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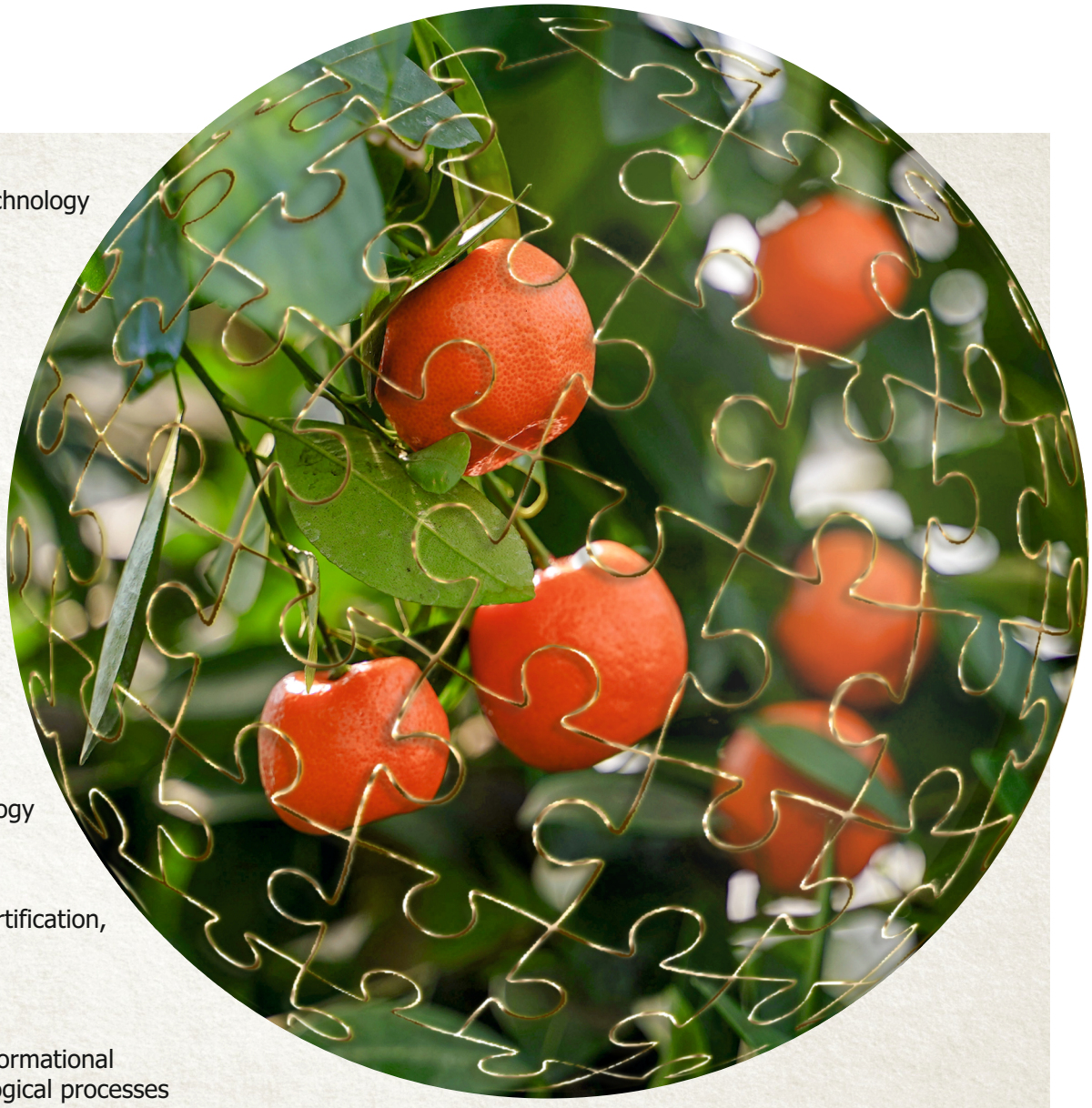

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Editorial
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Editor's column

The importance of the agro-industrial complex is immense: this economic segment is the backbone of industry and global food security. Fruits and vegetables are valuable food products because they contain a complex of vitamins, enzymes, and other biologically active substances. The total world fruit production is estimated as 800 million tons annually; it increases at an average pace of 3% per year. World production of vegetables and cucurbits is 1 150 million tons. The enormous potential of the agro-industrial sector attracts science, government, and business.

The current agri-food market is a territory of fierce competition, which has to deal with huge volumes of fruit and vegetable production. Therefore, long-term storage and delivery are its most pressing issues. The agri-food market has to develop new storage and processing technologies to minimize food losses, which has risen beyond 30%. Long-term storage products should maintain their appearance and useful properties throughout the entire shelf life. Effective storage methods can increase the storability of fruits and vegetables. A wrong choice of storage regime can cost producers their entire business.

Effective storage conditions support the vital activity of fruits, inhibit ripening and aging, maintain valuable consumer qualities, protect from parasitic and physiological diseases, etc.

The existing conventional, controlled and modified storage technologies very often fail to protect fruits from physiological and fungal diseases, as well as to maintain their original quality. Excessive ethylene is believed to trigger various diseases and decrease the quality of fruits. Ethylene can be exogenous if it comes from the environment or endogenous if it is synthesized inside the fruit. Even low concentrations of ethylene activate ripening and aging. Fruit storage under low oxygen conditions includes ultra-low oxygen or dynamic atmosphere. These technologies inhibit the accumulation of ethylene. Post-harvest treatment of fruits with 1-methylcyclopropene inhibits ethylene synthesis, reduces sunburn losses, and promotes quality retention. When stored under normal atmospheric conditions, the methods of ultra-low oxygen and dynamic atmosphere eliminate the shortcomings and enhance the capabilities of post-harvest treatment.

The storage of fruits and vegetables depend on temperature, relative humidity, and disinfection. Modern fruit and vegetable storage systems are fully automatic. They are regulated by special software, which creates optimal conditions inside storage chambers by adjusting the storage parameters to the type of product, temperature, humidity, gas composition, etc.

Various sectors of the economy, production, and business benefit from the proper storage of vegetables, root crops, potatoes, and fruits. What is more important, storage affects human health. Modern storage methods that improve the quality of products are in demand in the highly competitive environment of the agri-food market.

Vladimir Gudkovskiy, Member of the Russian Academy of Sciences, is known for his works on the scientific and technological foundations of long-term storage. In 1975, he set up the first soviet industrial storage facility with a controlled atmosphere. Its capacity was 500 tons and it was located in a pilot farm of the Kazakh Research Institute of Fruit Growing and Viticulture. In 1976, the Scientific and Technical Council of the USSR Ministry of Agriculture approved his industrial technology for controlled fruit storage, which was a scientific and technical breakthrough in agricultural science. For many years, this fruit storage has been a school of excellence in mastering new methods. Vladimir Gudkovskiy is the author of such fundamental publications as Industrial Fruit Storage in a Controlled Gas Environment and The Long-Term Fruit Storage. Vladimir Gudkovskiy is an outstanding expert recognized in Russia and abroad. His ideas are developed by modern scientists, government, and business.

Vladimir Gudkovskiy celebrates his 85th birthday on April 17, 2022. The editors of Foods and Raw Materials wish the honored scientist even greater professional achievements and talented students.

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

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Innovative use of date (*Phoenix dactylifera* L.) press cake in the food industry

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Abstract:
Introduction. Date press cake is a waste product of date juice making that can lead to potentially serious environmental problems if discarded in large amounts in open areas. Therefore, it needs to be utilized. Our study aimed to investigate the possibilities of using date press cake powder to formulate innovative ready-to-eat products – vegan biscuits and vegan protein bars.
Study objects and methods. The food products under study were subjected to a palatability test, a nutritional value evaluation, a texture profile analysis, and a scanning electron microscopy, as well as a microbiological assay performed during eight months of storage.
Results and discussion. The palatability test showed that the biscuits supplemented with 10% date press cake had the best acceptable preference, compared to control, 5 and 15% date press cake samples. Our innovative vegan protein bar scored highest in overall acceptability, flavor, taste, texture, and willingness to buy, compared to the commercial vegan protein bar. Overall, our study showed that both of our products were safe to consume within eight months. Additionally, our innovative protein bar and fortified biscuits had high proportions of the recommended dietary allowances for most nutrients for adolescents and athletes, especially for vegetarians.
Conclusion. Date press cake can be successfully used as a food ingredient to produce new formulations of vegan protein bars. Additionally, it can be used as an alternative ingredient to improve the nutritional quality of vegan biscuits.

Keywords: Date press cake, food industry, vegan products, biscuits, protein bars, chemical composition

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INTRODUCTION

Phoenix dactylifera L. is commonly known as Nakhel Al-Tamr in Arabian countries and as the date palm in English. A member of the genus Phoenix in the *Arecaceae* family, it grows in the hot desert regions of North Africa. In Egypt, dates have been an economically important food crop for thousands of years. According to the earliest records of predynastic Egypt (excavation of a vat in Hierakonpolis, Upper Egypt, 3450 BC), they were used as a beer sweetener. However, their cultivation started somewhat later than in Iraq (about 3000–2000 BC) [1, 2].

Egypt is at the top of the world's date-producing countries. In 2019, its production totaled 1.61 million tons, representing 16.41% of the world's production of 9.75 million tons [3]. Date fruits are a cheap and rich source of carbohydrates (70–80%) in the form of glucose and fructose, proteins, amino acids, and essential minerals (zinc, copper, selenium, potassium, calcium, magnesium, phosphorus, manganese, and iron), fiber, vitamins C and E, carotenoids, fatty acids, polyphenols, and flavonoids. Known as "emerging healthy foods" due to their health benefits, dates are commonly processed into juice, syrup, and paste, with many applications in other foods such as confectionary, bakery, and dairy products [2, 4–6]. As a result of agri-food production, large amounts of organic waste are produced as press cake that is mainly used as animal fodder and also in biofuel production [7].

Date press cake is a fibrous material that remains after date juice filtration. This by-product is a cause of disposal problems and environmental issues due to its bulky nature, high moisture, and carbohydrate content [2]. Date juicing accounts for 17–28% of date press cake that is dumped into open lands and drains or used as stock feed. However, it has been underutilized in the food industry, mainly due to the lack of technical knowledge about its nutritional value, health benefits, and possible effects on the quality of food products [2, 8]. In this context, we aimed to produce vegan biscuits

and vegan protein bars from date press cake and assess their quality and nutritional value.

STUDY OBJECTS AND METHODS

The objects of the study were date press cake powder in the production of innovative vegan protein bar and vegan biscuits (control sample and samples with 5, 10, and 15% of date press cake powder).

Materials. Date press cake was obtained from Al Tahhan Golden Dates Factory (New Valley Governorate, Egypt). Crisp rice (rice flour-corn flour-wheat flour 72%-calcium carbonate) was a gift from Caker Food Industries (New Damietta City, Egypt). Other dry ingredients and corn oil for our innovative vegan protein bars and vegan biscuits were purchased from the Metro Market (Egypt). The control was a commercial protein bar (Go Food Bar) (dates, almonds, cashew, cocoa) purchased from the Advanced Sport Nutrition Company (Cairo, Egypt). All the chemicals and equipment used in this research were of analytical grade.

Technological methods. ***Processing methods. Production of date press cake powder.*** After juicing fully matured Saidi dates, press cake was ground in a laboratory grinder, sieved through a 70 mesh sieve to obtain particles of 210 microns, and stored in sealed polyethylene bags at –18°C for technology application, as described in [6].

Preparation of vegan protein bars. Our innovative vegan protein bars were prepared in the following stages:
– good quality dried peeled split fava beans were rinsed, soaked in water for 12–13 h, boiled in plenty of water for 4 min until tender but not mushy; then drained, tossed in the oil, and spread out in a single layer on a baking sheet, and roasted at 185°C until golden and crispy;
– yellow split chickpeas and roasted split fava beans were individually milled in a laboratory mill (JKA-Labora technic, Janke and Kunkel Type: MFC, Germany);
– oat was roasted in an aluminum pan at 140°C in a low flame to get roasted aroma;
– 10 g of date press cake powder was mixed with 40 g of other dried ingredients (yellow split chickpeas, roasted split fava beans, instant coffee, oat, salt, coconut powder, crisp rice);
– glucose syrup (26 g) and molasses (24 g) were heated to 70°C for 2.5 min, mixed well with all dried ingredients and 2 mL of oil, molded into a bar shape (8×2.3×1.5 cm), and finally packaged in airtight polyethylene bags (HDPE) before measurements;
– the samples were stored at room temperature 25 ± 5°C for 8 months for microbiological assays.

Preparation of vegan biscuits. Control biscuits dough was prepared using 100 g of wheat flour (72% extraction), 25 g powdered sugar, 22 g corn oil, 1 g sodium chloride, 1 g ammonium bicarbonate, and 28 g water, as described in [9]. Experimental samples contained 5, 10, or 15% of date press cake powder instead. Biscuits were prepared in the following stages:
– sugar was mixed with corn oil and creamed intensively (speed 5) for 3 min in a KitchenAid mixer;
– wheat flour (72% extraction), sodium chloride, ammonium bicarbonate, and water were added to prepare dough;
– the dough was manually mixed for 15 min into a uniform smooth paste [10];
– biscuits were formed and baked in an oven at 180°C for about 25 min;
– the biscuits were cooled at room temperature 25 ± 5°C, packaged in airtight polyethylene bags before measurements, and stored at room temperature for eight months for microbiological assays.

Palatability test. Palatability evaluation of our products was carried out by ten experienced panelists from the Food Technology Research Institute (Agricultural Research Center, Giza, Egypt) according to the method described in [11]. Then, 30 consumers from the local market who were willing to buy the products were also tested according to [12].

Evaluation of the nutritional value. The samples were analyzed for moisture, ash, protein, crude fiber, and crude fat on a dry weight basis according to the standard procedures recommended by [13] while total carbohydrates were calculated by difference. %Available carbohydrates (on dry basis) = 100 – (%Ash + %Fat + %Protein + %Fiber), as mentioned by [15]. Energy (Kcal) was calculated by the formula of [14] as follows: Energy (Kcal) = [Protein (g)×4] + [Carbohydrate (g)×4] + [Fat (g)×9]. Minerals, i.e., calcium (Ca), iron (Fe), Magnesium (Mg), Potassium (K), Manganese (Mn), and Zinc (Zn) were determined using an Atomic Absorption Spectrophotometer (3300 Perkin-Elme), as described in [13].

Texture profile analysis. Texture properties of our products, such as chewiness, gumminess, springiness, cohesiveness, and hardness, were estimated using a CT3 Texture Analyzer (Version 2.1, 10 000 Gram unit, Brookfield, Engineering Laboratories, Inc., USA), according to the method of [16].

Scanning electron microscopy. Scanning electron microscopy (SEM) was used to visualize the microstructure of the bars, as described by [17].

Microbiological analysis. Total bacterial, yeast, and mold counts were carried out during different storage periods according to [18].

Statistical analysis. The statistical analysis was performed using SPSS One-Way ANOVA, version 22 (IBM Corp.) released in 2013. Data were treated as a complete randomization design according to [19]. Multiple comparisons were carried out applying the Duncan test. The significance level was $P < 0.05$.

RESULTS AND DISCUSSION

Chemical composition and minerals content. The chemical composition and mineral content of Saidi date press cake are shown in Table 1. As we can see, Saidi date press cake contained 6.11% moisture, 5.12% fat, 7.4% protein, 12.38% crude fiber, 2.78% ash, and 66.21% carbohydrates. Of the six minerals determined in the sample, Mg had the highest content, while Mn had the

Table 1 Chemical composition (dry weight) and mineral content of Saidi date press cake powder

Parameters	Content
Moisture, %	6.11
Protein, %	7.4
Crude fiber, %	12.38
Fat, %	5.12
Ash, %	2.78
Total carbohydrates, %	66.21
Minerals, mg/kg:	
Mn	10.9
Ca	502
Fe	78.45
Zn	19.2
Mg	625
K	27.4

Table 2 Sensory evaluation and willingness to buy vegan protein bars with data press cake powder

Parameters	Samples	
	Commercial bar	Innovative bar
Color	9.80 ± 0.95^{a}	7.14 ± 0.72^{b}
Flavor	6.95 ± 0.30^{b}	8.35 ± 0.23^{a}
Taste	7.73 ± 0.21^{b}	9.30 ± 0.20^{a}
Texture	7.83 ± 0.16^{b}	8.40 ± 0.20^{a}
Overall acceptability	8.18 ± 0.45^{b}	9.13 ± 0.20^{a}
Willingness to buy the vegan protein bar samples:		
Yes	25.00 ± 01.00^{b}	27.30 ± 0.58^{a}
No	5.0 ± 1.0^{a}	2.66 ± 0.57^{b}

[a] and [b]: If there is no significant difference ($P > 0.05$) between any two means, they have the same superscript letter within the same row

Table 3 Sensory preference test and willingness to buy biscuit with 10% of date press cake powder

Parameters	Samples			
	Control	5% DPC	10% DPC	15% DPC
Color	6.30 ± 0.71^{b}	8.06 ± 0.07^{a}	9.02 ± 0.15^{a}	5.40 ± 0.23^{b}
Flavor	7.18 ± 0.28^{b}	8.20 ± 0.23^{a}	8.68 ± 0.14^{a}	5.21 ± 0.26^{c}
Taste	7.54 ± 0.06^{c}	8.08 ± 0.08^{b}	9.07 ± 0.12^{a}	4.67 ± 0.18^{d}
Texture	7.96 ± 0.89^{a}	8.51 ± 0.10^{a}	8.17 ± 0.11^{a}	4.07 ± 0.22^{b}
Overall acceptability	7.73 ± 0.17^{c}	8.16 ± 0.18^{b}	8.86 ± 0.05^{a}	4.17 ± 0.15^{d}
Willingness to buy the vegan biscuit samples				
Samples	Control	5% DPC	10% DPC	15% DPC
Yes	20.20 ± 0.59^{c}	23.20 ± 0.51^{b}	27.00 ± 0.33^{a}	13.90 ± 0.86^{d}
No	9.80 ± 0.59^{b}	6.80 ± 0.51^{c}	3.00 ± 0.33^{d}	16.10 ± 0.86^{a}

[a–d]: If there is no significant difference ($P > 0.05$) between any two means, they have the same superscript letter within the same row. DPC – date press cake.

lowest content. These findings are in agreement with those reported by [2], who found that Shahani date press cake contained an average of 4.92% fat and 11.74% crude fiber, while Na, K, Cu, Zn, and Fe amounted to 2.05, 29.93, 9.06, 19.72, and 80.75 mg/kg, respectively.

Palatability tests. ***Vegan protein bars.*** Sensory evaluation is an important indicator of potential consumer palatability. Table 2 presents the sensory scores for the tested vegan protein bars, commercial and innovative. As we can see, the innovative protein bar obtained a significantly higher sensory score in flavor, taste, texture, and overall acceptability, while the commercial protein bar scored higher in color, which might be due to chocolate. However, the willingness to buy the vegan protein bars was significantly higher for the innovative samples (27.3 ± 0.58) than the commercial samples (25.00 ± 01.0), with 91 and 83.33% of the respondents, respectively.

Vegan biscuits. A preference experiment was designed to select the best ratio of raw materials for vegan biscuits. According to the palatability results (Table 3), the control biscuits and fortified biscuits with 5 and 10% date press cake powder (DPC) scored higher in color, flavor, taste, texture, and overall acceptability. The sample with 10% DPC and 90% wheat flour received the most significant scores in taste, and overall palatability. Additionally, most of the respondents (90%) were significantly willing to buy this sample, compared to the other samples. Since the 10% sample showed the best preference, we selected it for our further studies.

Nutritive values of investigated products. ***Vegan protein bars.*** The nutritive values of commercial and innovative vegan protein bars are shown in Table 4. We found that the innovative bar scored significantly higher in protein, fiber, ash, and minerals, compared to the commercial bar. However, fat, available carbohydrates, energy, and moisture were significantly lower. Snack bars with high energy are consumed by top athletes to improve their performance. Therefore, the commercial and innovative bars could be introduced in their diet, as described by [20].

