by asynanthropic game animals

Asynanthropic animals	Zoonotic disease	Pathogen
Hares (*Lepus europaeus* L., *Lepus timidus* L.)	Trichinosis	*Trichinella spiralis*
Bears (*Ursus arctos* L.)	Rabies	*Rabies lyssavirus*
Beavers (*Castor fiber* L.)	Paratyphoid	*Salmonella*
Badgers (*Meles meles* L.)	Listeriosis	*Listeria monocytogenes*
Marmots (*Marmota* L.)	Tularemia	*Francisella tularensis*

Glad *et al.* studied the intestinal microbiome of polar bears and profiled microorganisms that belonged to the phylum of *Firmicutes* [12]. They identified 160 sequences as *Clostridiales* and found a new, unclassified sequence of *Firmicutes*. Most of the sequences (70%) belonged to *Clostridium*. The aerobic heterotrophic cell count on chocolate agar ranged from 5.0×10^4 to 1.0×10^6 CFU/mL for rectal swabs and from 4.0×10^3 to 1.0×10^5 CFU/g for feces samples.

Franz *et al.* studied intestinal microbiomes from two polar bear populations and identified eight most common classes of bacteria: *Clostridia*, *Gammaproteobacteria*, *Actinobacteria*, *Coriobacteriia*, *Nogativicutes*, *Bacilli*, *Bacteroidia*, *Fusobacteria*, *Campylobacteria*, and *Saccharimonadia* [13]. The microbiomes were different and reflected the habitat, diet, sex, and age of the animals. The authors decided that *Bacilli* were especially important for restoring intestinal health and maintaining intestinal homeostasis.

Schwab *et al.* studied fecal microbiota from ten grizzly bears [14]. The samples that belonged to wild grizzly bears contained more eubacteria than those obtained from captive bears. *Enterococci* and *Enterobacteria* were numerous in all samples. Pathogenic *Clostridum perfringens* group I had a positive correlation with protein intake and a negative correlation with dietary fiber content. Although considered healthy, the wild bears that lived on a normal protein-based diet were more likely to carry *C. perfringens* than those wild bears that relied mostly on plant-based food. Three samples even contained *Clostridium sordellii*, which can cause toxic shock syndrome in humans. Thus, the count of pathogenic *Escherichia coli* depended neither on the diet nor on the habitat.

Watson *et al.* and Trujillo *et al.* reported that subpopulations of brown bears living in different national parks shared five types of bacteria: *Firmicutes*, *Proteobacteria*, *Epsilonbacteraeota*, *Bacteroidetes*, and *Actinobacteria* [15, 16]. The authors identified 16 major genera.

Therefore, bear intestinal microbiomes depend on the habitat, climate, age, sex, food diversity, and hunting strategy. Raw materials from game animals are to be checked for pathogenic and opportunistic pathogenic microflora before being processed into food products or biologically active additives.

Raw bear fat has a high lipid content, but it may also contain lipophilic microorganisms capable of synthesizing enzymes that hydrolyze lipids. Many bacteria, fungi, yeasts, and actinomycetes produce lipases that hydrolyze lipids at the water-fat phase boundary. Lipase-producing bacteria usually belong to *Acinetobacter*, *Bacillus*, *Burkholderia*, *Pseudomonas*, *Staphylococcus*, *Microbacterium*, *Lactobacillus*, *Stenotrophomonas*, *Arthrobacter*, *Serratia*, *Aeromonas*, *Thermosyntropha*, *Achromobater*, *Chromobacterium*, *Burkholderia*, *Streptomyces*, etc. [17–20].

Cooling, freezing, and thermal processing protect adipose tissue from bacterial contamination. Freezing and subsequent defrosting change the microbial quantitative and qualitative composition. Refrigerated storage gradually kills mesophilic microorganisms; however, some psychrophilic microorganisms remain viable for a long time.

Psychrotrophs proliferate on livestock products. Food-spoiling psychrotrophs are known to affect commercial foodstuffs. Zhang *et al.* identified microorganisms of 38 genera and 20 families, including Gram-negative bacteria [21]. Saprophytic *Pseudomonas* and especially *Pseudomonas fragi* had the highest count, followed by *Psychrobacter*, *Brochothrix*, *Serratia*, and *Stenotrophomonas*. Li *et al.* also reported other pathogenic and toxic microorganisms, such as *Salmonella*, *Staphylococcus aureus*, and *C. perfringens* [17]. Moschonas *et al.* detected psychrophilic and psychrotrophic anaerobic microflora in commercial Irish beef abattoir environments and vacuum-packed beef [22]. They tested 431 isolates and profiled 25 microbial species, with the most frequently recovered species being *Clostridium gasigenes*, followed by *Clostridium estertheticum* and *Clostridium algidixylanolyticum*. These species often cause spoilage in chilled lamb and vacuum-packed beef, which poses a significant commercial threat to the meat industry.

Pathogenic bacteria survive various methods of freezing and defrosting. Choi *et al.* studied the effect of freezing and defrosting on the microbiological quality and changes in the microstructure of chicken breasts inoculated with *Listeria monocytogenes* and *Campylobacter jejuni* [23]. They detected no differences in the count of *L. monocytogenes* under different freezing conditions. However, air freezing (–20°C) reduced the total aerobic bacterial count and *C. jejuni* in particular, compared to other freezing methods. Defrosting by hot/cold air flow, water immersion, and high pressure at 4 and 25°C caused no significant differences in the count of *L. monocytogenes*.

Metzger *et al.* reported that foodborne pathogens survived freezing in cheese [24]. They produced three samples of semi-soft cheese with milk inoculated with two pathogen mixes of *L. monocytogenes/S. aureus* and *E. coli/Salmonella typhimurium*. Storage at –20°C for 2, 7, or 30 days resulted in little to no reduction in *L. monocytogenes*. However, 90 days at –20°C reduced the count of *L. monocytogenes* significantly, while the count of *S. aureus* remained constant over the 90-day storage in the freezer. *E. coli* and *S. typhimurium* rapidly decreased at –20°C. The defrosting conditions were 4°C for 14 h and 20°C for 4 h, but these factors had no effect on the viability of microorganisms.

Apparently, game meat and offal have to undergo a number of microbiological tests before consumption.

The research objective was to determine the microbiological safety profile and assess the qualitative composition of the microflora of bear adipose tissue.

The list of tasks included:

– reviewing scientific publications;
– developing a scheme for microbiological safety studies;
– analyzing microbiological safety criteria;
– isolating pure microbial cultures;
– profiling the isolated microflora; and
– comparing the obtained results with available publications.

The identified microbiological safety indicators can be used in technical regulations on quality and safety requirements for foods and dietary supplements based on bear fat.

STUDY OBJECTS AND METHODS

The research featured subcutaneous fat of *Ursus arctos* L. The samples belonged to a bear shot by a licensed hunter in the Kemerovo Region in 2021. The adipose tissue was separated from the carcass within 2 h after the slaughter. The butchering took place at –15 ± 5°C. For veterinary and sanitary examination, the samples were frozen at –18 ± 2°C and delivered to the laboratory within three days.

The Technical Regulations of the Customs Union TR TS 021/2011 On Food Safety contain no requirements for the microbiological safety of wild animal raw fat. Thus, we used the requirements for pork fat as control. The pork fat was purchased from the market and frozen under similar conditions.

After 12 h of freezing, the samples were defrosted at 20 ± 2°C in open air for 4 h, crushed in a cutter to a particle size of 3–5 mm, and packaged.