The recommended dietary allowances (RDA) given by the Indian Council of Medical Research (ICMR) for female and male athletes, respectively, are as follows: energy 3600 and 4500 Kcal/day, protein 82.5 and 120 g/d, fat 30 and 40 g/d, carbohydrates 585 and 731.25 g/d, iron 21 and 17 mg/d, and calcium 600 mg/d for females and males, as reported by [21, 22]. Our

Table 4 Nutritive values of innovative protein bar vs. commercial protein bar

Parameters	Vegan protein bars	
	Commercial bar	Innovative bar
Moisture, %	13.46 ± 0.17[a]	6.89 ± 0.38[b]
Protein, %	12.30 ± 0.17[b]	19.32 ± 1.24[a]
Fat, %	8.26 ± 0.24[a]	6.25 ± 0.21[b]
Fiber, %	0.67 ± 0.06[b]	2.10 ± 0.07[a]
Ash, %	1.21 ± 0.05[b]	1.80 ± 0.07[a]
Available carbohydrates, %	77.50 ± 1.17[a]	70.60 ± 1.19[b]
Energy, Kcal/100 g	433.50 ± 2.05[a]	416.10 ± 4.87[b]
Iron, mg/100 g	1.46 ± 0.08[b]	2.50 ± 0.15[a]
Zinc, mg/100 g	1.30 ± 0.08[b]	2.10 ± 0.11[a]
Calcium, mg/100 g	36.00 ± 2.40[b]	45.70 ± 2.21[a]
Potassium, mg/100 g	199.66 ± 1.20[b]	214.66 ± 1.76[a]
Mg, mg/100 g	19.30 ± 0.41[b]	28.18 ± 0.61[a]
Mn, mg/100 g	1.34 ± 0.06[b]	1.83 ± 0.04[a]

[a] and [b]: If there is no significant difference ($P > 0.05$) between any two means, they have the same superscript letter within the same row

Table 5 Nutritive values for vegan biscuits fortified with 10% of date press cake powder vs. control vegan biscuits

Parameters	Vegan biscuits	
	Control	10% DPC
Moisture, %	3.89 ± 0.11[b]	4.76 ± 0.09[a]
Protein, %	7.40 ± 0.18[b]	8.70 ± 0.11[a]
Fat, %	11.72 ± 0.19[a]	9.49 ± 0.24[b]
Fiber, %	0.90 ± 0.01[b]	2.53 ± 0.14[a]
Ash, %	1.83 ± 0.06[a]	1.16 ± 0.12[b]
Available carbohydrates, %	78.15 ± 0.38[a]	78.12 ± 0.66[a]
Energy, Kcal/100 g	447.50 ± 3.60[a]	432.80 ± 2.47[a]
Iron, mg/100g	1.86 ± 0.03[b]	2.90 ± 0.24[a]
Zinc, mg/100g	0.51 ± 0.11[b]	1.21 ± 0.02[a]
Calcium, mg/100g	16.22 ± 0.56[b]	22.51 ± 1.73[a]
Potassium, mg/100g	135.66 ± 7.20[b]	158.66 ± 3.17[a]
Mg, mg/100g	14.60 ± 0.69[b]	19.50 ± 0.84[a]
Mn, mg/100g	0.37 ± 0.03[b]	1.67 ± 0.22[a]

[a] and [b]: If there is no significant difference ($P > 0.05$) between any two means, they have the same superscript letter within the same row. DPC – date press cake.

female and male athletes, respectively: energy 11.55 and 9.24%, protein 23.46 and 16.13%, fat 20.8 and 15.6%, carbohydrates 12.06 and 9.65%, iron 11.90 and 14.70%, and calcium 7.6% for both. The results for 100 g of the commercial bar were as follows: energy 12.04 and 9.63%, protein 14.90 and 10.25%, fat 27.5 and 20.7%, carbohydrates 13.24 and 10.59%, iron 6.95 and 8.59%, and calcium 6.0% for both females and males. The intake of total calories, carbohydrates, proteins and fats were normal in female athletes but less than RDAs [23].

Adolescence is a transition period between childhood and adulthood from 13 to 17 years of age. Therefore, adolescents need additional calories, protein, calcium, and iron [24]. According to [25], the recommended intake for female and male adolescents, respectively, is as follows: energy 2200 and 2900 Kcal/d, protein 46 and 58 g/d, iron 15 and 10 mg/d, calcium 1200 mg/d for both sexes, and zinc 12 and 15 mg/d. We found that 100 g of the innovative bar covered the recommended intake at the following levels in females and males, respectively: energy 18.91 and 14.34%, protein 42.08 and 33.30%, iron 16.67 and 25.00%, calcium 3.80% for both sexes, and zinc 17.5 and 14.0%. The results for 100 g of the commercial bar were as follows: energy 19.70 and 14.95%, protein 26.73 and 21.20%, iron 9.7 and 14.6%, calcium 3.0% for both sexes, and zinc 10.33 and 8.66%.

Vegan biscuits. The nutritive values for the vegan biscuits fortified with 10% DPC and the control vegan biscuits are given in Table 5. The 10% DPC sample contained higher moisture, protein, fiber, iron, zinc, calcium, potassium, magnesium, and manganese but lower ash and fat. In addition, 100 g of the 10% biscuits met the daily requirement in female and male adolescents, respectively, for energy 19.67 and 14.92%, protein 18.91 and 15.00%, iron 19.33 and 29.00%, calcium 1.88% for both sexes, and zinc 10.08 and 8.06%. The results for 100 g of the control biscuits were as follows: energy 20.34 and 15.43%, protein 16.08 and 12.75%, iron 12.4 and 18.6%, calcium 1.35% for both sexes, and zinc 4.25 and 3.40%, as reported by [25].

We found that a 100 g serving of the 10% DPC biscuits provided a substantial proportion of the RDAs established by the ICMR for female and male athletes, respectively, in relation to energy 12.02 and 9.62%, carbohydrates 13.35 and 10.68%, fat 31.63 and 23.73%, protein 10.54 and 7.25%, iron 13.80 and 17.05%, and calcium 3.75% for both sexes. The control biscuits (100 g) showed the following values: energy 12.43 and 9.94%, carbohydrates 13.36 and 10.68%, protein 8.97 and 6.16%, fat 39.06 and 29.30%, iron 8.86 and 10.94%, and calcium 2.7% for both sexes, according to [22].

Texture profile analysis and scanning electron microscopy. ***Vegan protein bars.*** Texture profile

Table 6 Texture profile of vegan protein bars (mean ± SE)

Samples	Texture profile analysis				
	Chewiness, mJ	Gumminess, N	Springiness, mm	Cohesiveness, mm	Hardness, N
Commercial bar	32.63 ± 0.81[a]	17.42 ± 0.54[a]	1.10 ± 0.32[a]	22.40 ± 1.93[b]	35.54 ± 1.00[b]
Innovative bar	25.97 ± 1.54[b]	14.33 ± 0.42[b]	1.96 ± 0.05[a]	31.73 ± 1.68[a]	43.58 ± 2.28[a]

[a] and [b]: If there is no significant difference ($P > 0.05$) between any two means, they have the same superscript letter within the same column

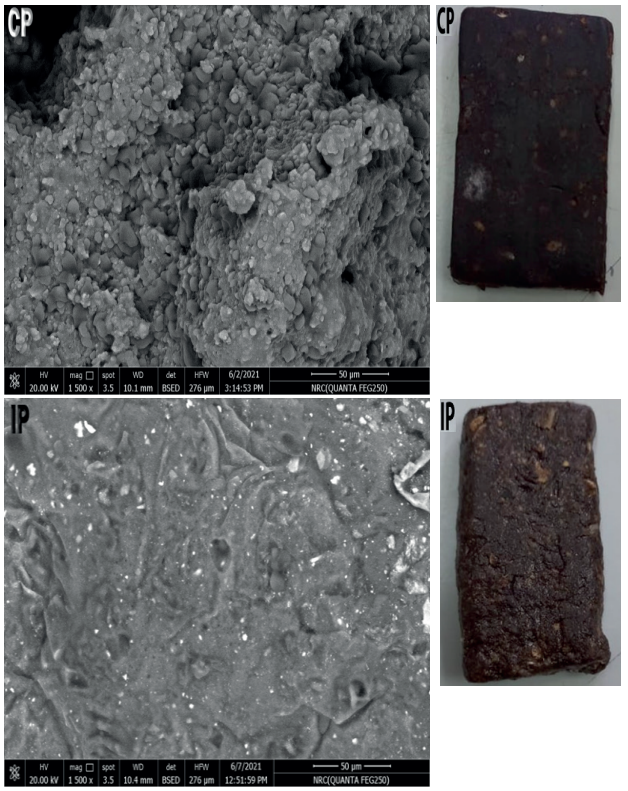

Figure 1 Scanning electron microscopy images for experimental vegan protein bars. IP – innovative protein bar. CP – commercial protein bar. Magnifications of 1500×

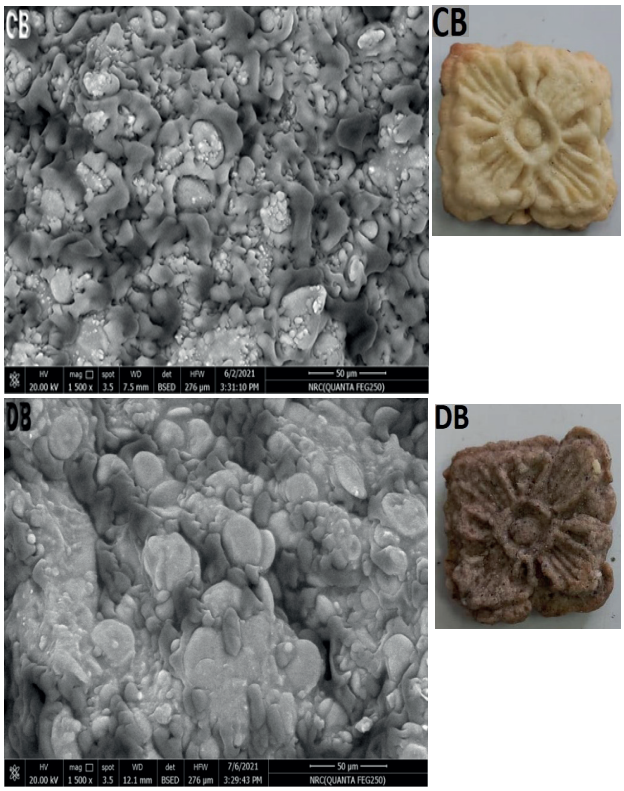

Figure 2 Scanning electron microscopy images of experimental vegan biscuits. DB – 10% DPC-fortified biscuits. CB – Control biscuits. Magnifications of 1500×

analysis is another important measure of a food product's overall quality and consumer acceptability. The results of texture analysis of the vegan protein bars (commercial and innovative) are shown in Table 6. We found that the DPC-fortified innovative bar showed significantly higher ($P \leq 0.05$) hardness and cohesiveness values, but significantly lower gumminess and chewiness values, compared to the commercial bar, which contained date and was coated with chocolate. The increased hardness of our innovative bars may be due to the migration of moisture between carbohydrates (such as starches, pectins, sugars, and maltodextrin) and proteins, or due to their protein content increase (19.32%), as shown in Table 4 [26]. However, the hardness of high-protein bars is quite high. It increases with the addition of protein and is characterized by a large variety of parameters [27].

The microstructures of cross-sectional areas of the commercial and innovative protein bars are shown in Fig. 1. As we can see, the innovative bar had a wavy structure, with few cavities and air pores, and relatively high hardness, while the commercial bar had a round structure with a number of cavities and air pores. A formation of large protein clusters (agglomerates) was probably the cause of the increased hardness of the innovative bar [27]. The formation of characteristic agglomerates in the innovative bar might be due to the presence of lectin sources in its ingredients. Lectins are glycoproteins known for their aggregation and high specificity binding with carbohydrates without initiating a modification through associated enzymatic activity. This leads to the formation of compact and hard structures, accounting for high hardness, factorability, and adhesiveness [27, 28].

Table 7 Texture profile of vegan biscuits (mean ± SE)

Treatment	Physical properties				
	Chewiness, mJ	Gumminess, N	Springiness, mm	Cohesiveness, mm	Hardness, N
Control biscuits	65.93 ± 1.74[a]	28.33 ± 0.88[a]	3.25 ± 0.07[a]	0.55 ± 0.06[b]	35.05 ± 4.04[b]
Biscuits fortified with 10% of date press cake powder	42.68 ± 1.24[b]	16.44 ± 1.26[b]	2.40 ± 0.04[b]	1.20 ± 0.08[a]	56.20 ± 3.53[a]

[a] and [b]: If there is no significant difference ($P > 0.05$) between any two means, they have the same superscript letter within the same column

Table 8 Total bacterial, yeast and mold count (CFU/g) of 10% date press cake vegan biscuits and innovative vegan protein bar during storage at room temperature 25 ± 5°C

Total bacterial count						
Storage period, month	Vegan biscuits				Innovative vegan protein bar	
	Control		10% DPC			
	Number of cells, CFU/g	Log survivor	Number of cells, CFU/g	Log survivor	Number of cells, CFU/g	Log survivor
0	n.d.	–	n.d.	–	n.d.	–
2	1.6×10^2	2.20	1.1×10^2	2.04	n.d.	–
4	3.7×10^2	2.57	2.2×10^2	2.34	n.d.	–
6	5.0×10^2	2.70	3.9×10^2	2.59	6.0×10	1.78
8	7.0×10^2	2.85	4.0×10^2	2.60	9.0×10	1.95
Yeast and mold count						
Storage period, month	Vegan biscuits				Innovative vegan protein bar	
	Control		10% DPC			
	Number of cells, CFU/g	Log survivor	Number of cells, CFU/g	Log survivor	Number of cells, CFU/g	Log survivor
0	n.d.	–	n.d.	–	n.d.	–
2	n.d.	–	n.d.	–	n.d.	–
4	4.0×10	1.60	n.d.	–	n.d.	–
6	6.0×10	1.78	3.0×10	1.48	n.d.	–
8	2.6×10^2	2.41	1.1×10^2	2.04	2.0×10	1.30

*n.d. – not detected

Vegan biscuits. Biscuits are a type of bakery products with minimal mixing and low water activity. Short dough has minimal, if any, gluten development that results in the production of smaller biscuits. In our study, the addition of date press cake caused a significant increase ($P \leq 0.05$) in hardness and cohesiveness, as well as a significant decrease in springiness, gumminess, and chewiness, compared to the control sample. This was due to the effect of fibers that have high water and oil adsorption capacity [29].

Majzoobi *et al.* indicated that date press cake can promote starch retrogradation and protein aggregation, which can also account for the increased hardness and reduced springiness [6]. So, the increase in biscuits hardness can be attributed to the dilution and weakness of the gluten network caused by data press cake.

The Scanning Electron Micrograph showed the effect of date press cake fibers on cross-sectional areas of the control biscuits and the DPC-fortified biscuits. As we can see in Fig. 2, their structures were slightly different. The cross-sectional area of the control biscuits showed gaps and air cells, compared to that of the fortified sample. Also, we found that the fortified vegan biscuits had an increased cell size, as well as bulged and thicker cell walls.

Our results agreed with those of Dar *et al.* who studied carrot pomace powder-based extrudates [30]. They reported that as the concentration of powder increased, the size of air bubbles and moisture droplets became smaller, resulting in a tough product. They also found that the presence of additives such as fiber and sugar had a pronounced effect on the expansion of cookies.

In another study, supplementing cookies with date and peach powder (2, 4, and 6 %) increased cell size and caused a rough structure with fractured fibers and starch granules that got reoriented to shape the cookies [31]. At a 6% supplementation, the cell walls became bulged and thicker.

Microbiological assay. Table 8 illustrates various microorganisms (total bacteria count, yeast and mold count, CFU/g) in the vegan biscuits and innovative vegan protein bars partially supplemented with date press cake powder over a storage period of 8 months. We did not detect any bacteria in the biscuits samples in the zero time since high baking temperatures killed all the microorganisms. After 2 and 8 months of storage, the bacterial counts were 1.6×10^2 and 7.0×10^2 CFU/g for the control biscuits, respectively, and 1.1×10^2 and 4.0×10^2 CFU/g for the DPC-fortified biscuits, respectively.

Yeasts and molds were not detected after 2 months of storage for the control and after 4 months of storage for the fortified samples. The yeast and mold counts for the control biscuits were 4.0×10 and 2.6×10^2 CFU/g after 4 and 8 months of storage, respectively, whereas for the fortified biscuits they amounted to 3.0×10 and 1.1×10^2 CFU/g after 6 and 8 months of storage, respectively. Microbial contamination can be caused by the worker's contaminated hands during handling or by increased temperatures during storage [32]. Our results were very low compared to the WHO Standard (1994), which established the maximum permissible limits for total plate count and yeast and mold count to be 2.0×10^5 and $< 1.0\times10^4$ CFU/g, respectively, in baked products (cake, bread, and

biscuits) [33]. Thus, our biscuits had a lower microbial profile and therefore were safe to consume.

The shelf life analysis of the innovative vegan protein bar detected no bacteria after 2 and 4 months. Bacterial growth was detected after 6 months, while yeast and mold were detected after 8 months of storage at room temperature 25 ± 5°C. This means that the quality of our innovative bars was quite stable and they may be considered safe to consume. Our findings were in agreement with Pratiwi *et al*. who indicated that formulated products were safe for consumption if yeast and mold were lower than 10^2 CFU/g and total bacterial count was lower than 1×10^3 CFU/g, as based on the Thai Community Product Standard (TCPS 709/2004) [34].

CONCLUSION

Our study clearly showed that using date press cake powder to produce vegan biscuits and protein bars is practical, economic, and healthy since these products have a high nutritional value and technological quality. They contained high proportions of the recommended dietary allowances for most of the studied nutrients for adolescents and athletes and are especially useful for vegetarians.

CONFLICT OF INTEREST

The author declares no conflict of interest regarding the publication of this research.

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White, beige and brown adipose tissue: structure, function, specific features and possibility formation and divergence in pigs

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Abstract:
Introduction. Traditionally, mammalian adipose tissue is divided into white (white adipose tissue – WAT) and brown (brown adipose tissue – BAT). While the functions of WAT are well known as the triglyceride depot, the role of BAT role in mammalian physiology has been under close investigation. The first description of the role of BAT in maintaining thermogenesis dates back to 1961. This article offers a review of structural and functional specificity of white, beige, and brown adipose tissue.
Results and discussion. This manuscript describes the differences and descriptions of adipocytes and their impact on the maintenance of the main functions of the mammalian body. In particular, thermogenesis, stress response, obesity, and type II diabetes. In addition to WAT and BAT, an intermediate form was also detected in the body – beige fat (BeAT or Brite). We also considered presented the opposite opinions regarding the presence of three types of adipose tissue in the human and animal bodies. Studies on the identification of uncoupling proteins 1 and 3 and their role in the transformation of white fat into beige/brown. Basically, the data on the factors of endogenous and exogenous nature on their formation are given on the example of the human body.
Conclusion. With an abundance of publications on the keywords: "white, brown fat", these studies, in the overwhelming majority, are devoted to the role of these fats in the formation of human thermogenesis, the assessment of the impact on obesity. Pigs have also been suggested to lack functional BAT, which is a major cause of neonatal death in the swine industry, therefore the focus on investigating role of different types of adipose tissue in pigs seems very promising to understand whether there is a compensating mechanism of thermogenesis.

Keywords: Fat, beige and brown adipocytes, uncoupling protein, thermogenesis, adipocyte, animal health, livestock

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INTRODUCTION

The adipose tissue tissue is a form of connective tissue, the cells of which are filled with a fat drop [1]. Adipose tissue is distributed throughout the body and plays the key role in the body energy homeostasis as a lipid reservoir. Moreover, adipocytes are endocrinologically active, especially visceral [2, 3]. The content of adipose tissue and its lipid composition is strongly depended on biological species, diet, climate, etc.

Historically, adipose tissue of mammals has been divided into two types, white adipose tissue (WAT) and brown adipose tissue (BAT) based on its visible different color, as well as on its different physiological functions [4]. Anatomically, WAT presented in two major depots, subcutaneous and visceral around internal organs and comprises the largest adipose tissue volume in most mammals [2, 4, 5]. WAT is specialized in handling fatty acids and triglycerides (TGs) and critical for energy storage, endocrine communication, and insulin sensitivity [4, 5]. In contrast, amount of BAT is strongly lower. BAT participates in non-shivering thermogenesis and largely present in mammals postnatally and during hibernation [2, 4]. Although BAT is readily observed in both infant and adult mammals, BAT is gradually replaced by WAT with aging [2]. Beige adipose (BeAT) tissue is the third type of adipose tissue and is a result of "browning" of WAT, when brown-like adipocytes appear at anatomical sites characteristic of WAT [6]. It is also called Brite (brown-in-white) [7, 8]. Originally, BeAT was observed to arise in response to

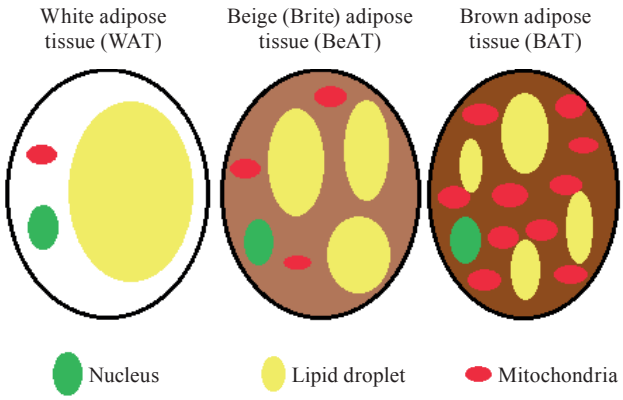

Figure 1 Comparison of white adipocytes beige adipocytes and brown adipocytes in morphology [11]

cold exposure; however, such factors, as diet, physical activity, pre-and probiotics, pharmaceutical and plant-based substances, etc. can also induce "beigeing" or "browning" of WAT [9].

Health status, survival of offspring, quality of pig processing products are strongly depended on proportion of all three types of adipose tissue. This article offers a review of structural and functional specificity of white, beige and brown adipose tissue, as well as possibility of its formation and divergence in pigs.

RESULTS AND DISCUSSION

Characteristics of white, beige and brown adipose tissue. The general classification of adipose tissue is based on its color, which is corresponded to lipid content, mitochondrial density and vascularization (Fig. 1). White adipocytes contain a single lipid droplet occupying approximately 90% of the cell space [9]. Brown adipose tissue (BAT) is highly vascularized, brown adipocytes contain a large number of mitochondria, but lipid droplet is smaller and presented in multiple vacuoles [9, 10]. Beige adipocytes display characteristics of both brown and white fat cells, the content of mitochondria is higher than in white adipose tissue (WAT), lipid droplet is not single, but bigger than in BAT [9].

Canonically, adipocytes are different in the origin and thought to arise from the *de novo* differentiation of precursor cells, particularly, white adipose stem cells originate from Myf5 (myogenic regulatory factor) negative progenitors, whereas brown adipose stem cells originate from myogenic lineage and express Myf5 [12, 13]. Otherwise, adipose precursors are heterogeneous, and the exact precursor population for adipogenesis may depend on the sex, location, age, or proadipogenic stimulus [14]. However, more than 95% of the precursors in brown fat are labeled with Myf5-Cre [15].

Beige adipocytes appears in WAT during white to brown transdifferentiation or can arise from adipogenic precursor cells in WAT through *de novo* differentiation. Additionally, preadipocytes in WAT give rise to mature white adipocytes with the potential to become brite adipocytes at a later point of time, which confirmed that after repeated stimuli most brite adipocytes localize to the same patches within the depot after the first stimuli. Such microenvironment factor, as density of vascularization, the types of stromal-vascular cells in the adipocyte's vicinity, the composition of the extracellular matrix, and the local innervation could also influence on this process [12, 16, 17].

Different morphology of adipose tissues types corresponds to other divergences (Table 1). WAT mainly maintains energy homeostasis and stores energy in the form of TGs, which are broken down into FFA and glycerol with following catabolism to generate energy when energy demands [10]. It also offers mechanical protection for muscles and internal organs and has an important role in conservation of body temperature [18].

Thus, subcutaneous WAT acts as a shock absorber, providing padding at various anatomic sites, whereas omental WAT is one of the visceral adipose tissue depots, surrounding and protecting inner organs from physical injury [19]. Subcutaneous WAT is more prone to expansion and represents a physiological buffer for excess energy intake during times of limited energy expenditure. When this storage capacity is exceeded, fat accumulates ectopically in areas outside the subcutaneous WAT [19, 20]. Some WAT has only biomechanical function, such as infrapatellar adipose tissue, which preserves even upon extreme starvation [21].

WAT, especially visceral, is also known as an endocrine organ producing adipokines, involved in lipid metabolism or transport, immune system, regulation of pressure, blood coagulation, glycemic homeostasis, angiogenesis, etc. [18, 22]. Adipose tissue also expresses receptors for most of these factors that are implicated in the regulation of many processes, including food intake, energy expenditure, metabolism homeostasis, as well as immunity and blood pressure homeostasis.

Both visceral fat and subcutaneous adipose tissue produce unique profile of adipocytokines, but visceral fat appears to be more active [23]. Excess of WAT is strongly correlated with obesity and insulin resistance [24]. Exceeded fat accumulation in areas outside the subcutaneous WAT, such as lipid accumulation in ectopic tissues (liver, skeletal muscle, and heart) as well as in the visceral depots lead to local inflammation, metabolic disorders and obesity-driven insulin resistance (IR) in WAT, liver, and skeletal muscle [25].

Brown adipose tissue was identified as a thermogenic organ in 1961, in 1978 BAT was shown to be the major site of thermoregulatory non-shivering thermogenesis [33]. However, beneficial effects of BAT could be also explained with its endocrine role through the release of endocrine factors, especially under conditions of thermogenic activation, such insulin-like growth factor I, interleukin-6, or fibroblast growth factor-21, which improve glucose tolerance and insulin sensitivity mainly by influencing hepatic and cardiac function [34].

Table 1 Characteristics of white, beige, and brown adipose tissue

Indicators	Types of adipose tissue			Ref.
	White	Beige (Brite)	Brown	
Function	Storage of energy and endocrine tissue	Adaptive thermogenesis	Heat production and endocrine tissue	16, 26–29
Mitochondria	Low, thin, elongated	Present (upon stimulation)	Abundant, bigger in size and contain more cristae	
Uncoupling protein	Nearly undetectable	Present (upon stimulation)	Present	
Iron content	Low	Upon stimulation (Abundant)	Abundant	
Correlation with insulin resistance	Positive	Negative	Negative	
Vascularization	Low	High upon stimulation	High	
Lipid composition	High level of TGs, DGs Decreased fractions of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), with longer (C > 36) and more polyunsaturated species, as well as lower levels of cardiolipin (CL)	Higher contents of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) fractions, with longer (C > 36) and more polyunsaturated species, as well ascardiolipin (CL), lyso-PC (LPC) Higher abundance of phospholipids such as PEs and PCs (predominantly composed by polyunsaturated LCFAs, especially DHA) Higher in FFAs		30, 31
Thermogenic mechanisms	–	UCP1-independent (Ca^{2+} cycling, creatine cycling)	UCP1-dependent creatine cycling	32

Comparatively large amounts of BAT are present in the newborns, and then reduced during aging [35].

There is a general proposal that brown adipose tissue is rapidly lost postnatally, the implication being that this process is normally concluded within the first (few) years of life, and that humans later in life do not possess more than vestigical amounts of brown adipose tissue [36].

BAT contains a lot of mitochondria, free fatty acids serve as substrates for lipid oxidation and as potent activators of the mitochondrial uncoupling protein 1 (UCP1), the crucial trans-membrane protein that catalyzes heat production at the mitochondrial level [37]. UCP1 is the only memberable to translocate protons through the inner membrane of brown adipocyte mitochondria, uncouples respiration from ATP synthesis, and therefore provokes energy dissipation in the form of heat while, also stimulating high levels of fatty acid oxidation.

UCP1 homologs were identified but they are biochemically and physiologically different from UCP1 [38]. The biochemical activities and biological roles of the identified UCP2 and UCP3 are poorly understood [39]. UCP2, widely expressed in tissues and cell types, could have particular importance in the regulation in lipid metabolism and contribute to resting metabolic rate, fat oxidation, while UCP3 could transport protons with a rate comparable to UCP1 [40–42]. Thus, it was shown that UCP3 is highly abundant in BAT and the sensitivity of the protein expression to temperature is similar to that of UCP1 [43]. It was also revealed that UCP3, expressed in both skeletal muscle and brown adipose tissue, may act as an inducible thermogenin *in vivo* and could indirectly mediate thermogenesis by increasing fatty acid oxidation and metabolite transport [44]. However, UCP2 and UCP3 are not generally responsible for adaptive thermogenesis, but nonetheless they may be significantly thermogenic when fully activated by endogenous or exogenous effectors [45].

BAT has negative correlation with obesity and insulin resistance, increasing BAT mass could improve glucose metabolism and metabolic health [46]. Thus, it was estimated that 50 g of BAT can burn as much as 20% of daily energy intake; therefore even though the BAT depots are present in small amounts, the activated tissue has the potential to substantially contribute to energy expenditure. In addition to using lipids, BAT also displays a very high rate of glucose uptake under cold exposure, glucose uptake increases by 12-folds, dissipating energy as a function of increased blood flow [47].

Beige – brown in white or brite (BeAT) – an intermediate type of fat, which is similar functionally to brown fat – it has a high thermogenicity and contains a significant number of mitochondria. Nevertheless, beige adipocytes may secrete certain factors that affect WAT function, systemic metabolism or both, has negative correlation with obesity and insulin resistance and appears upon the stimulation into WAT [29, 48, 49]. BeAT plays the key role in adaptive thermogenesis, subcutaneous WAT is particularly prone to browning [50]. Thermogenic capacity of beige fat cells depends on the presence of UCP1 [51].

Localization of beige and brown adipose tissue. Distribution of BAT and BeAT is different, localization is various in species; it is most studied in humans and rodents (Fig. 2). In human infants, BAT is located in interscapular and peri-renal areas, while in adults smaller BAT depots are located in the anterior cervical, supraclavicular, axillary, peri-aortic, paravertebral and suprarenal regions, while beige fat

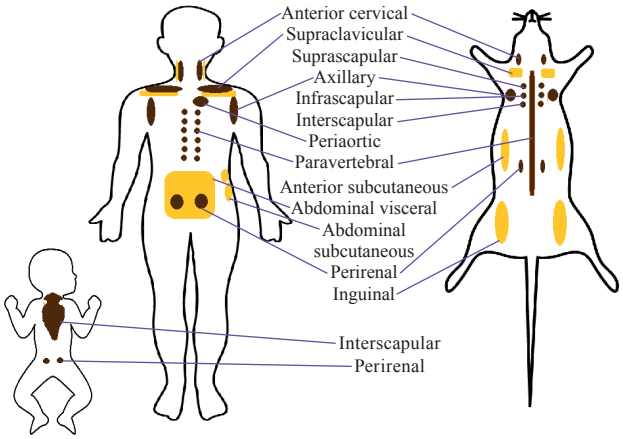

Figure 2 Localization of beige and brown adipose tissue in human and mouse [32, 52–55]

signature could be formed in supraclavicular, abdominal visceral and subcutaneous fat depots [32, 48, 52–57]. However, distribution of human BAT could be wider. Visceral BAT includes perivascular (aorta, common carotid artery, brachiocephalic artery, paracardiac mediastinal fat, epicardial coronary artery and cardiac veins, as well as internal mammary and intercostal artery and vein), periviscus (heart, trachea and major bronchi at lung hilum, esophagus, greater omentum, transverse mesocolon) and around solid organs (thoracic paravertebral, pancreas, kidney, adrenal, liver, hilum of spleen). Subcutaneous BAT includes depots lying between the anterior neck muscles and in the supraclavicular fossa, under the clavicles, in the axilla, in the anterior abdominal wall, and in the inguinal area [58]. Beige fat is could be also detected in cervical, parasternal, supraclavicular, para- and prevetebral areas [59].

In mouse BAT is located in anterior cervical, supraclavicular, axillary, interscapular, infrascapular, paravertebral and perirenal areas, while BeAT – in anterior subcutaneous WAT, supraclavicular WAT and inguinal WAT [32, 54, 56]. The main differences between human and mouse adipocytes are defined. Human BAT are dispersed and represented a mix of white, classical brown, and recruitable brite adipocytes, while murine the main BAT depots are in well-defined anatomic sites and homogeneously composed of brown adipocytes [60].

The ways of "browning" of white adipose tissue. Beige adipocytes were originally observed to arise in response to cold; however, studies have since identified that diet, exercise, pre- and probiotics, pharmaceutical agents, numerous plant-based bioactives, and even adipokines, can also induce "beigeing" or "browning" of WAT [61]. Thus, "beigeing" or "browning" of WAT could be caused by β-3 adrenergic receptor agonists, (CL 316243, BRL 26830A), short-chain fatty acids (acetate), dietary factors and organic compounds (capsaicin (and related capsinoids), plant-produced resveratrol, plant-derived berberine (BBR), fish oil, decaffeinated green tea extract, cinnamon extract, ginsenoside Rb1, curcumin, quercetin, ginger extract), nuclear receptors and ligands (farnesoid X receptor, liver X receptors), microRNAs (miRNA-32, miRNA-455), drug agents (Thiazolidinediones, Prostaglandin E2, Gleevec, Beta-lapachone, Slit 2 derived secretory product, Artepillin C, Adrenomedullin 2), inflammatory factors (IL-6, IL-4, IEX-1), hormonal factors (thyroid hormones, Glucagon-like peptide 1, leptin, melatonin, natriuretic peptides), genetic factors (PTEN, Cox2, Foxc2, folliculin, Gq, TGF-β/Smad3), batokines (FGF21, apelin), exercise, PPAR agonists (rosiglitazone,WY14643), bone morphogenetic proteins (BMP7, BMP4), metabolites (lactate, β-hydroxybutyrate, beta-aminoisobutyric acid (BAIBA), retinoic acid), bariatric surgery (physical reconstruction of the gastrointestinal tract) [10, 49].

Nevertheless, the most studied factor is cold exposure. Thus, cold is sensed by the skin and central signals result in increased noradrenaline release via sympathetic neurons and subsequent stimulation of various subtypes of β-adrenergic receptors (ADRBs, mainly subtype β3) and downstream cyclic adenosine monophosphate signaling, leading to the proliferation of brown adipocytes and activation of lipolysis and/or of thermogenesis [62–64]. However, cold-induced BAT from adult human neck area consists of classical brown adipocytes, as well as activated thermogenic fat in the supraclavicular region is composed of both classical brown and beige adipocytes [65].

Nutritional induction is also studied. WAT content is influenced by n-3 PUFA, polyphenols, vitamin D, vitamin E, vitamin A, carotenoids, BAT – by PUFA, especially n-3 PUFA, bile acids, BeAT – by amino acid restriction, capsaicin, bile acids, n-3 PUFA, retinoic acid [27]. Low protein diet results in activation of brown adipose tissue, as well as sucrose intake increase BAT activity. Some of the diet-derived small molecules increase BAT activity and browning of WAT, such as acetic, butyric and succinic acids, ketone bodies. Consumption of chilli peppers (capsaicin, non-pungent capsinoids), olive oil (oleic acid), green tea (catechins), raspberry (RB-ketone), grapes (resveratrol), fish (PUFAs) also stimulate BAT activation and browning [66].

Cannon and Nedergaard described the mechanism of transformation controlled by hypothalamus [67]. In the experiment on obese ob/ob mice consumed cafeteria diet (overfed) an activation of brown fat was observed – diet-induced thermogenesis (DIT) [33]. In general, macronutrient content of meals (carbohydrate, fat, protein amount and type) and dietary bioactive compounds (capsaicin and capsinoids; tea, caffeine and catechins; menthol; conjugated linoleic acid, casein protein, curcumin, garlic powder, procyanidin-rich extracts from black soybean seed, resveratrol and extracts from ginger family plants, etc.) could affect BAT and browning process [68]. Interestingly, that gut microbiota could contribute to upregulation of thermogenesis in the cold environment [69]. Zhang *et al.* reported that *Caulis spatholobi* can activate brown

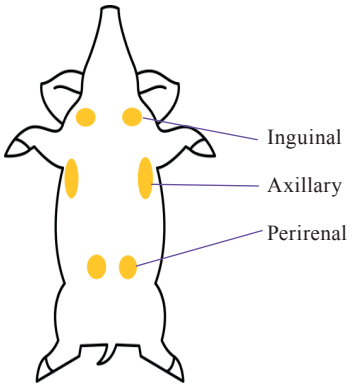

Figure 3 Anatomical locations of beige adipocytes in pigs [74]

adipose tissue and modulate the composition of gut microbiota, which is linked with normalization of thermogenesis during cooling [70].

Most of the research describe functions and role of WAT, BAT and BeAT in human body. Not many scientists deal with adipogenic features of white, beige, and brown adipose tissues in other mammalian, domestic farm animals in particular.

Adipose tissues types in pigs. Database search sciencedirect.com showed that according to the keywords "brown fat, white fat", the system issues 3475 publications for 2020, the number of publications has doubled in 10 years. 6557 scientific papers were published in 2020 for the keywords "brown fat, browning". When the keyword "pig" is added to these keywords, the number of publications is reduced to 356.

The analysis of these publications showed that the main scientific interest is directed on modification of fatty acid composition, but not on the study of the fat types and their distribution, although the directed modification of fatty acid profile is of considerable interest, taking into account correlation of the fatty acids amount with long chain activity of mitochondrial uncoupling protein 1 (UCP1), the activation of the mitochondria of brown fat and non-shivering thermogenesis.

Uncoupling protein 1 (UCP1), is a unique mitochondrial membranous protein devoted to adaptive thermogenesis, a specialized function performed by brown adipocytes [38]. The restricted interest to BAT and BeAT in pigs is explained that pigs (Suidae) have a predominantly tropical distribution and lost functional UCP1 in a genetic event that eliminated exons 3–5 ~ 20 million years ago [71–73]. They consequently have also been suggested to lack functional BAT, which is a major cause of neonatal death in the swine industry [71].

Despite these inconsistent findings, some pig breeds, such as the Tibetan pig found on the Qinghai-Tibetan plateau and the Min pig living in Northeast China, are well recognized to be cold resistant, and WAT browning was induced after cold exposure as well as UCP3 expression was significantly increased. Cold-resistant pig breeds (eight dominant pig breeds found across China) have evolved a novel mechanism involving UCP3 in beige adipocytes as the primary thermogenic mechanism, challenging the orthodoxy based on studies of mice that only UCP1 may act as a significant source of thermogenic heat [71]. Pigs do not have BAT, but beige adipocytes were found in inguinal subcutaneous WAT, axillary sWAT and perirenal fat from acute cold-stimulated cold-tolerant pig breeds in China, including Tibetan pigs and Min pigs (Fig. 3) [74]. Differentiated beige cells were also observed in subcutaneous fat of Tibetan pigs [71].

As beige adipocytes were observed at least in cold stimulated adipose tissues from cold-resistant pigs, UCP1-independent non-shivering thermoregulation might be justified with temperature maintenance in pigs or UCP3-dependent thermogenesis in beige adipocytes as a key evolutionary response in cold-adapted pig lineages [71, 74]. The studies in this area are important, especially concerning neonatal death in the swine industry and expanding the geography of pig farming.

CONCLUSION

White, brown, and beige/brite adipose tissues are considered mainly from the point of view of human health, paying special attention to their role in obesity and type II diabetes. Mechanisms and tools of white adipose tissue browning are intensively studied, as well as brown and beige/brite adipose tissues localization and features in different species. The phenotypic and genotypic study of various breeds of pigs in different conditions of housing, taking into account climatic zones, will help reveal the main qualitative characteristics of fat.

The new knowledge about beige adipose tissue with some similarity to brown, which is characteristic of the neonatal period and almost disappears in the adult body and has a thermogenic function, opens up new opportunities for the formation of qualitatively new characteristics of pig adipose tissue.

Using the knowledge about the influence of a number of endo- and exogenous factors on the formation of adipose tissue (white, beige, brown), it will be possible to control the molecular mechanisms of adipocyte differentiation to obtain not only high-quality pork fat, but also meat products, as well as to expand the geography of pig breeding.

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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Optimizing enzymatic hydrolysis for feed production from catfish by-products

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Abstract:
Introduction. The fisheries industry generates large amounts of fish by-products. Their utilization is one of the relative tasks for fish manufacturers. Hydrolysate from fish by-products is regarded as a valuable bioactive protein source for feed production. In this study, we aimed to optimize hydrolysis conditions for the industrial by-products of catfish.
Study objects and methods. We studied the by-products of industrially processed *Pangasius hypophthalmus* L. fillet using biochemical methods to find the optimum hydrolysis conditions (enzyme type, enzyme/substrate ratio, temperature, water amount, and time). Then we built a regression model and verified it experimentally.
Results and discussion. According to the Box-Behnken design model, the optimum hydrolysis conditions were determined as 10% of water, 0.48% of SEB-Neutral PL enzyme, 57°C temperature, and 6 h duration. We found no significant differences between the modelled and the verified experimental values. The resulting hydrolysate was rich in nitrogen from amino acids, and its other parameters complied with the current national standards. The microbal and sensory attribites satisfied quality requirements as an animal feed supplement.
Conclusion. The study results are commercially applicable in feed production, providing a solution for the fisheries industry in by-product treatment.

Keywords: Fish, protein, hydrolysate, *Pangasius hypophthalmus*, catfish, by-products, enzyme

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INTRODUCTION

Tra catfish (*Pangasius hypophthalmus* L.) accounts for an essential part of Mekong Delta's and Vietnamese fisheries' yield. In 2019, their production area totaled 6600 ha yielding 1.42 million tons, of which 60–70% were by-products [1–3]. The production expansion in order to meet the domestic and export demand has caused a growing concern about the fisheries' by-product treatment, especially from *Pangasius* processing. The Mekong Delta, where *Pangasius* production and processing are more developed, needs economical and environmentally friendly solutions for large amounts of by-products generated by the local manufacturing facilities [4]. Recent years have seen an interest in the utilization of *Pangasius* by-products to manufacture value-added products, such as fish powder, fish skin, and viscera.

Fish powder is the most popular by-product used as a primary protein supplement for animal feeds. Fish by-products are a major source of lipids, native proteins, and hydrolysates, accounting for 10–20% of the total fish protein [5, 6]. Presently, most fish powder manufacturers use the traditional procedure with high pressure and temperature, resulting in products with a low digestive and absorptive index.

Figure 1 *Pangasius* by-products used as study objects

Enzymatic protein hydrolysis of fish by-products is currently a promising approach to making products with various applications and high nutritional values [5, 7–10]. Proteases are among common enzymes used in this process [6]. They hydrolyze protein in fish by-products to smaller peptides that usually contain 2–20 amino acids. Fish hydrolysate is a liquid of amino acids – peptides produced by speeding up fish protein hydrolysis using proteases under controlled conditions. When applied in animal feed products, fish hydrolysate improves the digestion and absorption of proteins, as well as feed intake, efficiency, and protein utilization [11–14]. Furthermore, protein hydrolysate enhances the attractiveness and palatability of the feed, thus increasing its consumption [15].

There have been several studies in Vietnam on different aspects of enzymes for hydrolysis of catfish by-products. Nguyen Cong Ha *et al*. found that possible substrate concentrations for an optimal enzyme to substrate ratio were 108.4 g/L for neutrase, 36.2 g/L for papain, and 135.8 g/L for bromelain [16]. Nguyen Thi Thuy *et al*. investigated *Pangasius* by-product hydrolysis using commercialized papain as a protein source for animal feeds [17]. Dang Minh Hien *et al*. also studied papain for application in *Bacillus subtilis* cultivation with satisfactory results [18]. Phan Viet Nam *et al*. hydrolyzed catfish by-product using a combination of enzymatic hydrolysis and thermal treatment, having achieved over 30% hydrolysis, 80% nitrogen recovery, and a large amount of essential amino acids [3]. The researchers showed the potential of catfish by-product treatment with protease enzymes and possible application of hydrolysates in feed production. Therefore, an investigation of optimal hydrolysis conditions is essential for further commercial application.

This study assessed optimal conditions for *Pangasius* hydrolysis, such as the type of enzyme, enzyme/substrate ratio, temperature, time, and the amount of water added by analyzing total nitrogen and amino acids. As a result, we identified the initial conditions to produce hydrolysates from *Pangasius* by-products for the animal feed industry.

STUDY OBJECTS AND METHODS

Study objects. This study featured the by-products of industrially processed *Pangasius* catfish (*Pangasius hypophthalmus*) fillet.

Materials. *Pangasius* by-products, including heads, bones, and fins (Fig. 1), were collected from the processing factory of the Travel Investment and Seafood Development Corporation (Trisedco, Vietnam). They were minced into small pieces of 3–5 mm and stored at –20°C until use.