The sampling followed State Standard 31904-2012. We diluted 10 g of each sample in 90 cm^3 of saline in the ratios of 1:10, $1:10^2$, $1:10^3$, and $1:10^4$ by volume. Then, we dropped 1 cm^3 of the substance with sterile pipettes in sterile Petri dishes and into test tubes with the Kessler medium. The procedure followed the microbiological control scheme illustrated in Fig. 1.

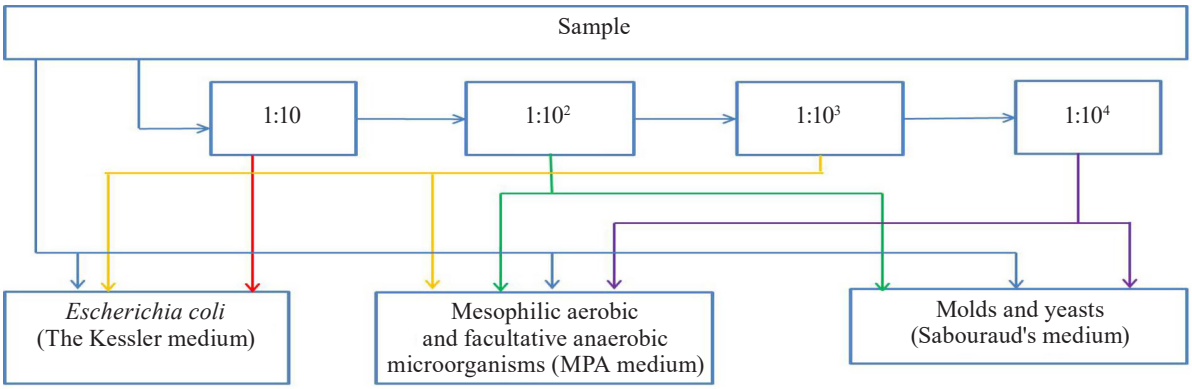


Figure 1 Microbiological test scheme

Table 3 Microbiological count of pork and bear raw fat

Indicator	Pork fat	Bear fat	TR CU 021/2011
Mesophilic aerobic and facultative anaerobic microorganisms, CFU/g	4.5×10^4	1.5×10^3	$\leq 5.0\times10^4$
Escherichia coli (coliforms), per 0.001 g	n.d.	n.d.	Unavailable for 0.001 g
Staphylococcus aureus, per 0,1 g	n.d.	n.d.	n.d.
Mold, CFU/g	n.d.	n.d.	n.d.
Yeasts, CFU/g	n.d.	n.d.	n.d.

n.d. – not detected

We profiled mesophilic aerobic and facultative anaerobic microorganisms in line with State Standard 10444.15-94. To study the QMAFAnM indicator, we used sterile meat peptone agar. After the nutrient medium solidified, Petri dishes were placed in a thermostat for cultivation at 37°C. After 48 h of cultivation, we counted the colonies and tested the indicators for the compliance with the requirements.

The procedure for *Escherichia coli* followed State Standard 31747-2012. We put the diluted product into a test tube with the sterile Kessler medium and a float and stored it in a thermostat at 37°C for 24 h. A bubble in the float indicated the presence of *E. coli*. The samples were tested for compliance with the Technical Regulations.

The mold and yeast tests corresponded with State Standard 10444.12-2013. We poured Sabouraud's sterile nutrient medium into Petri dishes. After it solidified, we put the Petri dishes in a thermostat for cultivation at 25°C and counted the colonies after 72 h of cultivation.

The *Staphylococcus aureus* test was in line with State Standard 31746-2012.

The cultural property test involved liquid and solid nutrient media. *Staphylococcus* bacteria were profiled using a VITEK 2 Compact biochemical analyzer (Bio-Mérieux, France) and a VITEK 2 Gram-positive identification card. This automated system provides 24-h microbial profiling and antimicrobial susceptibility testing. The software compares the test responses with the standard responses for each organism or group of organisms. A score and percentage probability indicate how the observed responses match the typical responses for each organism with a 99% probability.

RESULTS AND DISCUSSION

The Technical Regulations of the Customs Union provide no safety indicators for raw bear fat, so we used the requirements for pork fat as indicative indicators. Although they do not standardize the content of mold fungi and yeast in pork fat, we studied these indicators to assess the qualitative composition of the microflora, as well as the chance that these microorganisms might contaminate fat.

The pork fat and the bear fat complied with the TR CU 021/2011 in terms of microbiological safety criteria for pork fat. Table 3 shows the indicators of defrosted fat samples.

Pork fat and raw bear fat have a favorable chemical composition for microbial growth. The lipid part is 92% for pork fat and 72% for bear fat; they also contain 2–17% of proteins and 4–5% of moisture, respectively. Microorganisms can use these components as nutrients.

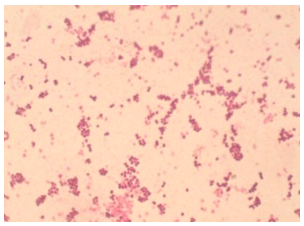
The total bacterial contamination was consistent with the data published by Maduka *et al.*, who linked the higher bacterial count in pork fat with its physical properties, i.e., mucous nature and high fat content [25].

To determine QMAFAnM for the samples grown on the media, we established the taxonomic affiliation of microorganisms based on cultural, morphological, and tinctorial properties. The microflora of raw bear fat was represented by *Bacilli*. The microorganisms could be natural microflora or contamination during butchering, transportation, and storage. Other studies on the intestinal microbiota of brown, black, and polar bears also reported *Bacilli* [11, 13, 15]. Their representatives are known to produce lipase [19].

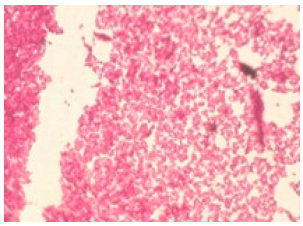
Colony 1

Colony 2

Figure 2 Colonies in bear fat samples



Colony 1



Colony 2

Figure 3 Microscopy of Gram-stained preparations in bear fat samples

Table 4 Identification results

Species	Probability, %,	Atypical results	
Staphylococcus lentus	96	Tyrosinarylamidase TyrA	+
		Alpha-Glucosidase AGLU	+
Staphylococcus sciuri	97	Alpha-Mannosidase AMAN	+

The research revealed some cocci that could be opportunistic pathogens for humans. Figures 2 and 3 show the colonies and the microscopy of bacterial preparations isolated from bear raw fat.

Colony 1 had a round shape with a diameter of 7 mm; it was white, flat, smooth, shiny, and opaque. Homogeneous in structure, it had a thick consistency, with serrated edges.

Colony 2 was round and slightly larger in diameter (11 mm). It was yellow, wavy, shiny, and opaque, with a thick and homogeneous consistency.

The microscopic image of the Gram-stained preparations made it possible to assess the shape and location of the microorganisms as cocci clustered like grape bunches. The cultural and morphological properties suggested that the microorganisms belonged to the genus of *Staphylococcus*.

Table 4 shows the identification of *Staphylococcus* isolated from the raw bear fat samples using the VITEK 2 Compact Bio-Mérieux automated system.

The isolated microorganisms were identified as Gram-positive, coagulase-negative, and oxidase-positive staphylococci consisting of clustered cocci.

Chervyakova *et al.* proposed to include the following parameters into the list of authenticity markers: utilization of *β*-galactosidase and *α*-glucosidase, resistance to polymyxin B and novobiocin, ability to alkalinize lactate and N-acetyl-d-glucosamine [26]. These indicators could provide more accurate intraspecific profiling of *Staphylococcus* bacteria. Tables 5 and 6 give a detailed biochemical information on how the isolated microorganisms utilize particular components.