The enzymes were obtained from ICFood Vietnam, including bromelain (active pH 5.5–7.0, 55–60°C, 500 IU/g), papain (pH 4.5–8.5, 60–70°C, 500 IU/g), protease (pH 4.5–8.5, 50–60°C, 500 IU/g), and SEB-Neutral PL (pH 5.5–7.5, 35–60°C, 750 IU/g). They are commercial enzymes commonly used in animal feed production.

Optimal hydrolysis conditions. The materials were defrosted, mixed with 20% water, and heated to 55°C before adding enzymes. Hydrolysis was performed in a pilot-scale hydrolysis equipment with a capacity of 80 kg/batch. It was terminated by heating to 85–90°C in 10 min.

After hydrolysis reactions, the mixtures were filtered and analyzed for total nitrogen (N_{aa}). The optimal hydrolysis conditions were obtained by optimizing a single condition at a time in consecutive order. The resulting condition in the previous experiments was used as a constant condition in the later experiments. The protein recovery efficiency was evaluated using the ratio of nitrogen amino acid (N_{aa}) to total nitrogen (N_t) in the hydrolysates.

Evaluating the chemical composition of the materials. We analyzed such parameters as crude protein, moisture content, lipid, ash, TVB-N, and total aerobic microorganisms.

Choosing the enzyme. We assessed four enzymes, namely protease, bromelain, papain, and SEB-Neutral PL. Hydrolysis reactions were performed with the enzyme/substrate (E/S) ratio of 0.3%, temperature of 55°C, and 20% water added in 5 h. The hydrolyzed products were then analyzed for total protein and N_{aa} to choose the most effective enzyme. The chosen enzyme was used in the later experiments.

Table 1 Chemical and microbiological parameters of the materials

Parameters	Results
Crude protein, %	12.76 ± 0.48
Moisture, %	59.27 ± 0.33
Lipids, %	21.19 ± 0.32
Ash, %	7.02 ± 0.24
TVB-N, mg/100 g	13.45 ± 0.37
pH	6.41 ± 0.17
Nitrogen from amino acid, %	0.07 ± 0.0028
Total aerobic microorganism, $\times 10^5$ CFU/g	1.13 ± 0.18

Choosing the optimal enzyme/substrate ratio. Seven enzyme/substrate ratios, namely 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6%, were analyzed during hydrolysis. The hydrolysis reactions were performed at a temperature of 55°C, with 20% of water added in 5 h. The chosen enzyme/substrate ratio was then used in the later experiments.

Choosing the optimal temperature. Five temperatures (45, 50, 55, 60, and 65°C) were assessed. The hydrolysis was performed with the optimal enzyme and enzyme/substrate ratio from the previous experiments and with 20% water added in 5 h.

Choosing the optimal amount of added water. Water in amounts of 0, 5, 10, 15, 20, 25, and 30% was used for hydrolysis under the optimal conditions chosen from the previous experiments in 5 h. The products were then analyzed to choose the optimal ratio of water required for hydrolysis.

Choosing the optimal hydrolysis time. Hydrolyses with the optimal conditions from the previous experiments were performed in five different periods, from 3 to 7 h. The products were then analyzed to choose the optimal reaction time.

Optimizing the hydrolysis procedure. A Box-Behnken design was used to investigate the effect of three factors: A – hydrolysis time, B – enzyme/substrate ratio, and C – temperature on the N_{aa}/N_t ratio [19]. An optimal scenario was obtained, and a verification experiment with the optimum conditions was performed in the lab.

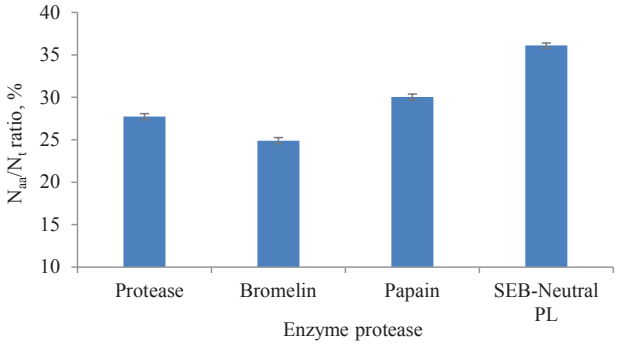


Figure 2 Proteases effect on N_{aa}/N_t ratio

Analysis methods. The following Vietnamese standards (abbreviated TCVN) were used to analyze the chemical quality parameters in this study: TCVN 3705:1990 for crude protein; TCVN 3708:1990 for amino acid nitrogen; TCVN 3700:1990 for water amount; TCVN 9215:2012 for vaporized acid-base; TCVN 3706:1990 for ammonia nitrogen; and TCVN 5165:1990/ TCVN 4884:2005 for total aerobic microorganisms.

Data analysis. Each experiment was done in triplicate, each time with three samples, and the results were averaged. The data were processed and charted in MS Excel 2007 and model analysis was performed in Design Expert (version 10).

RESULTS AND DISCUSSION

Materials' quality. The quality of the by-products used for hydrolysate production was evaluated through chemical and microbiological parameters, as shown in Table 1.

The materials' crude protein and moisture contents were 12.76 and 59.27%, respectively, similar to other research in Vietnam [20]. TVB-N, a freshness quality indicator, was 13.45 mg/100 g, which was much better than the best quality limit of 25 mg/100 g. Although the total microorganism count was 1.13×10^5, a large number relating to long storage before use, the TVB-N result indicated that the materials were still fresh and did not contain any spoilage compounds that could adversely affect the quality of hydrolysates [21].

Optimal hydrolysis conditions. ***Choosing the hydrolysis enzyme.*** The N_{aa}/N_t ratios obtained from the hydrolysis with four enzymes under study are shown in Fig. 2.

Under the same hydrolysis conditions, SEB-neutral PL resulted in the highest N_{aa} amount (36.12 ± 0.31%), equivalent to 1.4 times from bromelain hydrolysis and 1.3 times from protease or papain. The SEB-neutral PL enzyme was chosen to be used in the later experiments in this study.

Choosing the enzyme/substrate ratio. The effect of the enzyme/substrate ratios on the N_{aa}/N_t ratio is presented in Fig. 3.

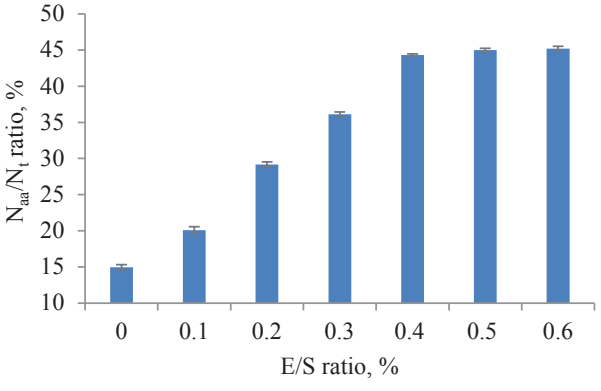


Figure 3 Enzyme/substrate ratio vs. N_{aa}/N_t ratio

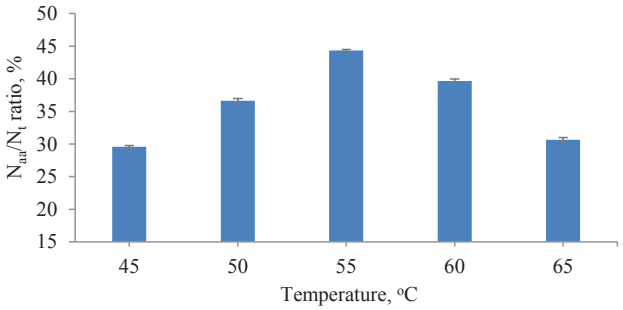


Figure 4 Hydrolysis temperature vs. N_{aa}/N_t ratio

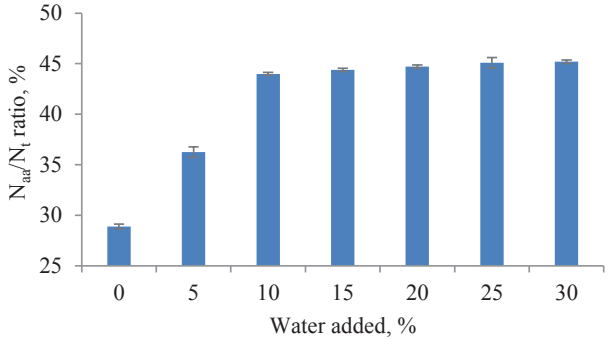


Figure 5 Effect of water amount on N_{aa}/N_t ratio

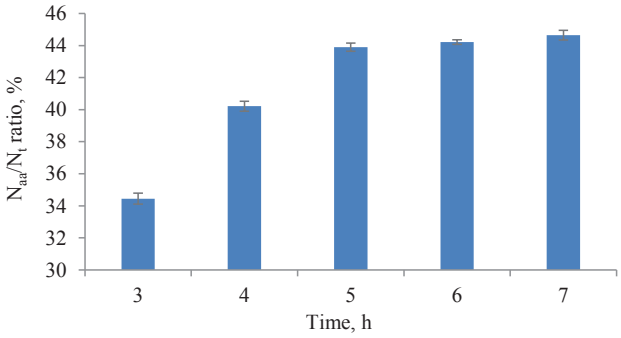


Figure 6 Effect of hydrolysis time on N_{aa}/N_t ratio

The lowest N_{aa}/N_t ratio demonstrated the reaction without enzyme (14.96 ± 0.38%), and the highest was from 0.6% enzyme added (45.22 ± 0.32%), though the ratios of 0.4 and 0.5% were not much different from 0.6%. The enzyme/substrate range of 0.3–0.5% was chosen for further analysis, and the enzyme/substrate ratio of 0.4% was chosen for the later experiments.

Choosing the hydrolysis temperature. Figure 4 shows the effect of hydrolysis temperatures on N_{aa}/N_t ratios.

The results showed that the higher the temperature, the better the hydrolysis performance till it reached optimum at 55°C. The effective ratio went down as the temperature increased beyond that. Thus, 55°C was chosen for use in later experiments, and the range of 55–60°C was used in the optimal analysis.

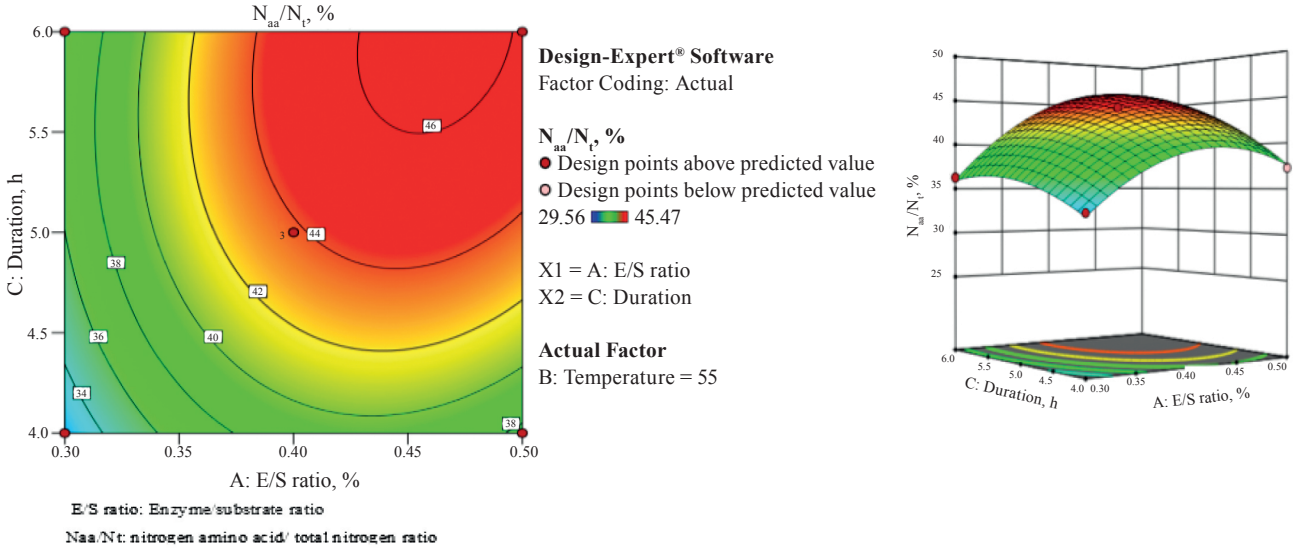
Choosing the amount of added water. The amount of added water influences the enzyme's dispersal and contact with substrates. As we can see in Fig. 5, hydrolysis performance increased steeply when the water amount went up from 0 to 10%. Then it slowed down considerably with water increasing from 10 onward to 30%, suggesting an equivalent efficiency. To economically use other substances in the hydrolysis reaction, we chose 10% added water to use in later experiments.

Choosing the hydrolysis time. The samples were hydrolyzed in 3, 4, 5, 6, and 7 h under the conditions from the previous experiments (0.4% SEB-neutral PL, 55°C, and 10% added water). The hydrolysis performance increased sharply from 34.44 ± 0.34% (3 h) to 43.90 ± 0.26% (5 h), then slowing down and reaching highest at 44.65 ± 0.30% (7 h). The difference between

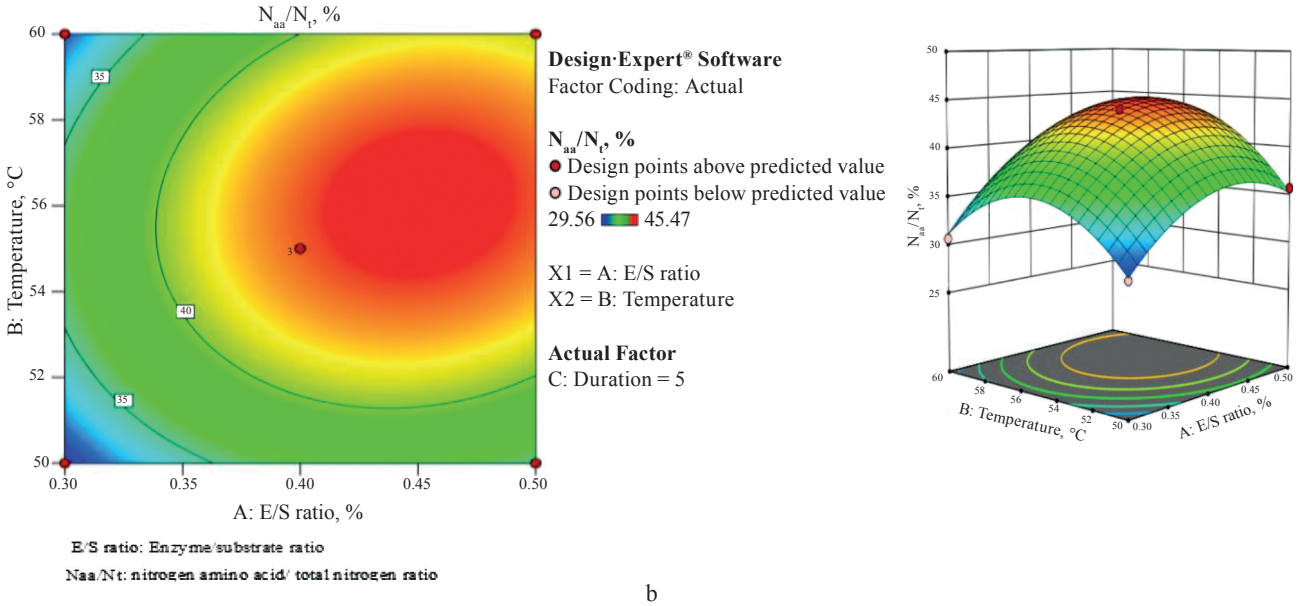
Table 2 ANOVA results

Factors	Sum of square	Degrees of freedom	Mean square	*F*-ratio	*P*-value ($P < 0.05$)	
Model	390.41	9	43.38	155.85	< 0.0001	significant
A	116.13	1	116.13	417.24	< 0.0001	
B	24.12	1	24.12	86.65	0.0002	
C	70.27	1	70.27	252.47	< 0.0001	
AB	4.82	1	4.82	17.31	0.0088	
AC	5.45	1	5.45	19.59	0.0069	
BC	4.67	1	4.67	16.76	0.0094	
A^2	58.95	1	58.95	211.81	< 0.0001	
B^2	112.44	1	112.44	403.98	< 0.0001	
C^2	12.68	1	12.68	45.57	0.0011	
Residual	1.39	5	0.2783			
Lack of fit	1.18	3	0.3934	3.72	0.2190	insignificant
Standard deviation	0.2115	2	0.1057			
R^2: 0.9964			Expected R^2: 0.9506			

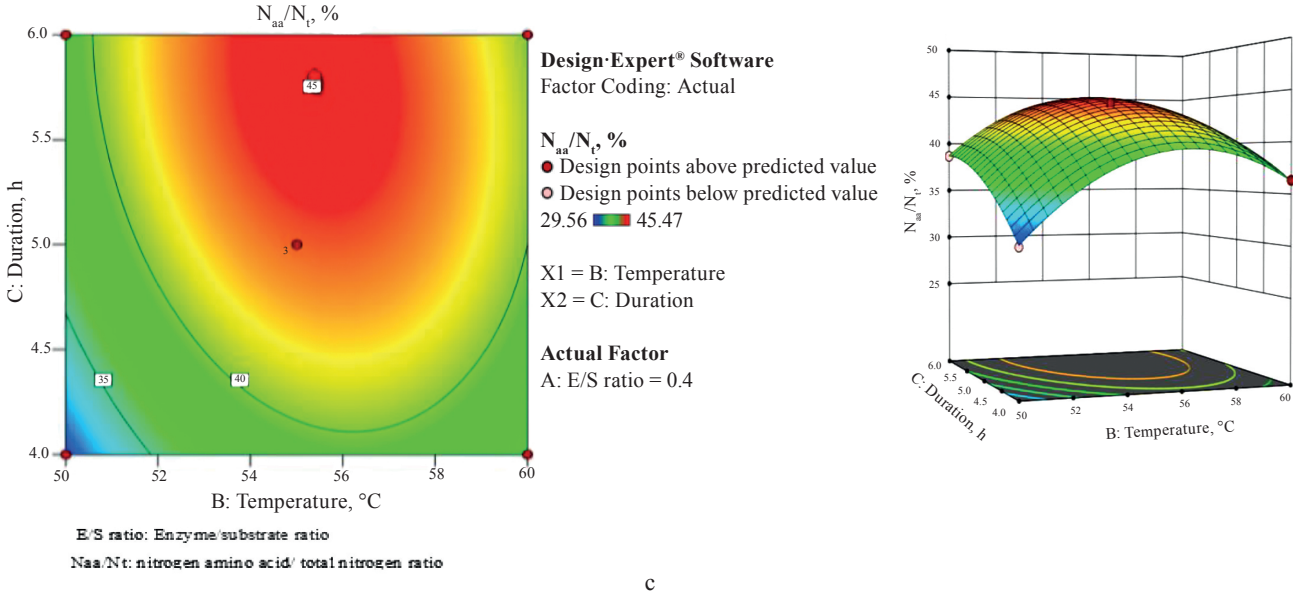
A – enzyme/substrate ratio, B – temperature, and C – hydrolysis duration



a



b



c

Figure 7 Factors relative influences on the dependent variable in the statistical model: (a) Influences of enzyme/substrate ratio and hydrolysis duration; (b) Influences of temperature and enzyme/substrate ratio; (c) Influences temperature and hydrolysis duration

Table 3 Predicted vs. experimental results

Value	Enzyme/ substrate ratio, %	Tempera-ture, °C	Duration, h	N_{aa}/N_t ratio, %
Predicted	0.48	56.52	5.77	46.25
Experimental	0.48	57.00	6.00	46.08 ± 0.31

the samples hydrolyzed in 6 and 7 h was not statistically significant ($P > 0.05$). Although nitrogen solubility increases with time in enzyme hydrolysis, after a certain period, the formation of hydrolysis products and the reduction of peptide bonds during hydrolysis inhibit enzyme activities and decrease hydrolysis rate [22, 23]. Therefore, the effective range of duration was determined to be from 4 to 6 h.

Optimizing Pangasius by-product hydrolysis with Seb-Neutral PL enzyme. We studied the influence of different variables on the outcome of hydrolysis by performing Box-Behnken Designs in Design-Expert. In particular, we used the following factors: A – enzyme/substrate ratio (0.3–0.4–0.5%); B – temperature (50–55–60°C); and C – hydrolysis duration (4–5–6 h).

The ANOVA results are presented in Table 2.

The model was significant with the *F*-ratio of 155.85 ($P < 0.0001$) and the lack of fit of 3.72 ($P = 0.2190$). The variables AB, AC, BC, A^2, B^2, and C^2 all had $P < 0.05$ and therefore were significant and included in the regression formula. The model to determine the relationship between the dependent variable N_{aa}/N_t and enzyme/substrate ratio, temperature, and duration, as well as their interactions, was presented in the following formula:

$$y = a + b_1 \cdot A + b_2 \cdot B + b_3 \cdot C + b_{12} \cdot AB + b_{13} \cdot AC + \\ + b_{23} \cdot BC + b_1^2 \cdot A^2 + b_2^2 \cdot B^2 + b_3^2 \cdot C^2$$

$$N_{aa}/N_{ts} \text{ ratio} = 43.79 + 3.81 \cdot A + 1.74 \cdot B + 2.96 \cdot C + \\ + 1.10 \cdot AB + 1.17 \cdot AC - 1.08 \cdot BC - 4.00 \cdot A^2 - \\ - 5.52 \cdot B^2 - 1.85 \cdot C^2 \quad (1)$$

In formula (1), b_1, b_2, and b_3 were positive, showing that the hydrolysis performance (N_{aa}/N_t) was directly proportional to the analyzed factors: enzyme/substrate ratio, temperature, and duration. $|b_1| < |b_3| < |b_2|$ suggested that the enzyme/substrate ratio had a more substantial influence on the performance of the hydrolysis reaction than the other factors. The coefficients b_1^2, b_2^2, and b_3^2 were negative, suggesting that the graphs were parabolic faces with concave surfaces facing downwards and having extreme points. The coefficients b_{12} and b_{13} were positive, showing a positive interaction between temperature and time, with the enzyme/substrate ratio increasing hydrolysis performance. At the same time, b_{23} was negative, indicating that the interaction between temperature and time was inversely proportional to the N_{aa}/N_t ratio. This could be explained by the nature of enzyme reactions, where high temperature and prolonged duration might cause unstable enzymes and reduce enzyme hydrolysis activities. The effects of these factors on the N_{aa}/N_t ratio were graphically presented in Fig. 7.