The experimental data suggested that *Staphylococcus lentus* and *Staphylococcus sciuri* belonged to the new genus of *Mammaliicoccus*, of which *Mammaliicoccus sciuri* is the type species. *Staphylococcus*

Table 5 Biochemistry of *Staphylococcus lentus*

2	AMY	+	4	PIPLC	–	5	dXYL	–	8	ADH1	+	9	BGAL	+	11	AGLU	+
13	APPA	–	14	CDEX	–	15	AspA	–	16	BGAR	–	17	AMAN	–	19	PHOS	+
20	LeuA	–	23	ProA	–	24	BGURr	–	25	AGAL	–	26	PyrA	+	27	BGUR	–
28	AlaA	–	29	TyrA	+	30	dSOR	+	31	URE	–	32	POLYB	–	37	dGAL	–
38	dRIB	+	39	iLATk	+	42	LAC	–	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	–	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	–
57	dRAF	–	58	O129R	–	59	SAL	+	60	SAC	+	62	dTRE	+	63	ADH2s	–
64	OPTO	+															

Table 6 Biochemistry of *Staphylococcus sciuri*

2	AMY	–	4	PIPLC	–	5	dXYL	–	8	ADH1	–	9	BGAL	–	11	AGLU	–
13	APPA	–	14	CDEX	–	15	AspA	–	16	BGAR	–	17	AMAN	+	19	PHOS	+
20	LeuA	–	23	ProA	–	24	BGURr	–	25	AGAL	–	26	PyrA	–	27	BGUR	–
28	AlaA	–	29	TyrA	–	30	dSOR	–	31	URE	–	32	POLYB	–	37	dGAL	–
38	dRIB	–	39	iLATk	–	42	LAC	–	44	NAG	–	45	dMAL	–	46	BACI	+
47	NOVO	–	50	NC6.5	–	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	–
57	dRAF	–	58	O129R	–	59	SAL	+	60	SAC	+	62	dTRE	+	63	ADH2s	–
64	OPTO	+															

fleurettii was also assigned to the new genus of *Staphylococcus stepanovicii* and *Staphylococcus vitilinus* [27].

M. sciuri and *Mammaliicoccus lentus* live on the skin and mucous membranes of many domestic, farm, and wild animals, as well as in foods of animal origin [28–33]. They occur in soil, sand, water, and marsh grass [34]. Adkins *et al.* found *M. sciuri* in milk and bedding on free-stall dairy farms [33]. *M. sciuri* were isolated from sick goats, cows with mastitis, dogs with dermatitis, cats with sepsis, and minks with urinary infections [35–37]. *M. sciuri* were also isolated from healthy and diseased humans [29].

CONCLUSION

The microbiological safety of raw bear fat complied with the requirements for pork fat listed in Technical Regulation of the Customs Union TR CU 021/2011 On Food Safety. The VITEK 2 Compact Bio-Mérieux automated system detected *Mammaliicoccus lentus* and *Mammaliicoccus sciuri* in the samples grown on the QMAFAnM test medium. These microorganisms were reported as pathogens in animals.

The microbial profiling of bear adipose tissues indicated that foods and biologically active substances based on raw bear fat require safety tests, and their quality indicators have to be introduced into the existing regulatory documents.

CONTRIBUTION

E.A. Vechtomova and I.V. Dolgolyuk supervised the research, analyzed the data, interpreted the results, and wrote the article. M.M. Orlova and A.V. Zaushintsena reviewed scientific publications and performed the research.

CONFLICT OF INTEREST

The authors declared no conflict of interests regarding the publication of this article.

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Quality index method to evaluate the quality of Jinga shrimp (*Metapenaeus affinis* L.) preserved in ice water

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Abstract:
The Quality Index Method (QIM) provides a quick and accurate quality assessment. It makes it possible to calculate preserving time and establish the freshness of sea food. This article introduces a quality assessment program based on the QIM scheme and quality index (QI) for the Jinga shrimp.
The research included Jinga shrimps (*Metapenaeus affinis* L.), a commercially valuable aquatic species widely distributed throughout the Vietnamese coast. The input data included the changes in appearance and sensory profile of sampled shrimps during 20 days of storage at 0–4°C in ice water.
They were used to construct a QIM scale, which was applied to other shrimp samples at different storage time to evaluate the relationship between the QI score and the storage time. After that, the QIM scale was tested on ten random shrimp samples to verify its shelf-life predictive power. This study managed to establish a correlation equation between the QI scores and the storage time with the coefficient of R_2= 0.97. This correlation proved highly reliable as verified by comparing the predicted and actual best shelf life of Jinga shrimps stored in ice water for 8–10 days.
The QIM program provides a practical and effective science-based tool that delivers fast and reliable results for customers, fishermen, food traders, and aquacultural enterprises.

Keywords: Jinga shrimp, sensory evaluation, quality index method, storage time, ice storage, shelf life

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INTRODUCTION

Consumers assess food products according to their sensory properties [1, 2]. Sensory evaluation is necessary to commercialize a food product. It is especially important for seafood, since freshness is the most important aspect of fisheries and aquaculture products [3, 4]. Wholesalers often use ice water to store shrimps in fishing vessels or processing facilities. However, the quality of fresh aquatic raw materials, especially raw shrimps, degrades very quickly during storage. Therefore, all stakeholders need a quick and efficient method to assess the freshness of aquatic products.

Sensory assessment is a scientific method used to evoke, measure, analyze, and interpret sensations that are perceived through sight, smell, taste, touch, and hearing [5]. For marine seafood, sensory assessment is fast, cheap, and effective. The list of contemporary aquatic sensory assessment methods includes the EU-scheme, the Quality Index Method, and the Torry Sensory Analysis [6]. The Quality Index Method (QIM) is an especially fast and reliable means of measuring

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the freshness of seafood [7, 8]. QIM tests the significant levels of such attributes as skin, slime, eyes, abdomen, smell, etc. of aquatic products. These attributes are indicated in the table of assessment guidelines with scores from 0 to 3. Quality Index (QI) is the total score. If the QI value is close to zero, the product is considered fresh, whereas a higher QI value indicates that the product has degraded. Each aquatic species has its own QIM [9, 10]. QIM has an obvious advantage over the conventional classification method commonly used for seafood raw materials: the QI is the sum of all attribute variations. As a result, food scientists can establish a linear relationship between the QI score and the ice-preserving time, thereby estimating the storage period [11, 12].

Shrimps go through a number of physical and chemical transformations from catch to death. These processes cause sensory changes in appearance, texture, and color during the storage period. Discoloration is a common phenomenon in aquatic products. In shrimps, suboptimal preservation might cause immediate black spots called melanosis. This phenomenon was first reported in 1951 [13]. Originally, scientist attributed melanosis to the activity of microorganisms although today it is concluded to be the oxidation of polyphenoloxidase enzymes in shrimp and crabs [14, 15]. In shrimps, the color depends on the close connection between pigments and proteins. This association makes shrimps shiny blue while alive. When boiled, shrimps turn reddish pink. When shrimps are alive, astaxanthin pigment exists in the form of a protein bond that creates the characteristic color. When shrimps die, this bond weakens and disappears under the impact of temperature and light, separating from the protein and creating a new red color [16].