Based on the Box–Behnken design model, the optimum hydrolysis conditions (enzyme/substrate ratio = 0.48%, temperature = 56.52°C, and duration = 5.77 h) was chosen to conduct a laboratory experiment. The results of the laboratory experiment in comparison with the predicted values are shown in Table 3.

There were no significant differences between the predicted and experimental values. Therefore, we proposed the following optimum hydrolysis conditions for *Pangasius* by-products: 10% added water, 0.48% SEB-Neutral PL enzyme, 57°C, and 6 h.

Table 4 Quality parameters of the resulting hydrolysate

Parameters	Results
Total nitrogen, g/L	26.94 ± 0.16
Nitrogen from amino acid, g/l	12.41 ± 0.08
N_{aa}/N_t ratio, %	46.08 ± 0.31
TVB-N, mg/100 g	42.73 ± 0.63
Lipid, %	2.6 ± 0.02
Escherichia coli	Not detected in 1 g
Salmonella	Not detected in 25 g
Sensory	
Color: deep brown	
Smell: aroma characteristic of fish protein hydrolysates, no off-aroma.	

Product quality. The results of the hydrolysate quality evaluation are presented in Table 4.

The resulting *Pangasius* hydrolysate was a high-quality source of nutrition with 12.41 g/L nitrogen from amino acids, accounting for 46.08% of total nitrogen (26.94 g/L). Its other parameters fully complied with the current national standard QCVN 01-190:2020/BNNPTNT, making it suitable for use as a supplement to animal feed to improve its amino acid content and aroma.

CONCLUSION

We investigated the initial hydrolysis conditions to produce hydrolysates from *Pangasius hypophthalmus* by-products. The regression model prediction and laboratory verification determined the following optimum hydrolysis conditions: 10% water amount, 0.48% SEB-Neutral PL enzyme, 57°C temperature, and 6 h hydrolysis time. The hydrolysates yielded from the proposed hydrolysis procedure satisfied the quality requirements for animal feed supplements and complied with the national standard QCVN 01-190: 2020/BNNPTNT. Thus, our study results were commercially applicable for feed production and provided a solution for by-product treatment in the fisheries.

CONTRIBUTION

B.T.T. Hien designed the study concept. P.T. Diem developed the methodology. L.A. Tung, T.T. Huong, and N.H. Hoang performed the validation experiment. N.H. Hoang and N.K. Bat conducted formal analysis. N.V. Nghia and B.T.T. Hien drafted, reviewed, and edited the manuscript. All the authors were involved in the investigation, as well as read and agreed to the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Structure and properties of antimicrobial peptides produced by antagonist microorganisms isolated from Siberian natural objects

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Abstract:

Introduction. Public healthcare urgently needs new pharmaceuticals – alternative to traditional antibiotics – that pathogens develop no resistance to. Of special interest in this regard are antimicrobial, ribosomally synthesized bacterial peptides or bacteriocins. In this work, we aimed to study the structure and properties of antimicrobial peptides produced by antagonist microorganisms isolated from the natural objects of the Siberian region.

Study objects and methods. The study objects were bacteria isolated from the natural sources of Kuzbass. After culturing bacteria, total protein was precipitated from the culture fluid and separated into fractions by gel permeation HPLC. Their amino acid sequences were determined by MALDI-TOF mass spectrometry. The antibacterial (against *Bacillus pumilus* and *Escherichia coli*) and fungicidal (against *Aspergillus flavus* and *Aspergillus niger*) properties of the peptides were studied by the disk diffusion method.

Results and discussion. Seven peptides with different amino acid sequences were isolated from the culture fluid of bacteria, five of which had no analogues in the PepBank and Uniprot data banks. The peptide with an amino acid sequence of VMCLARKCSQGLIVKAPLM (2061.66 Da) was homologous to the cysteine membrane protein *Giardia lamblia* P15, and the peptide with an amino acid sequence of AVPSMKLCIQWSPVRASPCVMLGI (2587.21 Da) showed a homology with the *Planctomycetes bacterium* I41 peptides. We found antibacterial (against gram-positive and gram-negative bacteria) and fungicidal (against *Aspergillus*) properties in the peptide fractions.

Conclusion. Antimicrobial peptides produced by bacteria isolated from the natural objects of the Siberian region can be used to create pharmaceuticals as an alternative to traditional antibiotics to treat infectious diseases.

Keywords: Antimicrobial peptide, bacteriocin, fungicide, antagonistic properties, antibiotic resistance, amino acid sequence, mass spectrometry, bacteria

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INTRODUCTION

Pathogenic microorganisms resistant to traditional antibiotics are a serious problem of modern healthcare. There is evidence that over 70% of all pathogenic bacteria are resistant to at least one of the most commonly used antibiotics. Therefore, there is an urgent need for new drugs and therapeutic approaches to overcome their resistance [1–5].

Antimicrobial peptides produced by various organisms from bacteria to mammals are an ideal alternative to antibiotics due to their antimicrobial, anti-inflammatory, angiogenic, and immunomodulatory properties, as well as low bacterial resistance [6]. However, their use is limited by toxicity and stability *in vivo* [7].

Antimicrobial peptides act against various types of pathogens, including Gram-positive and Gram-negative

Table 1 Study objects

Microorganism	Reference	Source of isolation
Bacillus subtilis	Bs-1	Soil (Peshcherka village, Kemerovo district)
Lactobacillus plantarum	Lp-7	Rhizosphere of plants (Voznesenka village, Yaya district)
Leuconostoc mesenteroides	Lm-8	
Pediococcus acidilactici	Pa-9	Rhizosphere of plants (Ursk village, Guryevsk district)
Pediococcus pentosaceus	Pp-11	Plant waste at Sukhovsky farm (Kemerovo city)
Lactobacillus casei	Lc-12	
Lactobacillus fermentum	Lf-13	Plant waste at Niva farm (Gorskino village, Guryevsk district)
Pediococcus damnosus	Pd-16	Plant waste at Veles farm (Yaya village, Yaya district)
Geobacillus stearothermophilus	Bs-19	Bottom sediments of the Kara-Chumysh reservoir (Prokopyevsk district)
Bacillus caldotenax	Bc-20	Bottom sediments of Lake Udai (Mariinsk district)

bacteria, viruses, and microscopic fungi, through the destruction of the cytoplasmic membrane, intracellular penetration, and immunomodulation [8, 9]. Structurally, antimicrobial peptides are classified into linear cationic amphipathic peptides and macrocyclic peptides [10]. As a rule, antimicrobial peptides are short peptides consisting of 10–50 amino acids [11, 12]. They have common features despite differing in length, amino acid sequences, and conformation [13]. Typical antimicrobial peptides are composed of positively charged residues such as arginine, lysine, and histidine [14]. Cationic peptides with a positive charge ranging from +2 to +11 can interact with the membranes of microbial cells. Besides, a significant part of antimicrobial peptides is hydrophobic, contributing to the formation of amphipathic secondary or quaternary structures [15].

Antimicrobial peptides have several advantages over traditional antibiotics [16]. First of all, they have a broad spectrum of antimicrobial activity, against even multidrug-resistant pathogens [8, 16]. Secondly, antimicrobial peptides are highly active against gram-negative bacteria, which are more serious targets than gram-positive bacteria [17]. Another advantage is a rather low likelihood of drug resistance.

Bacteriocins are antimicrobial, ribosomally synthesized peptides of bacteria with a low molecular weight [18]. Mostly studied are bacteriocins produced by lactobacilli. They can be roughly divided into four categories: lantibiotics (e.g., nisin); non-antibiotic bacteriocins with good activity against *Listeria monocytogenes*, as well as pediocins, which make up the largest group; thermosensitive macromolecular proteinaceous bacteriocins; and complex bacteriocins with carbohydrates, lipids, and proteins [19–23]. Of all well-studied bacteriocins of lactobacilli, only nisin is produced commercially [24].

Potential sources of bacteria producing bacteriocins are dairy products, cow rumen, feed, as well as natural objects such as soils, plant waste, rhizosphere of plants, bottom sediments of water bodies, etc. [18, 25, 26].

In our previous studies, we isolated 19 microorganisms from the natural sources of Kemerovo Region (Siberian Federal District, Russia), including 10 species of bacteria (*Geobacillus*, *Bacillus*, *Lactobacillus*, *Leuconostoc*, and *Pediococcus*) that showed high antimicrobial activity against *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus mycoides*, *Candida albican*s, and *Penicíllium citrinum* [27–29].

In this study, we aimed to examine the structure and properties of antimicrobial peptides produced by antagonist microorganisms isolated from the natural objects in Siberia.

STUDY OBJECTS AND METHODS

Our study objects were bacteria isolated from the natural sources of Kuzbass (Table 1).

Microorganism cultures. To obtain enrichment cultures of microorganisms, we crushed the samples of soil, bottom sediments, and plant waste under sterile conditions and rubbed their small amounts on Petri dishes with nutrient agar. The Petri dishes were incubated for three days at 26°C. Two nutrient media were used: lactobacilli were cultured on MRS agar; *Bacillus* and *Geobacillus* bacteria were cultured on a medium (pH 7.4 ± 0.2) containing 10.0 g/L casein hydrolysate, 2.5 g/L yeast extract, 5.0 g/L glucose, 2.5 g/L potassium hydrogen phosphate, and 12.0 g/L bacteriological agar.

Pure cultures of microorganisms were obtained from enrichment cultures by streaking. Microorganisms were cultivated on the media described above for 24 h: *Lactobacillus*, *Leuconostoc* and *Pediococcus* bacteria at 37°C, and *Bacillus* and *Geobacillus* at 30°C.

At the end of cultivation, cell debris was removed from all suspension cultures. The cultures were centrifuged at 3900 rpm in plastic flasks. The resulting supernatant was dried in a Labcocnco Triad freeze dryer (Labcocnco, USA) at a freezer temperature of –80°C, supernatant temperature of –20°C, and 0.05 mbar vacuum.

Protein fractions. To separate protein into individual fractions, the dried biomass was dissolved in 1 mL of 0.25 M phosphate buffer and the total protein was precipitated by adding 2 mL of concentrated

ammonium sulfate solution. The resulting protein suspension was separated by centrifugation at 8000 rpm. The protein precipitate was dissolved in 1 mL of 0.025 M Tris buffer solution (pH 4.5). The precipitate was applied to an Enrich 650 10 mm × 300 mm column (Biorad, USA) for a gel permeation high performance liquid chromatography (HPLC) at 280 nm using a direct injection system. Fractionation was performed using an NGC fraction collector (Biorad, USA).

Additionally, each protein fraction was purified on hydrophobic Amberlite XAD X-6 resins by chromatography. A glass column was filled with 10 g of Amberlite XAD-2 resin equilibrated with 10 mL of 20 mM trifluoroacetic acid solution. A protein solution in an acetate buffer was applied to the column and eluted in a methanol gradient from 0 to 15%, with a gradient rise of 5% for every 10 fractions. Fractions containing proteins were determined by taking 50 µL of each fraction and mixing it with a solution of Bradford's reagent in a 1:1 ratio. The resulting solution was measured on a Biorad SmartSpec Plus Spectrophotometer (USA). Fractions with an optical absorption of 0.06 or more were selected for further drying and identifying the amino acid sequence by the MALDI-TOF method using a MALDI TOF/TOF BRUKER Autoflex Speed mass spectrometer (Bruker Corporation, USA)

Trypsinolysis. Peptides were precipitated by adding an equal volume of methanol/chloroform mixture to an aliquot of a 200 µL fraction. The resulting precipitate was separated by centrifugation at 4000 rpm. The precipitate was dissolved in 100 µL of 6 M urea solution, to which 5 µL of dithiothreitol (DTT) solution was added to keep for 60 min at room temperature. Then, we added 20 µL of iodoacetamide solution and kept the mixture for 60 min at room temperature. After that, we added 20 µL of a DTT solution and kept the mixture again for 60 min at room temperature. After adding 775 µL of MiliQ H_2O and 50 µL of trypsin solution, the mixture was stirred by pipetting and kept in a thermostat at 37°C for 12 h. The enzyme was inactivated by adding 10 µL of trifluoroacetic acid. The peptides were purified by chromatography on C18 cartridges. The reaction mixture was applied to a cartridge and eluted with a solution of 0.1% trifluoroacetic acid in a 1:1 H_2O/acetonitrile mixture. Analysis and Top-Dawn sequencing were performed on 1 µL of a purified peptide solution.

Table 2 Peptides from the biomass of bacteria isolated from natural sources of Kuzbass

Microorganism	Isolated fractions	Microorganism	Isolated fractions
Bs-1	Bs-1_1	Lc-12	Lc-12_1
Lp-7	Lp-7_1	Lf-13	Lf-13_1 Lf-13_2 Lf-13_3
Lm-8	Lm-8_1 Lm-8_1	Pd-16	Pd-16_1 Pd-16_2 Pd-16_3 Pd-16_4
Pa-9	Pa-9_1 Pa-9_2	Bs-19	Bs-19_1 Bs-19_2
Pp-11	Pp-11_1 Pp-11_2 Pp-11_3 Pp-11_4 Pp-11_5 Pp-11_6 Pp-11_7 Pp-11_8	Bc-20	Bc-20_1

The antibacterial properties of the peptides against *Bacillus pumilus* and *Escherichia coli* were measured by the disk diffusion method. For this, we used suspensions of night cultures grown on a standard liquid nutrient LB medium with a titer of 0.5. The number of microorganisms (titer) in the suspension was determined by optical density at 595 nm. 200 µL of the pathogen culture was dropped onto a 90 mm Petri dish, rubbed with a sterile spatula by the spread plate method, and left to dry for 20 min under a laminar with the lid ajar. Then, 0.5 cm sterile filter disks soaked in the peptide solutions under study and dried at room temperature for 10 min were placed on the Petri dishes in the radial direction. The Petri dishes were left for 30 min at room temperature and then incubated in a thermostat at 37°C for 12 h. Then, we identified a bacterial inhibition zone around the disc and measured its diameter with a vernier caliper. Ampicillin at a concentration of 5 mg/mL was used as a positive control, and a disc soaked in a liquid medium was used as a negative control.

The fungicidal activity of the peptides against the microscopic fungi *Aspergillus flavus* and *Aspergillus niger* was measured by the disk diffusion method. The fungi were cultivated for 7 days, with an inoculation density of 6×10^7 conidia per 1 mL of medium. The results were analyzed with time intervals (3, 9, 12, 24, 48, 72 h, etc.) and by the fungus growth phase (stationary, accelerated growth, logarithmic), i.e., during the periods of exponential cell growth, decreased growth, and death or autolysis. At the end of the incubation, the inhibition zone around the disc was measured with a vernier caliper (mm), which indicated the degree of biocidal activity or its absence. A negative control was the samples with filters impregnated with the medium, and a positive control was the pharmaceutical preparation Irunin® (Veropharm, Russia) with itraconazole as an active ingredient.

Statistical data were analyzed in Microsoft Office Excel 2007. All the experiments were carried out in triplicate. Statistical analysis was performed using a one-sample Student's t-test. The differences were considered statistically significant at $P < 0.05$.

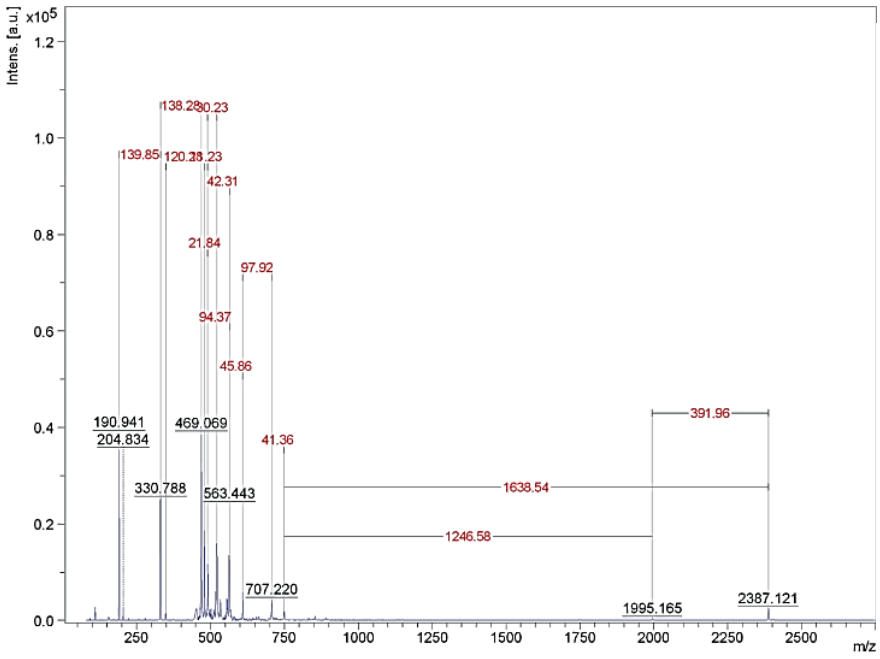


Figure 1 Mass spectrum of Bs-1_1 fraction

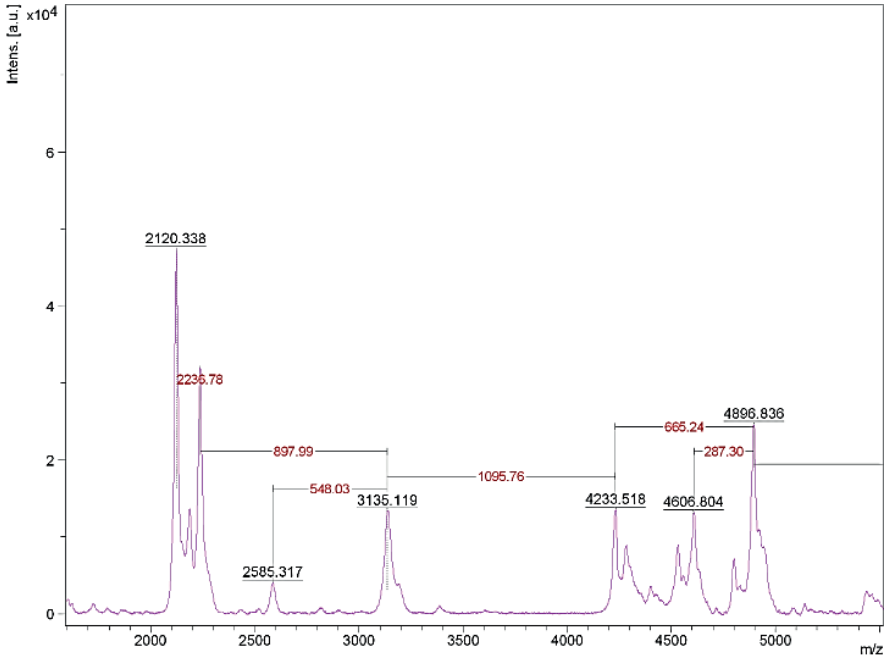


Figure 2 Mass spectrum of Bc-20_1 fraction (Lf-13_1, Lf-13_2, Lf-13_3)

RESULTS AND DISCUSSION

Several protein fractions were isolated from the culture fluid of all the studied samples (Table 2).

According to Table 2, one protein fraction was isolated from the culture fluid of *Bacillus subtilis*, *Lactobacillus plantarum*, *Lactobacillus casei*, and *Bacillus caldotenax*; two protein fractions from *Leuconostoc mesenteroides*, *Pediococcus acidilactici*, and *Geobacillus stearothermophilus*; three protein fractions from *Lactobacillus fermentum*; four protein fractions from *Pediococcus damnosus*; and eight protein fractions from the *Pediococcus pentosaceus* culture fluid.

The results of the MALDI TOF mass spectrometry of protein fractions are presented in Figs. 1–7. We found some identical mass spectra of protein fractions synthesized by different bacteria.

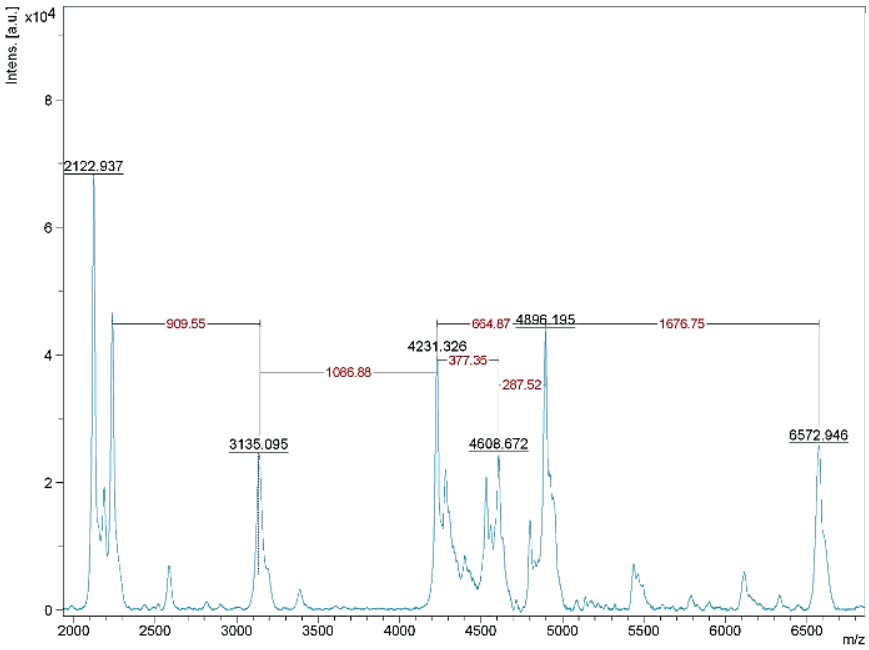


Figure 3 Mass spectrum of Bs-19_1 fraction (Lc-12_1)

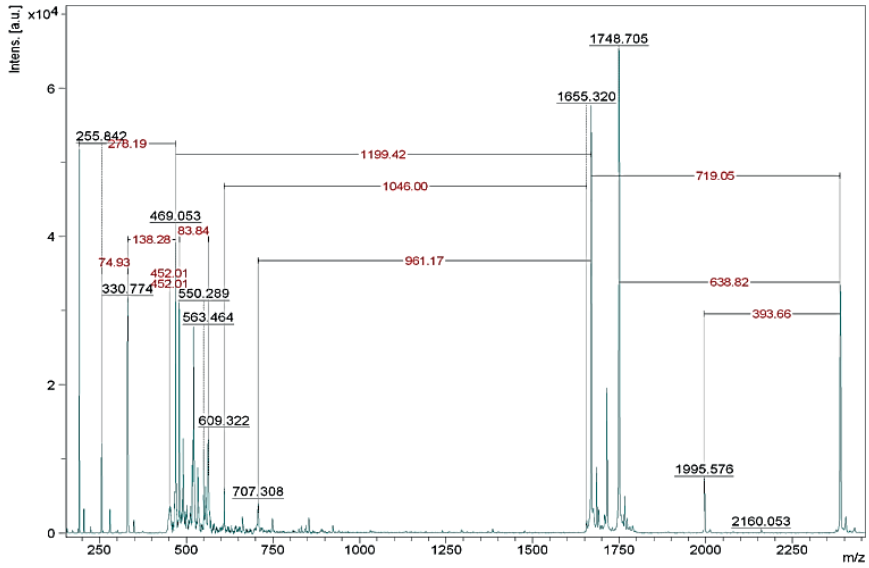


Figure 4 Mass spectrum of Bs-19_2 fraction

Having analyzed the mass spectra, we determined the molecular masses and amino acid sequences of seven peptides (Table 3).

Table 3 also shows the presence of analogues for the studied peptides in the PepBank and Uniprot databases. We established a homology of fractions Pp-11_1, Pp-11_2, Pp-11_3, Pp-11_4, Pp-11_5, Pp-11_6, Pp-11_7, Pp-11_8, Lp-7_1, Pd-16_1, Pd-16_2, Pd-16_3, and Pd-16_4 with the cysteine membrane protein *Giardia lamblia* P15 (Fig. 8), as well as a homology of peptides Pa-9_1 and Pa-9_2 with the *Planctomycetes bacterium* I41 peptides (Fig. 9). The rest of the peptides had no analogues in the PepBank and Uniprot databases.

The antibacterial properties of the studied peptides against gram-positive (*Bacillus pumilus*) and gram-negative (*Escherichia coli*) bacteria, as well as their fungicidal properties against the microscopic fungi *Aspergillus niger* and *Aspergillus flavus* are presented in Tables 4–5 and Figs. 10–11.

According to Table 4 and Fig. 10, of the seven peptides under study, only one (Bs-19_2) exhibited no antagonistic activity against *E. coli* and *B. pumilus* strains. Peptide fraction Pp-11_1 (and peptides with identical amino acid sequences Pp-11_2, Pp-11_3, Pp-11_4, Pp-11_5, Pp-11_6, Pp-11_7, Pp-11_8, Pd-16_1, Pd-16_2, Pd- 16_3, Pd-16_4, and Lp-7_1) showed

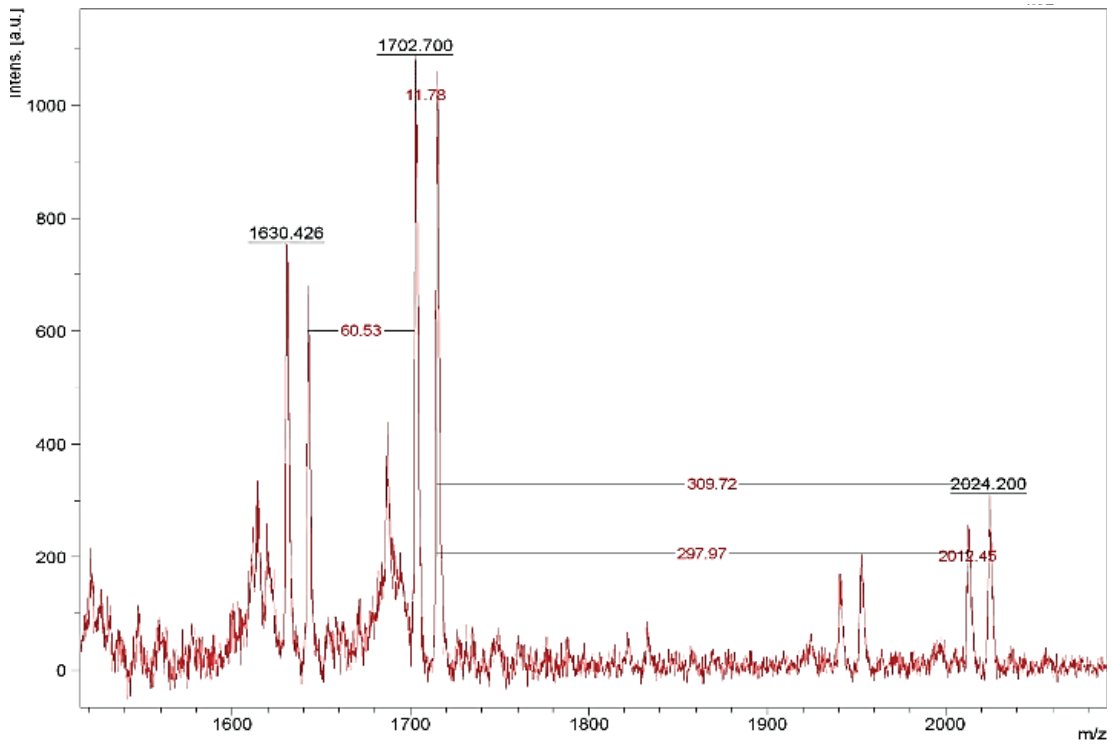


Figure 5 Mass spectrum of Pp-11_1 fraction (Pp-11_2, Pp-11_3, Pp-11_4, Pp-11_5, Pp-11_6, Pp-11_7, Pp-11_8, Lp-7_1, Pd-16_1, Pd-16_2, Pd-16_3, Pd-16_4)

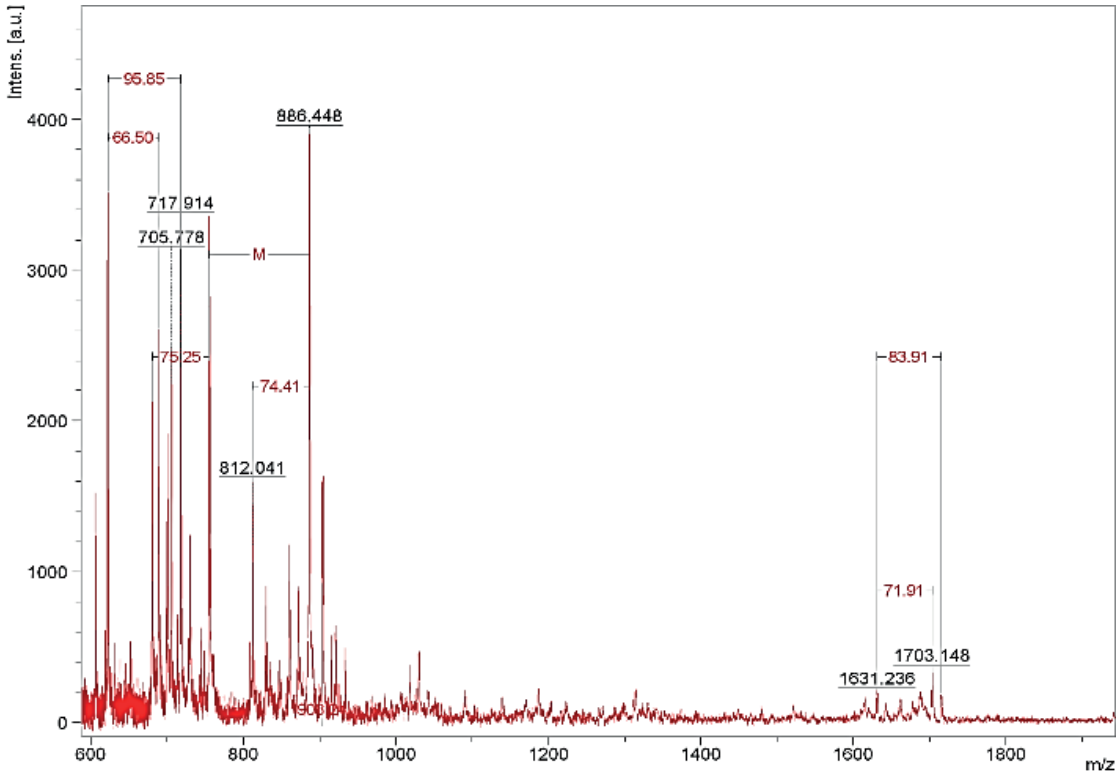


Figure 6 Mass spectrum of Lm-8_1 fraction (Lm-8_2)

high antagonism against *B. pumilus* and pronounced antibacterial activity against *E. coli*. Peptides Bs-1_1 and Bc-20_1 (identical Lf-13_1, Lf-13_2, and Lf-13_3), Lm-8_1 (identical Lm-8_2), and Pa-9_1 (identical Pa-9_2) had moderate and pronounced antagonistic activity against *B. pumilus*, but no activity against *E. coli*. Finally, peptide Bs-19_1 (identical Lc-12_1) showed bacteriostatic activity only against *E. coli*.

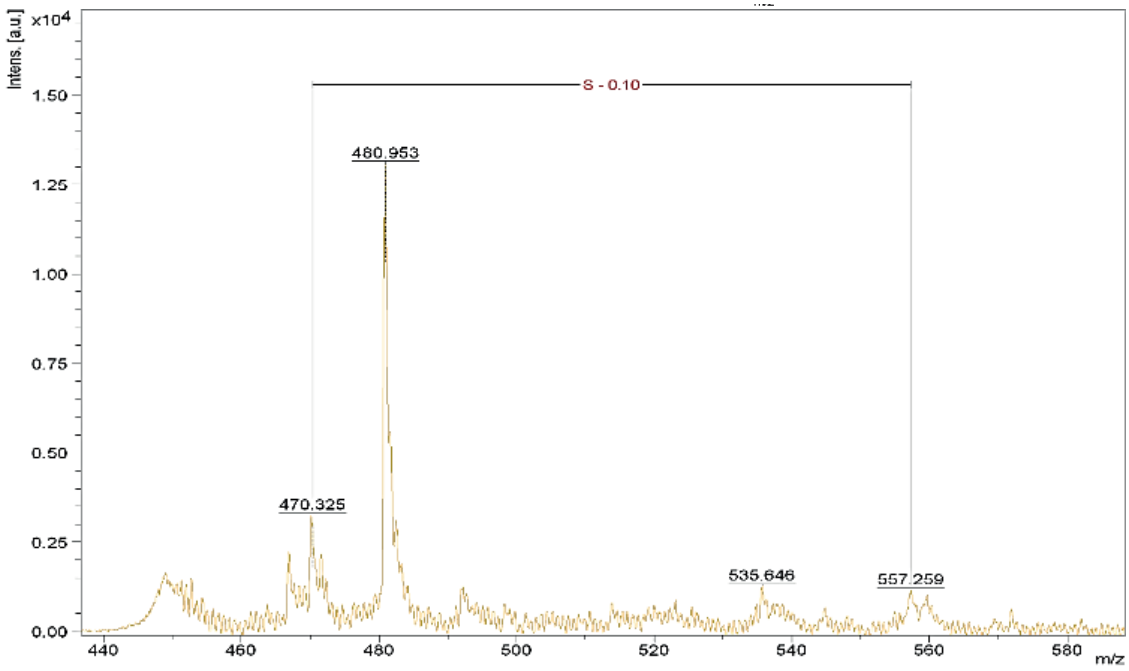


Figure 7 Mass spectrum of Pa-9_1 fraction (Pa-9_2)

Table 3 Molecular masses and amino acid sequences of peptides from the culture fluid of bacteria isolated from the natural sources of Kuzbass

Code of peptide	Molecular mass of peptide, Da	Amino acid sequence	Analogues in PepBank or Uniprot
Bs-1_1	13140.97	AFGKHVLIPVSCGFTYVWKCTLIPHISARPHYCFH RQHCDYKINQVSFEDAWHTPC	No analogues
Bc-20_1 Lf-13_1 Lf-13_2 Lf-13_3	6577.63	FLAFAYLPIPGWHPDYNGRAMKWANRPFTYICHGR DLKLRQMLYSGATIGHAEMR	No analogues
Bs-19_1 Lc-12_1	6572.00	PHQGHAFNFSCDMETAGFKGTQFWTFKSV SPHLATFKLGHMSTYAILGFAGCH	No analogues
Bs-19_2	6290.80	FVKGFHPSMTARGVVSDEADGRCDRFV KGFHPSMTARGVVSDEADGRCDR	No analogues
Pp-11_1 Pp-11_2 Pp-11_3 Pp-11_4 Pp-11_5 Pp-11_6 Pp-11_7 Pp-11_8 Lp-7_1 Pd-16_1 Pd-16_2 Pd-16_3 Pd-16_4	2061.66	VMCLARKCSQGLIVKAPLM	High homology with cysteine membrane protein *Giardia lamblia* P15
Lm-8_1 Lm-8_2	35571.18	MOPRKLCQSP VAILKMCVPA RQKVPSILKM OPRKLCQSPV AILKMCVPAR QKVPSILKMO PRKLCQSPVAILKMCVPARQ KVPSILKMOP RKLCQSPVAI LKMCVPARQK VPSILKMOPR KLCQSPVAIL KMCVPARQKV PSILKMOPRK LCQSPVAILK MCVPARQKVP SILKMOPRKL CQSPVAILKM CVPARQKVPS ILKMOPRKLC QSPVAILKMC VPARQKVPSILKMOPRKLCQ SPVAILKMCV PARQKVPSIL KMOPRKLCQS PVAILKMCVP ARQKVPSILK MOPRKLCQSP VAILKMCVPA RQKVPSILK	No analogues
Pa-9_1 Pa-9_2	2587.21	AVPSMKLCIQWSPVRASPCVMLGI	High degree of homology with *Planctomycetes bacterium* I41 peptides

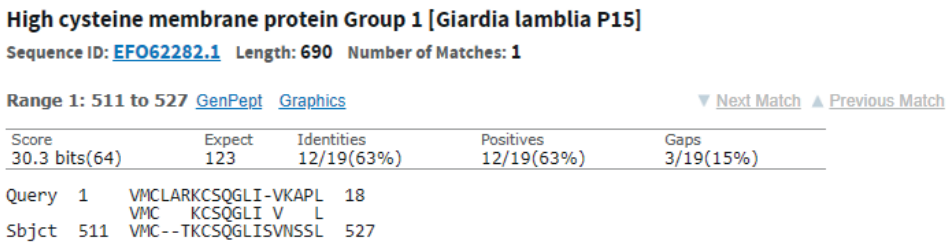


Figure 9 The closest analogues for peptides Pa-9_1 and Pa-9_2 according to BLAST NCBI

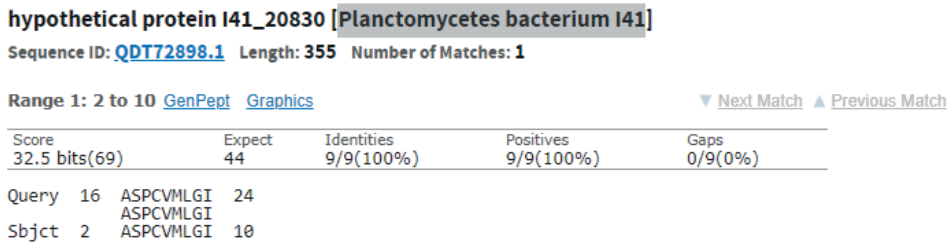


Figure 8 The closest analogues for peptides Pp-11_1, Pp-11_2, Pp-11_3, Pp-11_4, Pp-11_5, Pp-11_6, Pp-11_7, Pp-11_8, Lp-7_1, Pd-16_1, Pd-16_2, Pd-16_3, and Pd-16_4 according to BLAST NCBI

Table 4 Antibacterial properties of peptides from the culture fluid of bacteria isolated from the natural sources of Kuzbass (M ± m)

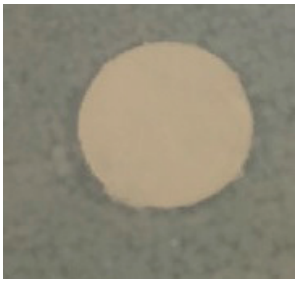
Peptide code	Test strain	Lysis zone diameter, cm	Degree of activity
Negative control	*Escherichia coli*	0	–
	Bacillus pumilus	0	–
Ampicillin (positive control) 0.5 mg/mL	*Escherichia coli*	0.90 ± 0.05	–
	Bacillus pumilus	2.40 ± 0.10	–
Bs-1_1	*Escherichia coli*	0	Absent
	Bacillus pumilus	0.60 ± 0.03	Moderate
Bc-20_1 Lf-13_1 Lf-13_2 Lf-13_3	*Escherichia coli*	0	Absent
	Bacillus pumilus	0.80 ± 0.04	Pronounced
Bs-19_1 Lc-12_1	*Escherichia coli*	0.60 ± 0.03	Moderate
	Bacillus pumilus	0	Absent
Bs-19_2	*Escherichia coli*	0	Absent
	Bacillus pumilus	0	Absent
Pp-11_1 Pp-11_2 Pp-11_3 Pp-11_4 Pp-11_5 Pp-11_6 Pp-11_7 Pp-11_8 Pd-16_1 Pd-16_2 Pd-16_3 Pd-16_4 Lp-7_1	*Escherichia coli*	0.70 ± 0.04	Pronounced
	Bacillus pumilus	1.00 ± 0.05	High
Lm-8_1 Lm-8_2	*Escherichia coli*	0	Absent
	Bacillus pumilus	0.70 ± 0.04	Pronounced
Pa-9_1 Pa-9_2	*Escherichia coli*	0	Absent
	Bacillus pumilus	0.60 ± 0.03	Moderate

Unlike biocidal properties, which do not depend on the pathogen growth phase and naturally decrease over time, fungicidal properties need to be determined at each stage of the fungus life cycle since fungal pathogens have a complex growth cycle. We found that the peptide fractions under study did not stop fungal growth, but only inhibited it, which was indicated by a change in the mycelium color. The results were analyzed with time intervals (3, 9, 12, 24, 48, 72 h, etc.) and by the fungus growth phase (stationary, accelerated growth, logarithmic), i.e., during the periods of exponential cell growth, decreased growth, and death or autolysis. The samples with filters impregnated with a nutrient medium were used as a control.

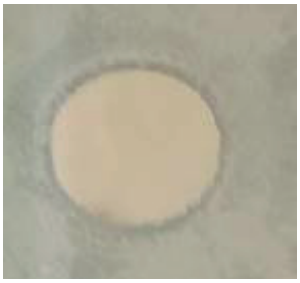
Having analyzed the peptides' fungicidal activity (Table 5, Fig. 11), we identified those peptides which could inhibit *Aspergillus* growth, rather than stop it completely. They were Bs-1_1, Bc-20_1 (identical Lf-13_1, Lf-13_2, and Lf-13_3) and Bs-19_2, with a lysis zone diameter of 0.1–0.2 mm. The maximum fungicidal activity against *A. niger* (0.3–0.5 mm lysis zone) was demonstrated by peptides Bs-19_1 (identical Lc-12_1), Pp-11_1 (identical Pp-11_2, Pp-11_3, Pp-11_4, Pp-11_5, Pp-11_6, p-11_7, Pp-11_8, Pd-16_1, Pd-16_2, Pd-16_3, Pd-16_4, and Lp-7_1), and Pa-9_1 (Pa-9_2). The highest activity against *A. flavus* (0.3–0.4 mm lysis zone) was revealed by peptides Pp-11_1 (identical Pp-11_2, Pp-11_3, Pp-11_4, Pp-11_5, Pp-11_6, p-11_7, Pp-11_8, Pd-16_1, Pd-16_2, Pd-16_3, Pd-16_4, and Lp-7_1), Lm-8_1 (identical Lm-8_2), and Pa-9_1 (identical Pa- 9_2).

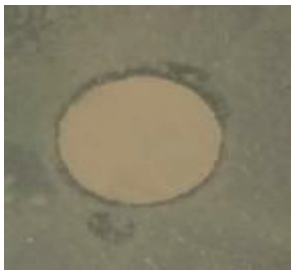
Based on the study of antimicrobial activity, we selected peptides with maximum antibacterial (against *B. pumilus*) and fungicidal (against *A. niger* and *A. flavus*) properties: Pp-11_1 (identical Pp-11_2, Pp-11_3, Pp-11_4, Pp-11_5, Pp-11_6, p-11_7, Pp-11_8, Pd-16_1, Pd-16_2, Pd-16_3, Pd-16_4, and Lp-7_1), Lm-8_1 (identical Lm-8_2), and Pa-9_1 (identical Pa-9_2).