Many contemporary studies apply QIM to evaluate the transformation of sensory quality in Northern shrimps (*Pandalus borealis* L.), white-leg shrimps (*Lipopenaeus vannamei* L.), and giant tiger prawns (*Penaeus monodon* L.) [17–22].

Jinga shrimps (*Metapenaeus affinis* L.) are harvested by shrimp trawls. In Vietnam, Jinga shrimps are widely distributed throughout the North-South coast [23]. Jinga shrimps have a water content of 77–79% and lipids of 1–2%, which is higher than in other shrimp species. These two characteristics affect the spoiling process, thus, requiring appropriate post-harvest preservation methods [24]. Considering the importance of maintaining the quality, we developed a program using the quality index method to evaluate the sensory quality transformation of the Jinga shrimp (*M. affinis*) during ice-water storage.

STUDY OBJECTS AND METHODS

Study objects. This study introduces a new program that applies the Quality Index Method (QIM) to the sensory assessment of Jinga shrimps (*Metapenaeus affinis* L.) during ice water storage.

Research materials. The research involved fresh Jinga shrimps, 30–35 shrimps/kg, harvested near Do Son at Hai Phong Port, the biggest port city in Northern Vietnam, in October 2019 and November 2020.

The shrimps were stored in insulated ice crates with a shrimp/ice ratio of 1:2 (w/w) and transported to the Marine Science Laboratory of the Seafood Research Institute (Hai Phong, Vietnam). There, the shrimps were put into plastic boxes, which were filled with ice up to the ratio of 1:2 (w/w) and water up to the ice surface. The boxes were kept in a refrigerator at 0–4°C. Ice supplements and water withdrawal were made every two days. Samples for analysis and sensory evaluation were taken daily, from fresh (day 0) until spoiled (day 20).

Methodology. ***Methodology for developing the QIM program for Jinga shrimps.***

Developing terms to describe changes in sensory profile of shrimps stored in ice water. Panelists were selected and trained according to TCVN 12388-2:2018 Sensory analysis – General guidance for the staff of a sensory evaluation laboratory – Part 2: Recruitment and training of panel leaders. The terms to describe the changes in texture, smell, and color were collected from direct observation of the study samples and from previous studies (TCVN 11182:2015 Sensory analysis – Vocabulary, TCVN 12614:2019 Frozen – Black tiger, Vannamei shrimp) [20–22, 25]. The trained panelists selected the terms by extensive discussion. According to the selection criteria, the terms were to be concise, clear, familiar, and easy to understand.

Formulating and developing the QIM scheme for Jinga shrimps. The QIM program included the following steps:

Step 1. Preliminary program: three to five experts of the Seafood Research Institute were tasked to observe and record all changes in quality attributes (color and structure of head, legs, and body; meat texture; smell) and set the terms in the preliminary program. Each attribute was scored from 0 to 3, with the lowest score for the best quality.

Step 2. QIM program and panel training: the shrimps were stored at 0–4°C and evaluated daily for 20 days. The shrimps were stored at different intervals in ice, as described in Section 2.1. Six experts participated in the training sessions for the panelists. During the training, the panelists received the information on the storage time to associate the attribute changes with the time. Next, they performed the assessment without information about the storage time to guarantee reliable and accurate evaluation results.

Step 3. Practicing the QIM program: the panelists collected ten samples of Jinga shrimps (samples T_{01}–T_{10}) stored in ice water and evaluated them according to the QIM program, as described in Step 2. The correlation equation between the storage time and the quality score was used to determine the stored time and the remaining shelf life of the shrimps to be compared with the actual storage shelf life.

Quality index (QI) scoring method. The method of calculating QI followed the procedure described in [26]. The quality index was calculated according to the following Eq. (1):

$$\mathrm{QI} = \frac{\sum_{i=1}^{n}\left(QI_1 + \ldots + QI_n\right)}{n} \sum_{k=1}^{m}\left(t_1 + \ldots + t_m\right) \quad (1)$$

where QI was the sensory quality index (average total sensory quality score); QI_1,..., QI_n stood for the total sensory quality score of each member of the evaluation board; n denoted the number of panelists; i was the number of the panelist; m was the number of shrimp attributes recorded in the QIM table (m depends on the species); t denoted the number of sensory scores of the panelist for each shrimp attribute; and k_1... k_m were shrimp attributes.

Data analysis methods. The data collected were analyzed using descriptive statistics (mean, standard deviation). The difference in the factors between the modal tests was analyzed by ANOVA 1 factor ($p < 0.05$) using the Statgraphic XV software and the Excel software. Each experiment was conducted three times, resulting in an average of total experiments.

RESULTS AND DISCUSSION

Results of recording the transformation and selection of terminology according to the storage time. The goal of the research was to document the changes in the sensory properties of Jinga shrimps stored in ice, including color, texture, smell, etc. Preference belonged to the terms with the highest frequency of occurrence in a given period.

Term selection results for smell. The shrimp smell changed at different stages.

Stage 1 (days 0–4). The shrimps still retained the characteristic fresh smell described as "the smell of the sea", with a high frequency of occurrence > 80% (Fig. 1). After this period, the shrimp smell began to change.

Stage 2 (days 4–8). Most samples were reported to have a slightly fishy smell of seaweed.

Stage 3 (days 8–14). The shrimps smelled fishy or of weak ammonia. The unmistakable fishy smell was more frequent, but both descriptions appeared for a long time and in the same period. Both descriptions were found fit to describe the shrimp smell at this stage.

Stage 4 (days 14–20). The clear ammonia and putrid odor clearly indicated that the shrimps went bad.

The terms selected to describe the smell of shrimps ranked as follows: characteristic fresh shrimp smell → smell of seaweed/mild fishy → clear fishy/mild ammonia → clear ammonia/putrid.

Term selection results for color. *Shrimp head color.* The shrimp head color change occurred in four stages (Fig. 2):

Stage 1 (days 0–2). The color was almost unanimously described as a clear and shiny yellow. The description of a bright, pink color (< 5%) was not selected due to the low frequency, and it was registered only on day 0.

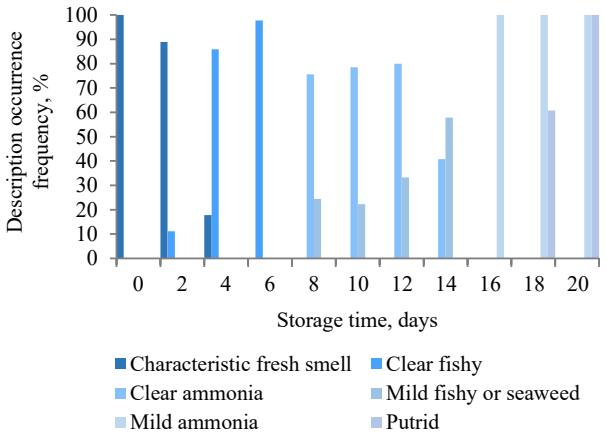

Figure 1 Frequency of shrimp smell descriptions by storage time

Stage 2 (days 2–8). The two most frequent descriptions included a less clear color (light yellow) and reduced gloss (> 80%).

Stage 3 (days 8–14). The shrimp heads were described as pale pink-orange. This description had a high frequency and appeared on many days. Black grey also was reported during this period; however, its frequency was low.

Stage 4 (days 14–20). The shrimp heads were orange-pink, black, grey, orange, and orange-red. The descriptions were persistent and appeared during the spoilage stage.

The terms selected to describe the color of shrimp heads ranked as follows: clear color (yellow)/shiny → less clear color (light yellow)/reduced gloss → pale orange pink → clear orange-pink, orange, orange-red, black grey.