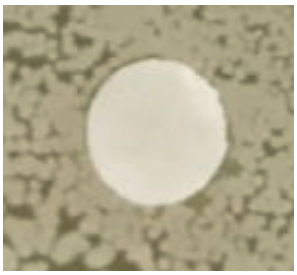

Bs-1_1 *Escherichia coli*


Bs-1_1 *Bacillus pumilus*

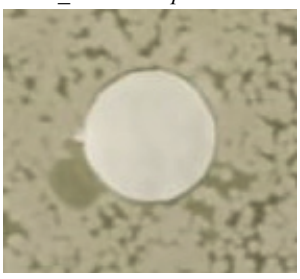

Bc-20_1, Lf-13_1, Lf-13_2, Lf-13_3 *Escherichia coli*

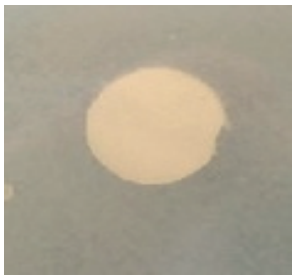

Bc-20_1, Lf-13_1, Lf-13_2, Lf-13_3 *Bacillus pumilus*

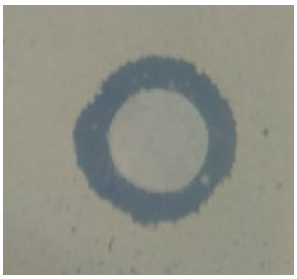

Bs-19_1, Lc-12_1 *Escherichia coli*

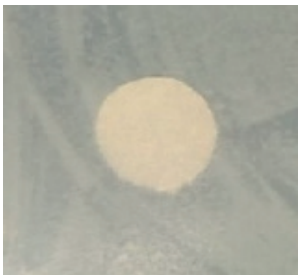

Bs-19_1, Lc-12_1 *Bacillus pumilus*

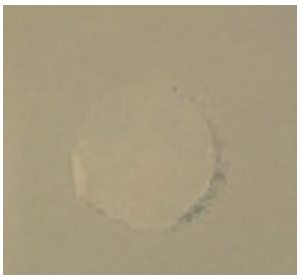

Bs-19_2 *Escherichia coli*


Bs-19_2 *Bacillus pumilus*

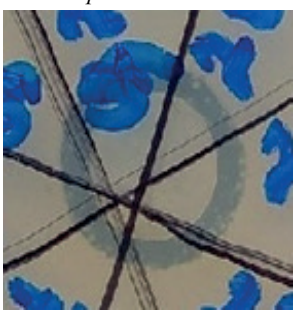

Pp-11_1, Pp-11_2, Pp-11_3, Pp-11_4, Pp-11_5, Pp-11_6, Pp-11_7, Pp-11_8, Lp-7_1, Pd-16_1, Pd-16_2, Pd-16_3, Pd-16_4 *Escherichia coli*


Pp-11_1, Pp-11_2, Pp-11_3, Pp-11_4, Pp-11_5, Pp-11_6, Pp-11_7, Pp-11_8, Lp-7_1, Pd-16_1, Pd-16_2, Pd-16_3, Pd-16_4 *Bacillus pumilus*

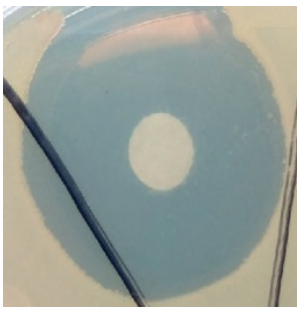

Pa-9_1, Pa-9_2 *Escherichia coli*

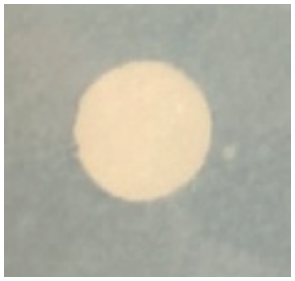

Pa-9_1, Pa-9_2 *Bacillus pumilus*


Lm-8_1, Lm-8_2 *Escherichia coli*


Lm-8_1, Lm-8_2 *Bacillus pumilus*


Amp *Escherichia coli*


Amp *Bacillus pumilus*


– *Escherichia coli*

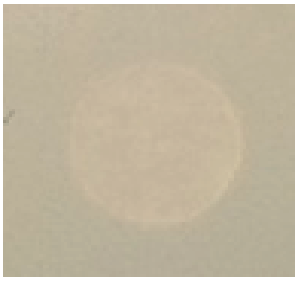

– *Bacillus pumilus*

Figure 10 Antibacterial properties of peptides from the culture fluid of bacteria isolated from the natural sources of Kuzbass

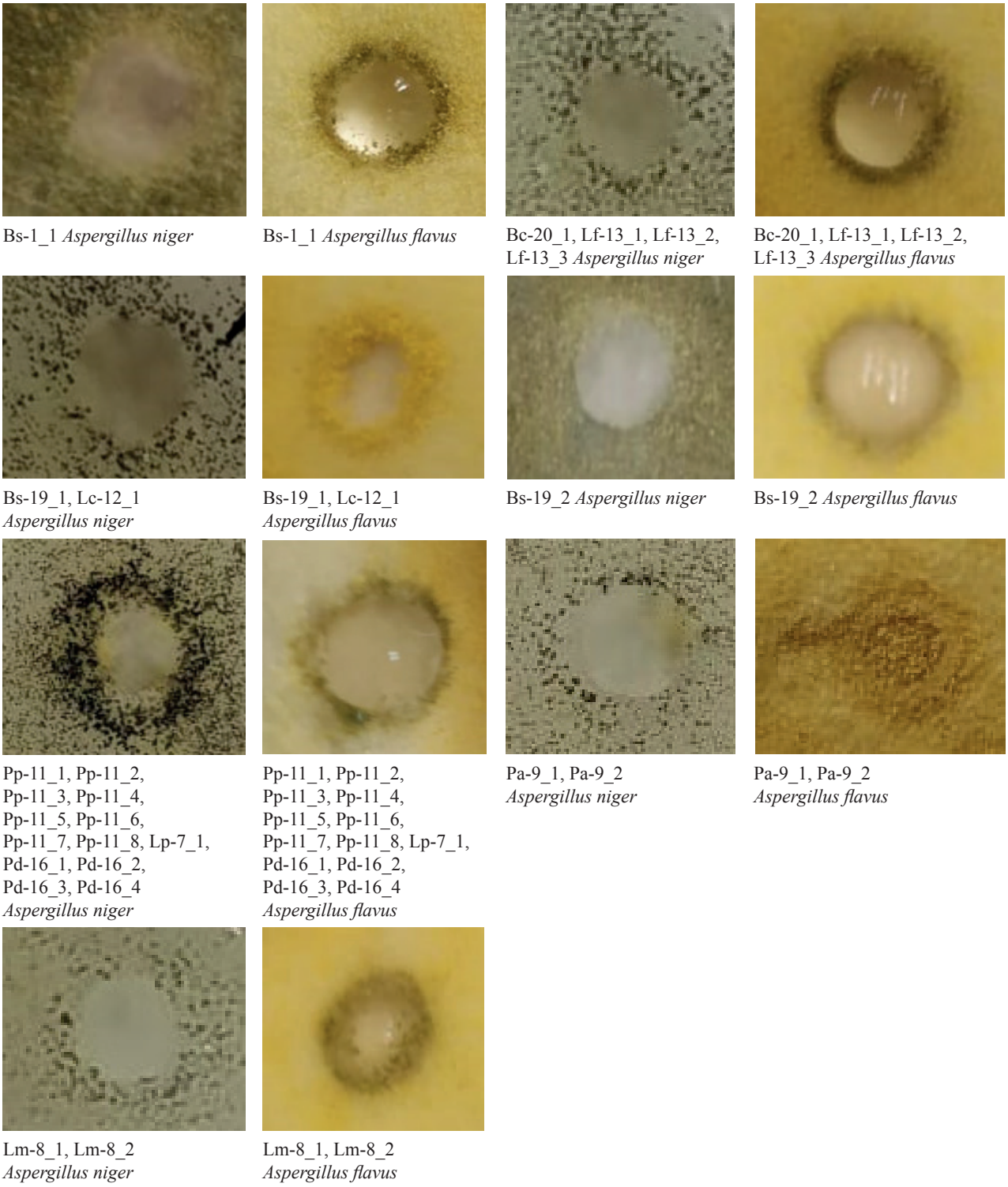


Figure 11 Fungicidal properties of peptides from the culture fluid of bacteria isolated from the natural sources of Kuzbass

Thus, the fact that peptides produced by microorganisms inhabiting the natural ecosystems of Kuzbass exhibit antagonistic activity against opportunistic strains opens up prospects for their use in the production of pharmaceutical substances with antimicrobial action, alternative to traditional antibiotics.

CONCLUSION

We identified amino acid sequences and molecular masses of peptide fractions produced by bacteria (*Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Bacillus*, and *Geobacillus*) isolated from the natural objects of the Siberian region (soil, rhizosphere of plants, bottom

Table 5 Fungicidal properties of peptides from the culture fluid of bacteria isolated from the natural sources of Kuzbass (M ± m)

Peptide code	Lysis zone diameter by growth phase, mm						
	Exponential cell growth, h			Decreased growth, h		Death or autolysis, days	
	3	9	12	48	72	6	12
Aspergillus niger							
Bs-1_1	+	+	+	0.100 ± 0.005	0.200 ± 0.010	0.100 ± 0.005	0.100 ± 0.005
Bc-20_1, Lf-13_1, Lf-13_2, Lf-13_3	+	+	+	0.200 ± 0.010	0.100 ± 0.005	0.100 ± 0.005	0.100 ± 0.005
Bs-19_1, Lc-12_1	+	+	+	0.500 ± 0.025	0.500 ± 0.025	0.400 ± 0.020	0.400 ± 0.020
Bs-19_2	+	+	+	0.100 ± 0.005	0.100 ± 0.005	0.200 ± 0.010	0.200 ± 0.010
Pp-11_1, Pp-11_2, Pp-11_3, Pp-11_4, Pp-11_5, Pp-11_6, p-11_7, Pp-11_8, Pd-16_1, Pd-16_2, Pd-16_3, Pd-16_4, Lp-7_1	+	+	+	0.100 ± 0.005	0.200 ± 0.010	0.400 ± 0.020	0.400 ± 0.020
Lm-8_1, Lm-8_2	+	+	+	0.100 ± 0.005	0.100 ± 0.005	0.100 ± 0.005	0.100 ± 0.005
Pa-9_1, Pa-9_2	+	+	+	0.100 ± 0.005	0.300 ± 0.015	0.400 ± 0.020	0.400 ± 0.020
Aspergillus flavus							
Bs-1_1	+	+	+	0.100 ± 0.005	0.100 ± 0.005	0.100 ± 0.005	0.100 ± 0.005
Bc-20_1, Lf-13_1, Lf-13_2, Lf-13_3	+	+	+	0.200 ± 0.010	0.100 ± 0.005	0.100 ± 0.005	0.100 ± 0.005
Bs-19_1, Lc-12_1	+	+	+	0.100 ± 0.005	0.100 ± 0.005	0.100 ± 0.005	0.100 ± 0.005
Bs-19_2	+	+	+	0.100 ± 0.005	0.100 ± 0.005	0.100 ± 0.005	0.100 ± 0.005
Pp-11_1, Pp-11_2, Pp-11_3, Pp-11_4, Pp-11_5, Pp-11_6, p-11_7, Pp-11_8, Pd-16_1, Pd-16_2, Pd-16_3, Pd-16_4, Lp-7_1	+	+	+	0.100 ± 0.005	0.100 ± 0.005	0.300 ± 0.015	0.400 ± 0.020
Lm-8_1, Lm-8_2	+	+	+	0.100 ± 0.005	0.300 ± 0.015	0.300 ± 0.015	0.400 ± 0.020
Pa-9_1, Pa-9_2	+	+	+	0.100 ± 0.005	0.300 ± 0.015	0.400 ± 0.020	0.400 ± 0.020
Positive control	+	+	+	0.100 ± 0.005	0.100 ± 0.005	0.100 ± 0.005	0.100 ± 0.005

sediments of reservoirs, and plant waste). In total, we isolated 25 protein fractions, some with identical mass spectra. Thus, we obtained seven peptides with different amino acid sequences, five of which have no analogues in the PepBank and Uniprot databases. One of the peptides (VMCLARKCSQGLIVKAPLM, 2061.66 Da) was homologous to the cysteine membrane protein *Giardia lamblia* P15, and another one (AVPSMKLCIQWSPVRASPCVMLGI, 2587.21 Da) was homologous to the *Planctomycetes bacterium* I41 peptides.

The peptides obtained from the culture fluid of bacteria isolated from natural sources of the Siberian Federal District were analyzed for antibacterial properties against *Bacillus pumilus* and *Escherichia coli*. We identified one peptide that exhibited no antagonistic activity against either gram-negative or gram-positive bacteria. One peptide fraction showed high antibacterial properties against both *B. pumilus* and *E. coli*. One peptide was active against *E. coli*, but not against *B. pumilus* (gram-positive bacteria). Finally, four out of seven peptides under study exhibited moderate and pronounced antagonism against *B. pumilus*, but no antibacterial activity against *E. coli*.

Our study of the peptides' antifungal activity revealed three peptides that could inhibit the growth of the microscopic fungi *Aspergillus niger* and *Aspergillus flavus*, without stopping it completely (0.1–0.2 mm lysis zone). Four peptide fractions showed high fungicidal activity against *Aspergillus* (0.3–0.5 mm lysis zone).

According to our results, antimicrobial peptides produced by bacteria isolated from the natural objects of the Siberian region can be used as promising agents in the production of pharmaceutical substances and drugs (after safety trials) to treat infectious diseases, such as gastrointestinal, respiratory, blood and skin, as well as fungal infections.

CONTRIBUTION

The authors are equally responsible for the research results and the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Muffins fortified with *Dacryodes macrophylla* L. fruit: quality and sensory evaluation

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Abstract:
Introduction. Due to the increasing demand for natural and functional products, scientists together with industries are conducting research to improve the nutritional quality of food. One of the ways to enhance the functionality of food is to add fruits or vegetables to their formulations. In this study, we attempted to develop muffins fortified with *Dacryodes macrophylla* L. fruit as a value-added ingredient.
Study objects and methods. Our study objects included *D. macrophylla* L. extract and six muffins: three eggless samples and three egg-containing samples. Each group included control and experimental samples. The experimental samples containing 0.5 and 1% of *D. macrophylla* extract instead of wheat flour were evaluated for muffin-making properties. All the samples were analyzed for their physicochemical, antioxidant, and sensory properties, as well as rheological parameters.
Results and discussion. We found that *D. macrophylla* L. reduced the water activity, color values (L^*, a^*, b^*), and firmness of muffins. It had no significant effect on baking loss, height, moisture, cohesiveness, springiness, gumminess or chewiness, but tended to decrease the specific volume of muffins. However, *D. macrophylla* fruit increased the specific gravity, improved rheology properties, and tended to increase adhesiveness and mineral contents. Na and K varied from 5.93 to 7.75 and 2.88 to 7.35 mg/g, respectively. Furthermore, *D. macrophylla* fruit significantly improved the muffins' antioxidant activities. According to sensory evaluation, the muffins made with egg solids and 0.5% of *D. macrophylla* fruit had higher sensory scores than the other experimental samples.
Conclusion. D. macrophylla L. fruit is a good potential ingredient for enriching muffins and developing new functional bakery products. However, further research is needed to improve the color reproduction of muffins and determine the optimal concentration of *D. macrophylla.*

Keywords: *Dacryodes macrophylla* L. fruit, minerals, moisture content, muffins, rheology, sensory, specific gravity, texture analysis, water activity

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INTRODUCTION

The growth in diet-related illnesses such as obesity, cardiovascular diseases, and some types of cancer led the World Health Organization (WHO) and other related organizations to encourage the consumption of fortified food [1]. Fortification is a deliberate addition of essential nutrients to a product to conserve its nutritional quality, enhance its added value, provide it with some functions, as well as to prevent or correct a particular nutritional deficiency in the population [2, 3]. However, one of the essential requirements of fortification is an appropriate food vehicle. Food vehicles should be widely consumed by a large proportion of the population to be able to meet the nutritional needs of the target group [4].

Baked food products are good potential vehicles of micronutrients and bioactive compounds because they are consumed all over the world by children and adults. Muffin is one of the most common bakery

products appreciated by people due to its taste and soft texture. Muffins are ready-to-eat snack food, similar to cupcakes, which are usually eaten at breakfast, as evening snacks, for tea, or at other meals. Muffins are also served as snacks during many celebrations. A special feature of muffins is their porous structure that leads to high volume and spongy texture [5, 6].

In response to the increasing demand for healthy, natural, and functional products, scientists are doing tremendous work in collaboration with industries to improve the nutritional quality of food products. Since fruits and vegetables are rich in natural nutrients, phytochemicals, and phenolic compounds with biological properties, incorporating them in muffins is a good way to fulfill the desires of consumers [7]. Furthermore, natural antioxidants from fruits and vegetables may inhibit lipid peroxidation in food and improve food quality and safety [5].

Dacryodes macrophylla L. is a fruit tree belonging to the *Buseraceae* family that is widespread in Cameroon, Equatorial Guinea, and Gabon. The fruits are commonly consumed directly or used to make natural juices and jelly [8]. *D. macrophylla* has red color that indicates the presence of phenolic compounds (e.g., anthocyanin) and some minerals (e.g., iron).

To the best of our knowledge, there are no available published data on *D. macrophylla* fruits as a potential value-added ingredient of muffins. Nevertheless, in our previous work, we studied the dyeability and bacterial resistance of these fruits on woolen fabric [9]. Ngondi *et al.* also showed that hydroethanolic extract of *D. macrophylla* fruits could have anti-obesity and antioxidant properties [10].

Therefore, we aimed to develop value-added muffins fortified with *D. macrophylla* fruits and to study the impact of that incorporation on the quality and acceptability of muffins. To achieve this aim, we fortified muffins with 1% of *D. macrophylla* fruit. Then, we evaluated their physicochemical properties, rheological parameters, and sensory characteristics. In addition, we determined the antioxidant properties of fortified muffins to assess their functionality.

STUDY OBJECTS AND METHODS

Study objects. We studied two groups of muffins: with egg and without egg. Each group contained a control and experimental samples with 0.5 and 1% of *Dacryodes macrophylla* L. extract instead of wheat flour.

Materials. Wheat flour (maida), sugar, baking soda, baking powder, egg, vegetal oil (soybean), and liquid milk (green packet Verka) were purchased from a local supermarket (Amritsar, India). 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchronan-2-carboxylic acid (Trolox), and ascorbic acid were obtained from Sigma-Aldrich Company Ltd. (St Louis, MO, USA). Analytical grade methanol, NaOH, NaCl, HNO_3, H_2SO_4, and $HClO_4$ were provided by Sisco Research Laboratories Ltd. (Mumbai, India). We used such equipment as an orbital shaker (Remi, Mumbai, India), a rotary evaporator (IKA Werke GmbH and Co. KG, Staufen, Germany), and a freeze dryer (Christ Beta 2-8 LD plus, Germany). Freeze-dried *D. macrophylla* L. was used to enhance the antioxidants and color of muffins.

Preparation of *D. macrophylla* extract. The seeds of fresh *D. macrophylla* L. fruit were discarded and the rest of the pulp was dried in a freeze dryer, followed by an extraction with 70% ethanol in an orbital shaker for 2 h at 200 rpm. It was then centrifuged at 4000 g for 10 min at 25°C and the supernatant was collected. The residue was re-extracted and the supernatant was collected and concentrated in a rotary evaporator under reduced pressure at 45°C. The remaining water was eliminated in the freeze dryer and the DME was kept in a fridge at –70°C in sealed plastic containers for the following experiments.

Preparation of muffins. Sugar was first powdered with a mixer and eggs were manually beaten in a bowl with a spoon (just for mixing purposes) for 1 min before weighing. All the ingredients were then weighed to prepare six different muffins (Table 1). Preliminary baking was done to standardize the formulation of muffins and to find the sensorily acceptable concentration of *D. macrophylla* extract.

Then, the required number of eggs was mixed with sugar using an electric hand mixer until creamy. Sunflower oil was added to the creamy mixture, which was continuously mixing, followed by the required amount of liquid milk. After about 4 min of mixing, wheat flour was gradually added to the emulsified gel during continuous stirring in the same direction. Baking powder was the last ingredient to be added to the formulation. The dough was then introduced into greased muffin molds and baked in the preheated oven at 210°C for 8 min. The muffins were allowed to stand for 2 min in the oven and then taken out to cool down for about 30 min at room temperature.

The samples were then kept in sealed plastic food-grade bags at room temperature for further analysis. For eggless muffins, the first step was to mix sugar with oil and the last step was to add baking soda after baking powder. For fortified muffins, *D. macrophylla* extract was dissolved in liquid milk before being added to the mixture (with egg and without egg).

Rheology of dough. Rheological tests of muffin dough were performed with a rheometer (MCR-102, Anton Paar Austria) as reported by Jantider *et al.* [11]. The dough sample was loaded between two parallel plate geometric probes of 40 mm in diameter (PP40) and kept for 5 min (for equilibration). The gap between the plates was 1 mm and the sample was run at 25°C. Stress was set at 0.1 Pa and frequency at 1 rad/s according to the linear viscoelastic region. The measurements of storage modulus (G', solid component) and loss modulus (G'', liquid component) were recorded.

Specific gravity of dough. The specific gravity of each type of muffin dough was determined gravimetrically by dividing the weight of a known

Table 1 Formulation of muffins

Ingredients, g	Eggless muffins			Egg-containing muffins		
	Control	1% DME	0.5% DME	Control	1% DME	0.5% DME
Wheat flour	149	148.5	149.25	150	148.5	149.25
Sugar	85	85	85	85	85	85
Vegetal oil	75	75	75	75	75	75
Milk	75	75	75	75	75	75
Baking powder	5.1	5.1	5.1	5.1	5.1	5.1
Eggs	0	0	0	75	75	75
Baking soda	1	1	1	0	0	0
Dacryodes macrophylla L.extract	0	1.5	0.75	0	1.5	0.75

volume of dough by the weight of an equal volume of water. A standard container was used for measurements [12].