Shrimp body color. Figures 3 demonstrates the color variation of the shrimp body:

Stage 1 (days 0–2). The color remained clear and shiny. The green dots on the body were distinct.

Stage 2 (days 2–10). The shrimp body color became less clear; the gloss decreased, and the green dots on the body grew slightly faded.

Stage 3 (day 10–16). The shrimp body turned orange; the dots became faint.

Stage 4 (days > 16). The body lost its color and turned dark orange; the green dots became blurred, and black spots started to appear.

Multiple descriptions were selected for each stage to provide a detailed description of shrimp body. The terms depicting the color variation of shrimp body were selected as follows: shiny clear color, green dots on the body are distinct → color is less apparent; gloss is reduced (slightly shine); green dots are slightly blurred → appear orange-pink/orange starts to appear; green dots are slightly blurred, with tiny black dots → clear orange-pink/dark orange; the green dots are blurred; many large, clear black spots start to appear.

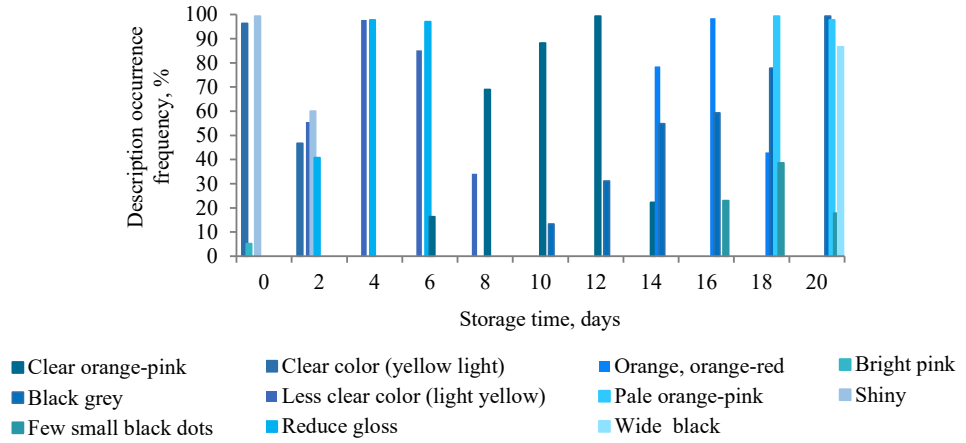

Figure 2 Frequency of shrimp head color descriptions by storage time

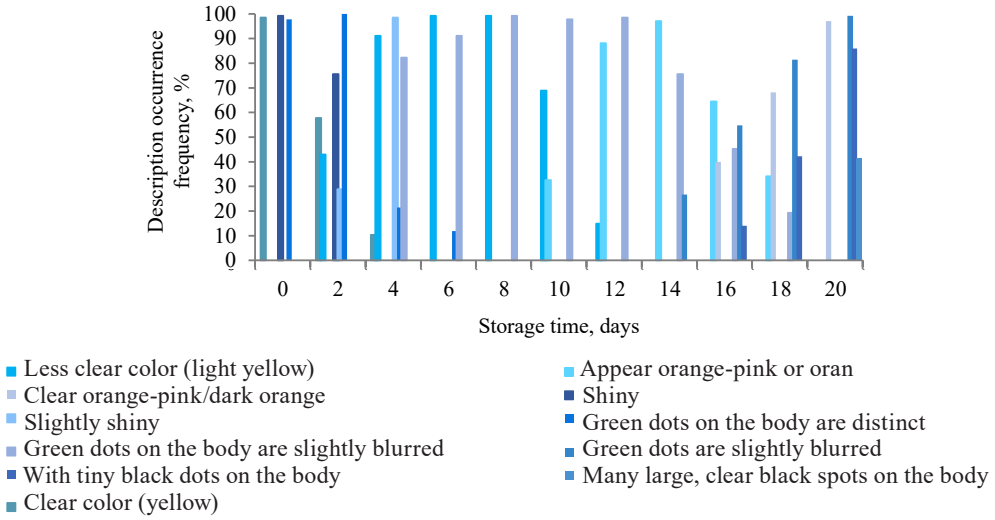

Figure 3 Frequency of shrimp body color descriptions by storage time

Shrimp leg color. Figure 4 shows the color terms that depict the changes in shrimp legs:

Stage 1 (days 0–2). The legs were bright yellow.

Stage 2 (days 2–8). The yellow color grew lighter (> 60%).

Stage 3 (days 8–14). The shrimp legs showed signs of blackening.

Stage 4 (days 14–20). They turned black.

The terms to describe the change in the color of the shrimp legs were selected as follows: bright orange-yellow → pale orange-yellow → signs of turning black black.

Shrimp meat color. Figure 5 shows the terms that be the color of shrimp meat in four stages:

Stage 1 (days 0–4). The shrimp meat was delicate white (> 60%).

Stage 2 (days 4–10). The meat lost its translucency and turned white.

Stage 3 (days 10–16). The meat was opaque white.

Stage 4 (days 16–20). The shrimp meat was pale yellow or pale pink, and small black spots started to appear.

The terms to describe the changes in the color of shrimp meat ranked as follows: translucent white/ transparent → loss of clarity, turning white → opaque white → pale pink, pale yellow, with some small black spots.

Term selection results for structure/state. *Shrimp head structure*. Figure 6 shows the way shrimp head structure changed with storage time:

Stage 1 (days 0–4). The shrimp heads were intact and firmly attached to the bodies; all the samples got this description with a very high frequency.

Stage 2 (days 4–6). The heads began to show signs of loosening from the body (> 70%).

Stage 3 (days 6–16). The shrimp heads began to loosen, with signs of tomalley dilution.

Stage 4 (days 16–20). The shrimp heads demonstrated crushed tomalley and fell off.

The terms to describe the structure of shrimps were selected as follows:intact; head fastened to body intact, signs of loosening → tomalley dilution → crushed tomalley, head off.

Shrimp body structure. The term depicts the four stages of body structure transformations (Fig. 7):

Stage 1 (days 0–4). The shrimp body was intact from, and the abdomen segments were firmly attached (> 80%).

Stage 2 (days 4–8). the shrimps showed signs of loosening (> 50%), along with firmly attached abdomen segments.

Stage 3 (days 8–14). one to two abdomen segments loosened; the term appeared with a high frequency of occurrence.

Stage 4 (days 14–20). the shrimps loosened in most segments; this description increased in occurrence frequency.

The terms to describe the state of the shrimp body were selected as follows: intact, firmly attached segments → signs of slight loosening in 1–2 segments → loosening in most abdomen segments.

Shrimp meat texture. Figure 8 shows the terms that describe the textural change in shrimp meat:

Stage 1 (days 0–4). the shrimp meat was described as firm and elastic, with high frequency (> 80%).

Stage 2 (days 4–10). the meat was still elastic (> 50%), followed by the description of less elastic and soft meat. The shrimp meat in this period began to transform and was not as good as at Stage 1.

Stage 3 (days 10–16). the meat was described as less elastic, which showed a reduction in the shrimp meat quality.

Stage 4 (days 16–20). The meat lost its elasticity and gained softness and flaccidity (> 60%).

The terms to describe the changes the shrimp meat texture were selected as follows: firm, elastic → elastic → slow elastic and soft → not elastic, soft and flaccid.

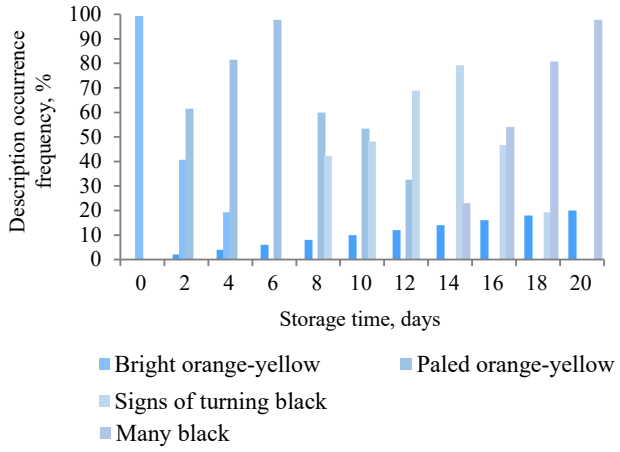

Figure 4 Frequency of shrimp leg color descriptions by storage time

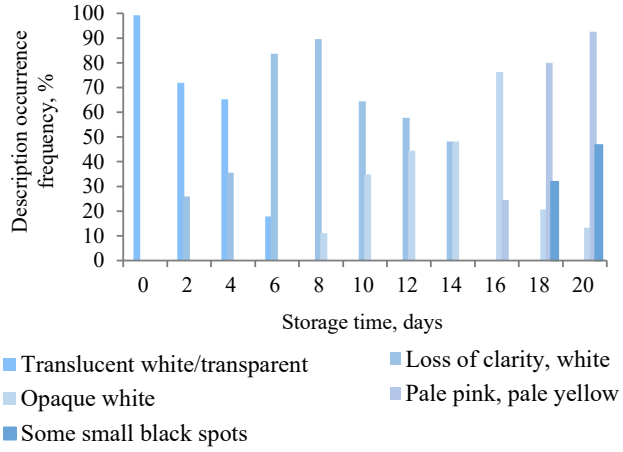

Figure 5 Frequency of shrimp meat color descriptions by storage time

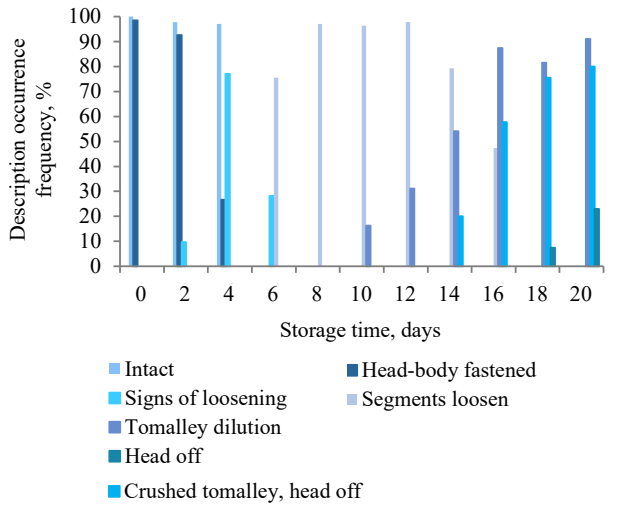

Figure 6 Frequency of shrimp head structure descriptions by storage time

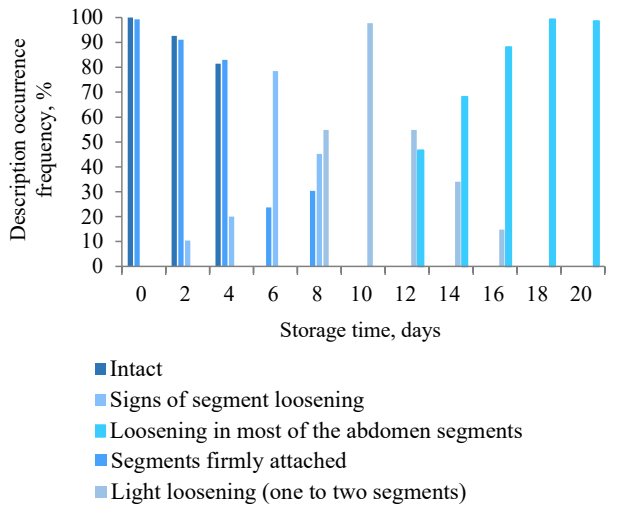

Figure 7 Frequency of shrimp body structure descriptions by storage time

Shrimp shell status. Figure 9 illustrates the frequency of terms selected to depict the transformation of shrimp shells:
Stage 1 (days 0–4). The shells were described as shiny, hard, and firm, tightly attached to the meat;
Stage 2 (days 40–14). The shells grew slightly tender and started loosening from the meat; and
Stage 3 (days 14–20). The shell became soft and loosely attached to the meat.

The terms to describe the shrimp shells selected ranked as follows: shiny, hard shells; firmly attached shell and meat → slightly soft shells; meat and shells not firmly attached → soft shells; shells loosen from meat.

Figure 10 illustrates the changes in the overall appearance of the shrimps during 20 days of storage in ice water.

QIM program for shrimp sensory evaluation. Table 1 shows scores from 0 to 3 that marked the changes in sensory properties of different parts of shrimp body. The QIM scheme was based on nine attributes observed to have changed during storage: smell; color of the head, body, and legs; body and head status; meat texture. These nine attributes were included in the QIM assessment program with scores from 0 to 26 points: QI = 0 corresponded to the best quality shrimps, and QI = 26 corresponded to the lowest quality possible.

The QIM scheme (Table 1) was designed to assess the sensory quality of Jinga shrimps preserved in ice water. Compared to the QIM program developed for giant tiger prawns and white-leg shrimps, the attributes of Jinga shrimps were different in terms of head and

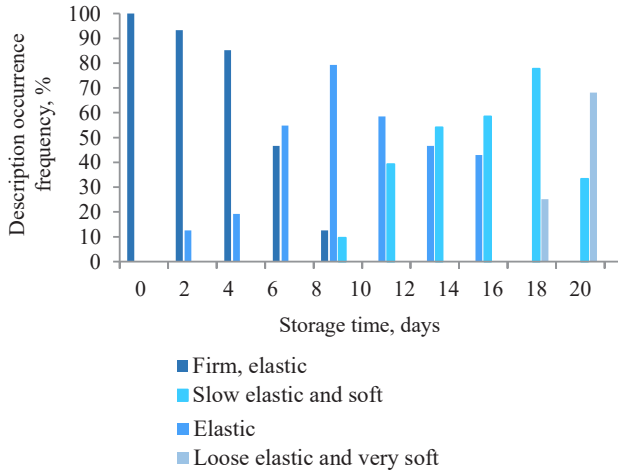

Figure 8 Frequency of shrimp meat texture descriptions by storage time

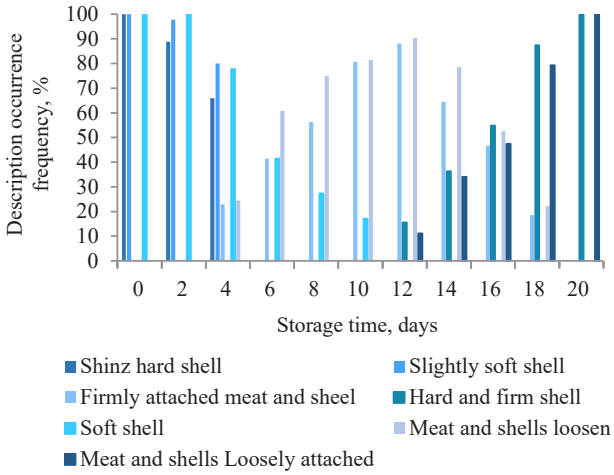

Figure 9 Frequency of shrimp shell descriptions by storage time

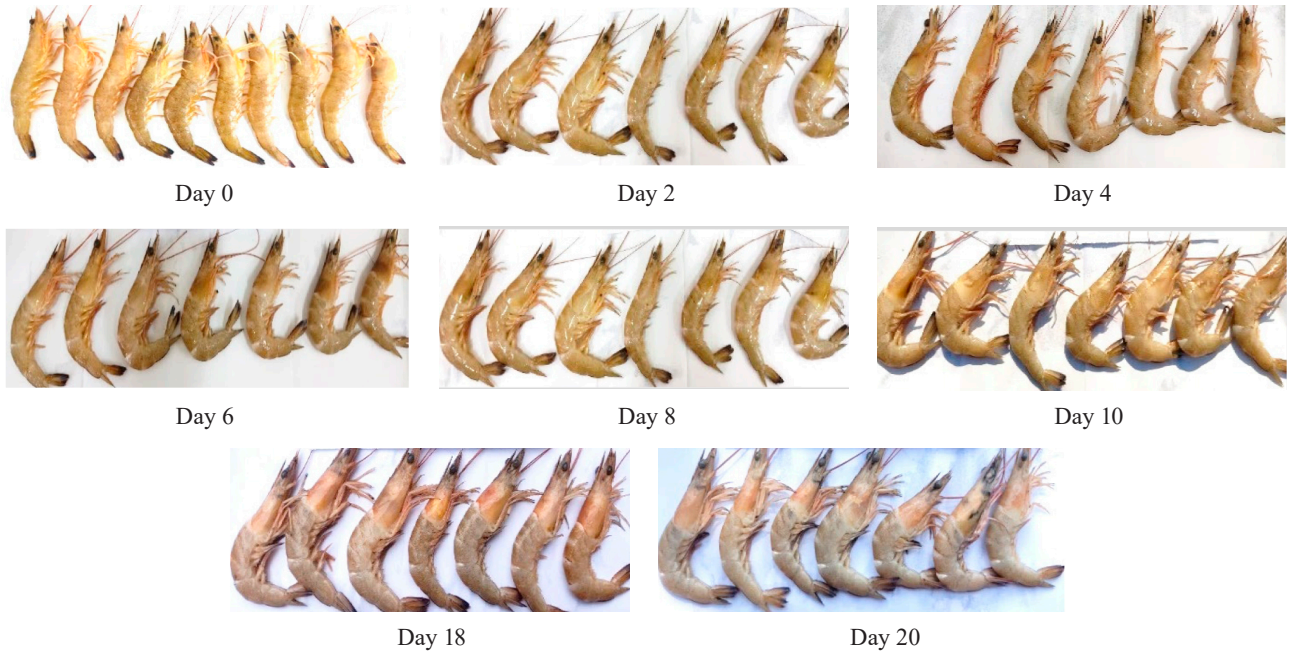

Figure 10 Transformations on shrimps stored in ice water for 20 days

Table 1 QIM Scales for Jinga shrimps (*Metapenaeus affinis* L.)

Attributes		Descriptions	QI
Smell		Characteristic fresh shrimp smell	0
		Seaweed smell, mild fishy	1
		Clear fishy, mild ammonia	2
		Clear ammonia, putrid	3
Shrimp Head	*Color*	Clear color (yellow light), shiny, translucent, visible inner organs	0
		Less clear color (light yellow); orange-pink appears; poor gloss	1
		Color changes into clear orange-pink, black-grey, or brown; opaque, with a few small black spots	2
		Light orange, orange-red, with wide black areas	3
	State	Intact; head fastened to body	0
		Intact; signs of segment loosening	1
		Loose segments; tomalley dilution	2
		Crushed tomalley; head falls off; loose segments	3
Shrimp body	*Color*	Clear color (yellow light), shiny, translucent; distinct green dots	0
		Light yellow, slightly shiny, with no black spots; slightly blurred green dots	1
		Discolored, orange-pink or dark orange; a few small black spots; blurred green dots	2
		Complete discoloration, dark orange-pink, black-grey	3
	State	Intact; firmly attached segments	0
		Intact; slightly loosened segments	1
		One to two loose segments	2
		Most segments are loose	3
Shrimp Legs	*Color*	Bright orange-yellow	0
		Pale orange-yellow	1
		Pale yellow-orange, signs of blackening	2
		Black	3
Shrimp shell status		Shiny, hard, and sturdy; firmly attached to meat	0
		Slightly soft; starts loosening from meat	1
		Soft, loose from meat	2
Shrimp meat	*Color*	Translucent white	0
		Clear, white	1
		Opaque, white	2
		Opaque white, pale pink, and pale yellow, with some small black spots	3
	Texture	Good elasticity, firm	0
		Elastic	1
		Soft, low elasticity	2
		Not elastic, soft and flaccid	3
Total			26

body color, leaning toward yellow, orange, and black. In terms of smell, ammonic smell grew clear over time. The color of giant tiger prawns changes from blue to green and the smell grows sour at the end of shelf life. White-leg shrimps change from grey to green and emit an unmistakable smell of trimethylamine, as reported by Le *et al.* [22, 25]. Thus, each type of shrimp has different sensory variations depending on the species and habitat, and each of them needs a tailored QIM scheme.

Results of developing the correlation equation between QI and storage time for Jinga shrimps. Table 2 sums up the changes in the sensory properties of Jinga shrimp's over preserving time.

The temporal transformations were consistent with the terminology developed by the panelists, and the progress of the changes was apparent over time. On day 2, the shrimps had almost the same properties as on day 0, thus keeping the original value; the head color and the shell gloss reduced but not significantly. On day 4, the shrimps developed evident changes: a fishy smell, dull color, opaque white meat, loose segments, etc. On day 6, the properties associated with freshness were almost gone: the heads turned orange, the meat grew white, the shell loosened from the meat, and the head separated from the body. On day 8, the shrimps began to show signs of deformation: the head turned orange or pink-orange while the legs turned black; the shell grew soft, and the meat and the shells started to loosen. On day 10, the shrimps developed a smell of ammonia, and the tomalley started to dilute while the head and body segments loosened. On days 12–20, the shrimps showed obvious signs of spoilage and emitted a robust putrid smell. We observed discoloration of the whole shrimp body, appearance of black spots, complete body segment loosening, and shell softening. The meat turned from poorly elastic to soft pasty, the head fell off the body, and the tomalley diluted to watery.

Table 2 Sensory changes and QI of shrimp stored in ice water

Day	Expression of sensory properties	QI score	Remarks
0	The characteristic fresh scent, or "the smell of the sea". The head and body of the shrimp have a clear light-yellow color, and shiny, transparent flesh; body segments are firmly attached. The shell is sturdy and rigid. The meat and shell are firmly attached. The shrimp is firm and elastic.	[a]	Most sensory attributes get 0 points
2	Shrimps retain their characteristic fresh scent, or "the smell of the sea". The state remains almost original; the head color and gloss are not significantly reduced.	$7.5^{b} \pm 0.51$	Most sensory attributes get 1 point
4	The shrimp smell is slightly fishy, like seaweed. Its head, body, and leg color are light yellow, and the gloss reduces. The shrimp meat loses its clarity and begins to turn white. The shrimp shows signs of loosening on the head and some body segments. The shrimp shell does not change much, and the meat remains firm and elastic.	$9.78^{c} \pm 0.55$	Most sensory attributes get 1 point
6	The shrimp smells slightly fishy, like seaweed. Its head starts to turn orange, while the body and legs go pale. The meat turns white. The head segment loosens, with signs of body segment loosening. The shell feels soft. The meat and shell do not attach firmly.	$11.72^{d} \pm 0.46$	Most sensory attributes get 2 points
8	The shrimp smell is fishy. The legs are pale orange, with signs of blackening. The meat is white. The head segment loosens, and one or two body segments start to loosen. The shell feels soft. The meat and shell do not attach firmly. The meat is still elastic but not as good as it used to be.	$13.39^{e} \pm 0.78$	Most sensory attributes get 2 points
10	The shrimp has a fishy, mild ammonia smell. The head is pale pink-orange, with the dilution of tomalley. The body is pale yellow. The legs show signs of blackening. The meat is white. The head segment and one to two body segments loosen. The shell feels soft. The meat and shell do not attach firmly; the meat is still elastic.	$16.17^{f} \pm 0.51$	Most sensory attributes get 2 points
12	The shrimp starts to emit a mild smell of ammonia. The head color is pink-orange; the tomalley is diluted. The body turns pale orange, and the green dots are slightly blurred. The leg color is dark and turns black. The meat is white. The head and body segments loosen. The shell is slightly soft. The meat and the shell are not firmly attached. The flesh starts to lose elasticity and becomes slightly soft.	$19.17^{g} \pm 0.51$	Most sensory attributes get 2 points
14	The shrimp emits ammonic smell. The head goes apparent orange-pink, accompanied by black-grey. The body turns pale orange, and the green dots are slightly blurred. The legs are black. The meat is white. The head loosens, and the tomalley dilutes. Segment loosening happens all over the body. The shell is slightly soft, and the meat and the shell are not firmly attached. The meat is soft and slowly elastic.	$20.83^{h} \pm 0.86$	Most sensory attributes get 2–3 points
16	The ammonic smell is clear. The head and body are completely discolored. The flesh is opaque white or slightly pale pink. The head segment loosens, and the tomalley dilutes. Whole body segments loosen. The shrimp shell is slightly soft; the meat and the shell are not firmly attached. The meat is soft and slowly elastic.	$20.56^{i} \pm 0.51$	Most sensory attributes get 2–3 points
18	The shrimp starts to smell putrid. Small black spots appear on the body. The legs are black. The meat is pale pink and yellow, with some small black spots. The head falls apart, and the tomalley dilutes. Whole body segments loosen. The shell grows soft. The meat and the shell are loosely attached. The meat is not elastic, but soft and flaccid.	$23.33^{k} \pm 0.91$	Most sensory attributes get 2–3 points
20	The shrimp is thoroughly spoiled and smells rotten and putrid. The head color appears clear orange with broad black areas; the body is orange, grey, or black, and the legs are black. The meat is opaque white, pale yellow, or pale pink. The tomalley is crushed, and the head falls apart. All body segments loosen. The shell is soft. The meat and the shell are loosely attached. The meat is not elastic, but soft and flaccid.	$26.0^{m} \pm 0.0$	Most sensory attributes get 3 points

[a, b, c,..., m] – represent the statistically significant difference of QI values between the dates; TGBQ stands for storage time

The results in Table 2 show that QI increased with storage time, and the increase was significant with $p < 0.05$. The regression equation takes the following Eq. (2):

$$Y = 1.16\text{Xtg} + 4.32\ (R^2 = 0.97)\ (*) \qquad (2)$$

Other researchers also reported that the quality index (QI) increases linearly with the storage time in ice water in seafood, giant tiger prawns, and white-leg shrimps [10, 20, 22, 25]. Figure 11 illustrates the correlation between the shrimp quality scores and the storage date.

The graph shows the linear relationship and the proportional correlation between storage time and QI score with $p < 0.05$ and angle coefficient $a > 0$. Based on Table 3, Jinga shrimps stored in ice water should be consumed within 8–10 days when the QI is still < 18

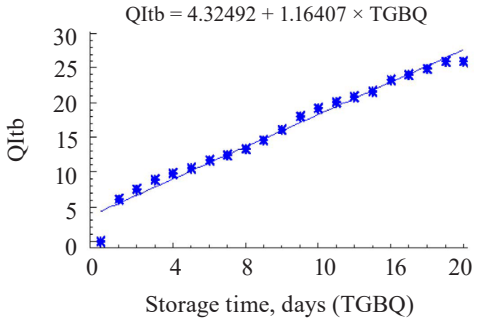

Figure 11 Linear correlation between QI score and shrimp storage time in ice water

points. These results were similar to those published on giant tiger prawns (8 days) and white-leg shrimps (9 days) [22, 25].

Verification results of QIM scheme for Jinga shrimps. Ten random shrimp samples with different freshness (unknown) were collected at Do Son port. The panelists scored them using the developed QIM scheme (Table 1). Table 3 shows the results of determining the storage time, predicting the remaining shelf life according to the equation (*), and the actual shelf life.

Table 3 shows no statistically significant difference between the predicted and actual storage dates. The residual value of the actual shelf life was close to the residual shelf life calculated using the regression equation (*). The obtained results indicate the possibility of estimating the preservation limit and predicting the remaining storage time of Jinga shrimp stored at 0–4°C. The result suggested that the QIM scheme developed for Jinga shrimp quality assessment provided good results and proved suitable for practical evaluation. It can be used as a reliable scientific tool to assess freshness and quality of raw Jinga shrimps.

Table 3 Determining the estimated shelf life according to the QIM program and the remaining actual shelf life of Jinga shrimps stored in ice water

Sample	Initial QI	Precalculated storage, days	Predicted remaining shelf life, days	Actual storage date based on observation, days
1	7.00	2.31	7.69	8.20 ± 0.45
2	9.00	4.03	5.97	6.40 ± 0.55
3	11.33	6.05	3.95	2.80 ± 0.84
4	12.33	6.91	3.09	3.60 ± 1.14
5	13.00	7.48	2.52	2.40 ± 0.89
6	14.67	8.92	1.08	1.20 ± 0.84
7	16.00	10.07	–0.07	–
8	17.33	11.22	–1.22	–
9	18.33	12.08	–2.08	–
10	19.67	13.23	–3.23	–

CONCLUSION

This study featured the transformations of Jinga shrimp sensory profile during storage in ice water. We developed a Quality Index Method (QIM) scheme to assess the sensory quality of Jinga shrimps based on nine sensory properties, with an Quality Index score of 0–26. We also developed a correlation equation between the QI score and the storage time with the coefficient of $R^2 = 0.97$. The best shelf life of Jinga shrimps stored in ice water was predicted to be between 8 and 10 days. The QIM program is a user-friendly and effective science-based tool that delivers fast and reliable results. It can help customers, fishermen, traders, aquaculture enterprises, and quality control officials to specify the storage time and estimate the remaining shelf life of Jinga shrimps. However, to improve the effectiveness and persuasiveness of the quality index method, it is possible to combine it with the chemical indicators of freshness, this creating a complete program for the Jinga shrimp quality assessment.

CONTRIBUTION

B.T.T. Hien: research concept, methodology, formal analysis, research, original draft, review and proofreading. D.T. Pham: methodology, research, and original draft. L.P. Vu: validation and research. P.H. Dao: validation and research. P.V. Tuyen: research. N.V. Nghia: research, review and proofreading. N.K. Bat: formal analysis, research, and original draft. All the authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication if this article.

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