Moisture content. The gravimetric method was used to determine the moisture content in muffin crumb. For this, 2 g of a sample was dried in an air oven at 105°C until no further weight change, using a clean, dry, and pre-weighed aluminum moisture dish. The moisture content was calculated as follows:

$$\text{Moisture content (\%)} = 100 - \frac{(W_1 - W_2)}{W_1} \times 100 \quad (1)$$

where W_1 is the weight of samples before drying; W_2 is the weight of samples after drying (in grams).

Weight loss. The baking loss of muffins was determined in percentage based on the weight of muffin after baking and the weight of muffin dough by using the following formula [13]:

$$\text{Weight loss (\%)} = \frac{(W_d - W_m)}{W_d} \times 100 \quad (2)$$

where W_d is the weight of dough; W_m is the weight of muffin.

Muffin height and diameter. A digital caliper was used to measure the height of muffins (from the highest to the lowest point) and their diameters (mm).

Water activity. The water activity of the samples was measured by placing about 2 g of muffin crumb on a plastic dish of a water activity meter (AquaLab TE, series 3B, version 3.4, Decagon). After calibration with water, values were recorded at 25°C in triplicate.

Muffin volume. The volume of muffins was determined by the millet-seed displacement method as described by Rashida *et al.*, with slight modification [5]. An empty baker was filled with millet seeds and then the seeds were transferred into a container. Then, a muffin was placed in the center of an empty baker and the seeds were loaded back from the container. The remaining seeds were put in a measuring cylinder and their volume (in mL) represented the volume of the muffin. The specific volume was then calculated by dividing the volume recorded by the weight of the muffin (mL/g).

Crude fat. Crude fat of the muffins was estimated gravimetrically on the Soxhlet apparatus [13]. The samples were weighed (W_1) and lipid was extracted with hexane for 6 h at 65°C. The lipid extract was then dried in the oven at 102°C till constant weight. Crude fat was expressed in percentage and calculated as follows:

$$\text{Crude fat (\%)} = \frac{W_2}{W_1} \times 100 \quad (3)$$

where W_1 is the weight of a sample in grams before lipid extraction; W_2 is the weight of the dried lipid extract.

Ash content. Total ash was determined by the incineration method in a muffle furnace. The samples were weighed in porcelain crucibles and incinerated for 1 h at 550 ± 10°C. White ash was cooled and weighed. Ash content was expressed in percentage by using the following formula:

$$\text{Ash (\%)} = \frac{W_2}{W_1} \times 100 \quad (4)$$

where W_1 is the weight of a sample in grams before incineration; W_2 is the weight of the sample after incineration.

Mineral content. ***Preparation of samples.*** The defatted muffins and extracts were digested using a mixture of tri-acid [14]. Three milliliters (3 mL) of tri-acid (HNO_3:H_2SO_4:$HClO_4$ = 5:1:1) was added to 0.5 g of a sample and the mixture was heated at 80°C. After about 2 min, two milliliters (2 mL) of tri-acid was added again under continuous heating until the fume of the mixture became transparent. The digested samples were then cooled at room temperature and the volume was made up to 20 mL with double distilled water. After filtration with Whatman filter paper, the solution was diluted to 100 mL with double distilled water and stored at room temperature as a stock sample solution for mineral estimation.

Calcium. To quantify calcium content, 5 mL of the stock sample solution was diluted to 50 mL with double distilled water. 2 mL of NaOH 1N was added and then a pinch (about 100 mg) of the murexide indicator (a mixture of grind 0.2 g of ammonium purpurate with 100 g of NaCl) to turn the solution pink.

The pink sample solution was then titrated with EDTA solution, 0.01 M (3.723 g of EDTA dissolved in 1000 mL of water) until the pink color turned dark purple. The endpoint of titration was determined by comparing the endpoint color of the sample to the one

obtained with the blank (titration with 50 mL of water). The calcium content (mg/g) was calculated as follows:

$$\text{Calcium content} = \frac{\text{Volume of EDTA used}}{\text{Volume of sample used}} \times 100 \quad (5)$$

Magnesium. To determine the magnesium content, we first estimated the hardness (Ca + Mg) of the samples. For this, 5 mL of the stock solution was diluted to 50 mL with water in a conical flask, followed by the addition of 1 mL of the buffer solution and about 100 mg of the EBT indicator (a mixture of grind 0.40 g of Erichrome with 100 g of NaCl). The wine red color developed and the titration was done with 0.01 M of EDTA. The endpoint was reached by comparing the blue color of the sample solution with the one obtained with the blank (water). Then, magnesium was measured in mg/mL by subtracting the volume of EDTA used to determine hardness to the one used to quantify calcium:

$$\text{Mg} = \frac{(Y - X) \times 400.8}{Vs \times 1{,}645} \quad (6)$$

where Y is the volume of EDTA used to estimate hardness, mL; X is the volume of EDTA used to quantify calcium, mL; and Vs is the volume of a sample, mL. The result was expressed in mg/g of the sample.

Phosphate. The phosphate content was determined spectrophotometrically at 625 nm. Five milliliters (5 mL) of the stock solution was diluted to 50 mL with water and then mixed with 2 mL of ammonium molybdate reagent (prepared by mixing 25 g ammonium molybdate dissolved in 175 mL water and 280 mL H_2SO_4 diluted with 400 mL of water and making the final volume up to 1000 mL with distilled water) and 0.5 ML of stannous chloride (2.5 g $SnCl_2$ dissolved in 100 mL water). The mixture was kept for 15 min and then used to record optical density against the blank on a microplate reader.

Potassium, Sodium and Zinc. These elements were analyzed by atomic absorption spectrometry [15]. KCl, NaCl, and $ZnSO_4$ were used as a standard to quantify K, Na, and Zn, respectively. A serial dilution of each standard was performed to make a calibration curve for each element. Subsequently, the filtrated liquor from mineralization of each sample was diluted with double distilled water and the content of minerals was determined at 766.5 nm for K, 330.2 nm for Na, and 213.9 nm for Zn with an AA 6300 spectrometer (Shimadzu, Tokyo, Japan) against the blank by extrapolation of absorbance on the calibration curve of each element. The final amount (dry weight) was then calculated in mg/g of the sample.

Muffin color. The color of the muffins was determined the next day after preparation by recording the L^*, a^*, and b^* values of crust and crumb. A spectrophotometer with spectra match software was used according to the CIE Lab color scales, where L^* goes in a range of 0 to 100 from dark to light, a^* from green to red, and b^* from blue to yellow. Color values were measured three times at three different points on each muffin and then averaged.

Texture analysis. The texture profile of crumb cubes (12.5 mm^3) from the middle of the muffins was determined using a texture analyzer (Model EZ-SX, Stable microsystems, Shimadzu, UK) equipped with a 5-kg load cell [16]. A double compression test was performed by putting a crumb cube sample in the center of a heavy-duty platform (HD P/90) and subjecting it to compression (50%) with an aluminum 75-mm cylindrical probe (P/75) at 1 mm/s. The texture parameters (firmness, cohesiveness, gumminess, chewiness, and springiness) were calculated based on the texture profile graphic [17].

Antiradical activity. ***Preparation of extract.*** To prepare the extract, 100 mg of a defatted powdered muffin (muffins defatted with hexane were dried in the oven at 40°C and powdered in a porcelain container) was mixed with 1 mL of 80% methanol in an Eppendorf tube. The extraction was performed in the orbital shaker for 2 h at 25°C followed by centrifugation at 500×g for 15 min. Supernatants were pooled in an empty Eppendorf tube for antiradical analysis.

DPPH assay. Free radical scavenging of the muffin samples was determined according to the method described by Uswa and Rabia, with slight modification [18]. 100 µL of a muffin extract was added to 3.9 mL of the DPPH solution (2.4 mg of DPPH in 100 mL of 80% methanol) and vortexed thoroughly. The mixture was then incubated for 30 min in the dark and the absorbance was read at 515 nm by using a spectrophotometer against 80% methanol as the blank. The control was 3.9 mL of DPPH + 100 µL of the solvent. A calibration curve of trolox was plotted, with the result expressed in µM trolox equivalent/mg of the sample.

Sensory evaluation. The overall acceptability of the fortified muffins was evaluated on the 9-point hedonic scale [19]. Muffin samples were given randomly to a panel of 100 untrained volunteers from Guru Nanak Dev University, Amritsar (India). They were requested to score their appreciation from extremely unpleasant (1) to extremely pleasant (9) based on color, odor, texture, taste, and overall assessment. The panelists were also asked to rinse their mouths with water before tasting each sample.

Data management and statistics. The results were analyzed with Statgraphics Plus program Version 2.1. Data were presented as mean values of triplicate reading ± standard deviation subjected to one-way analysis of variance (ANOVA). Tukey test was used to compare the means, and a significant difference was determined at $P < 0.05$.

RESULTS AND DISCUSSION

Table 2 shows the physical properties of the muffins fortified with *Dacryodes macrophylla* L. We observed that the baking loss in the eggless muffins (9.00–9.56%) was statistically the same but significantly ($P < 0.05$) lower than in the muffins with eggs (11.22–11.67%).

Similarly, the moisture content in the muffins with eggs was higher than in the eggless samples. This might be related to the weaker dough consistency of the muffins with eggs, leading to higher viscosity. When the viscoelasticity of dough is high, air bubbles incorporated during the creaming step of preparation tend to increase and rise to the surface of the muffin, getting lost at the beginning of baking. Moreover, carbon dioxide and vapor pressure produced during baking might escape and increase the baking loss and moisture content. Larger cells also increase baking loss and usually quicken moisture migration during baking [20].

Specific gravity gives general information about air bubbles that are incorporated in the dough during mixing and have a direct effect on the muffin height. Higher specific gravity means less incorporation of air and a lower muffin height. We found the specific gravity values for the eggless muffins (1.12–1.14) to be higher than that for the muffins with eggs (1.03–1.07). Therefore, the height of the eggless muffins was lower (33.97–34.37 mm) that that of the muffins with eggs (41.00–41.40 mm). Table 2 also shows a slightly higher specific gravity, and therefore a lower height, in the samples fortified with the *D. macrophylla* fruit extract. These results might be explained by the presence of eggs which provide the dough with water and protein (an egg contains 74% of water and 12.8% of protein), thereby increasing its viscoelasticity.

Another reason might be the amount of air incorporated in the egg-containing dough compared to the eggless dough. Potential fibers present in *D. macrophylla* fruits might have increased the dough viscosity and consequently decreased air bubbles. Similar results were reported by Rashida *et al.* and Manuel *et al.* who found that using fibers in bakery increased the specific gravity and viscosity of the dough, which might further lead to a lower height and volume of muffins by obstructing air incorporation during mixing [5, 17].

At the same wavelength, the specific volume of the eggless muffins (1.66–1.70 mL/g) was significantly lower than that of the muffins with eggs (2.18–2.36 mL/g). Specific volume indicates the number of air bubbles retained in the final product after baking. The higher specific volume of the muffins with eggs could be explained by higher dough viscoelasticity (due to protein and water from eggs) which might have enhanced the expansion of air bubbles by carbon dioxide and vapor pressure during baking.

Besides, Shevkani and Singh reported that higher dough viscoelasticity ensured air bubbles stability during baking [21]. They also found that the incorporation of proteins in muffin dough increased the specific volume and height of the final products. In our study, however, the specific volume of the muffins with eggs was slightly lower due to the *D. macrophylla* fruits extract.

Similar results were found by Singh *et al.* and Prerna *et al.* who fortified muffins with Jambolan fruit pulp and red capsicum pomace powder, respectively [12, 16]. Our results might be justified by the presence of fibers in *D. macrophylla* fruits which might have inhibited the expansion of muffin by weakening the ability of the gluten matrix to retain carbon dioxide during baking [13].

Water activity (A_w) is an important parameter that enhances the shelf life of dry foods when their value is low. It represents free water in the food and can be defined as a ratio of vapor pressure of the food to the vapor pressure of pure water. The water activity of the eggless muffins (0.81–0.83) was lower than that of the muffins with eggs (0.87–0.90). Consequently, the shelf life of the former samples was higher.

In contrast, Table 2 shows a slight decrease in water activity of the egg-containing muffins fortified with the *D. macrophylla* fruit extract. It might be attributed to fibers in *D. macrophylla* fruits absorbing more water and thereby reducing unbound water in muffins.

Moisture, fat, and ash contents (Table 2) in the control muffins with eggs (25.33, 18.61, and 1.27) were significantly higher than those in the control eggless samples (19.17, 16.82, and 1.07). Higher moisture might be attributed to egg yolk phospholipids acting as emulsifiers and thereby holding moisture in emulsified form.

Table 2 Physical properties of muffins with *Dacryodes macrophylla* extract

Physical properties	Eggless muffins			Egg-containing muffins		
	Control	1% DME	0.5% DME	Control	1% DME	0.5% DME
Baking loss, %	9.00 ± 0.33^a	9.56 ± 0.48^a	9.11 ± 0.11^a	11.67 ± 0.19^b	11.22 ± 0.11^b	11.56 ± 0.29^b
Specific gravity	1.12 ± 0.00^c	1.14 ± 0.00^d	1.13 ± 0.00^d	1.03 ± 0.00^a	1.07 ± 0.00^b	1.06 ± 0.00^b
Specific volume, mL/g	1.70 ± 0.03^a	1.66 ± 0.01^a	1.69 ± 0.02^a	2.36 ± 0.01^d	2.18 ± 0.01^b	2.27 ± 0.01^c
Water activity	0.83 ± 0.00^a	0.81 ± 0.00^a	0.82 ± 0.00^a	0.90 ± 0.00^c	0.87 ± 0.00^b	0.89 ± 0.00^c
Moisture, %	19.17 ± 1.64^a	19.67 ± 0.73^a	19.33 ± 0.88^a	25.33 ± 0.44^b	26.00 ± 1.04^b	25.50 ± 0.76^b
Crude fat, %	16.82 ± 0.13^a	16.84 ± 0.46^a	16.82 ± 0.22^a	18.61 ± 0.34^b	18.63 ± 0.24^b	18.63 ± 0.31^b
Height, mm	34.37 ± 0.50^a	33.97 ± 0.30^a	34.23 ± 0.27^a	41.40 ± 0.35^b	41.00 ± 0.21^b	41.33 ± 0.33^b

Values are mean ± standard deviation of triplicate experiments. The values carrying the same letter on the same row are not statistically significant ($P \geq 0.05$)

Table 3 Mineral and ash contents of muffins fortified with *Dacryodes macrophylla* extract

Component	Eggless muffins			Egg-containing muffins		
	Control	1% DME	0.5% DME	Control	1% DME	0.5% DME
Ca, mg/g	3.58 ± 0.53^{a}	5.18 ± 0.53^{ab}	4.38 ± 0.27^{a}	4.11 ± 0.53^{a}	6.79 ± 0.53^{b}	5.72 ± 0.27^{ab}
Mg, mg/g	2.27 ± 0.32^{a}	2.92 ± 0.56^{a}	2.76 ± 0.32^{a}	2.60 ± 0.32^{a}	3.90 ± 0.56^{a}	3.73 ± 0.43^{a}
P, mg/g	0.66 ± 0.13^{a}	0.87 ± 0.01^{abc}	0.83 ± 0.02^{ab}	1.00 ± 0.02^{bc}	1.11 ± 0.02^{c}	1.05 ± 0.02^{bc}
Na, mg/g	5.03 ± 0.03^{a}	7.19 ± 0.01^{d}	5.82 ± 0.02^{b}	5.93 ± 0.06^{b}	7.75 ± 0.01^{e}	6.75 ± 0.02^{c}
K, mg/g	1.52 ± 0.02^{a}	3.61 ± 0.01^{d}	2.40 ± 003^{b}	2.88 ± 0.11^{c}	5.36 ± 0.03^{f}	3.87 ± 0.05^{e}
Zn, $\times 10^2$ mg/g	0.39 ± 0.03^{a}	1.53 ± 0.08^{bc}	1.13 ± 0.09^{b}	1.67 ± 0.31^{c}	3.36 ± 0.06^{e}	2.45 ± 0.21^{d}
Ash, %	1.07 ± 0.07^{a}	1.12 ± 0.06^{ab}	1.11 ± 0.06^{ab}	1.27 ± 0.03^{ab}	1.34 ± 0.03^{b}	1.29 ± 0.05^{ab}

Values are mean ± standard deviation of triplicate experiments. The values carrying the same letter on the same row are not statistically significant ($P \geq 0.05$)

Similarly, the increment of fat and ash in the control muffins with eggs may be due to the inherent presence of fat and minerals in the egg. The incorporation of *D. macrophylla* did not have any significant effect on moisture or fat, although it slightly increased the ash content. These results might be due to lower fat and ash contents in *D. macrophylla*.

Furthermore, the mineral content (Table 3) in the control muffins with eggs was higher than that in the control eggless muffins, particularly phosphorus, sodium, potassium, and zinc, which showed a significant difference. This result was expected because of the inherent presence of minerals in the egg. Also, both samples clearly illustrated the enhancement of minerals in the muffin fortified with the *D. macrophylla* extract, thereby showing this extract as a rich source of minerals.

Our results were in line with those found by Sheetal *et al.*, who reported increased mineral contents in muffins fortified with dried Moringa Oleifera [1].

The rheology parameters of muffin dough s are presented in Table 4 as G', G'', and tan δ, where G' (storage modulus) represents dough elasticity meaning a solid-like behavior, G'' (loss modulus) represents dough viscosity meaning a liquid-like behavior, and tan δ (ratio of G'' over G') tends to zero for solids and to infinity for liquids.

We observed that the storage modulus of all doughs was greater than the loss modulus, indicating a typical elastic dough behavior required for good quality muffins. Besides, Nazanin and Mostafa reported that the viscosity of cake dough should be optimum to hold air bubbles in the final product, since too low dough viscosity inhibits air incorporation and too high dough viscosity inhibits expansion of air bubbles [22].

In our study, the control muffin with egg exhibited the highest tang δ, indicating very soft gel dough. As can be seen in Table 4, the moduli of the eggless doughs were lower than the moduli of the doughs made with eggs. This was due to the functional role of an egg as a good emulsifier increasing dough viscoelasticity.

The moduli G' and G'' increased both for the eggless and egg-containing muffins fortified with 1% *D. macrophylla* This might be attributed to the capacity of potential fibers in *D. macrophylla* to absorb water in the dough, thereby lowering the free water level available to facilitate the movement of particles in the matrix. The direct consequence of this process was higher dough viscoelasticity. This finding was also supported by Jantinder *et al.* and Felicidad *et al.* who found that adding proteins and Jambolan fruit pulp increased muffin dough viscosity and viscoelasticity, respectively [16, 23].

The color of bakery products is one of the most important parameters that influences consumers' purchasing choices. Crumb color highly depends on the formulation ingredients, as well as the duration and temperature of baking, whereas crust color depends on caramelization and Maillard reactions.

The color data for our muffins are given in Table 5 as L^*, a^*, b^* and DE corresponding to lightness, redness, yellowness, and different color. We observed that the L^* and a^* values of crumb and crust color for the control muffins with egg were slightly lower than those for the control eggless muffins but the difference

Table 4 Rheology parameters of muffins with 1% of *Dacryodes macrophylla* extract

Rheology parameters	Eggless muffins			Egg-containing muffins		
	Control	1% DME	0.5% DME	Control	1% DME	0.5% DME
G'	103.90 ± 9.38^{a}	120.90 ± 9.39^{a}	–	664.00 ± 22.62^{b}	804.00 ± 23.13^{c}	–
G''	41.00 ± 3.56^{a}	42.29 ± 3.13^{a}	–	286.11 ± 7.47^{b}	299.9 ± 8.02^{b}	–
Tang delta	0.39 ± 0.03^{a}	0.35 ± 0.031^{a}	–	0.43 ± 0.01^{b}	0.37 ± 0.01^{a}	–

Values are mean ± standard deviation of triplicate experiments

Table 5 Color parameters of muffins with 1% of *Dacryodes macrophylla* extract

Color data	Color parameters	Eggless muffins			Egg-containing muffins		
		Control	1% DME	0.5% DME	Control	1% DME	0.5% DME
Crust	*L**	47.67 ± 0.58^{b}	38.17 ± 1.43^{a}	–	46.85 ± 0.40^{b}	34.74 ± 1.32^{a}	–
	*a**	3.80 ± 0.27^{d}	2.16 ± 0.09^{c}	–	0.95 ± 0.03^{b}	–0.17 ± 0.03^{a}	–
	*b**	22.38 ± 0.15^{b}	19.01 ± 0.49^{a}	–	28.55 ± 0.25^{c}	21.76 ± 0.32^{b}	–
	DE	46.56 ± 1.18^{a}	52.24 ± 0.13^{b}	–	47.70 ± 1.50^{a}	50.70 ± 0.63^{b}	–
Crumb	*L**	69.31 ± 1.13^{c}	46.70 ± 0.30^{b}	–	52.36 ± 1.75^{b}	36.21 ± 2.59^{a}	–
	*a**	10.33 ± 0.51^{c}	6.73 ± 0.58^{b}	–	6.16 ± 0.27^{b}	0.90 ± 0.13^{a}	–
	*b**	29.52 ± 0.21^{c}	23.49 ± 0.49^{b}	–	38.51 ± 0.16^{d}	18.03 ± 0.89^{a}	–
	DE	40.40 ± 2.72^{a}	57.40 ± 1.80^{c}	–	46.22 ± 0.07^{b}	69.62 ± 0.97^{d}	–

Values are mean ± standard deviation of triplicate experiments. The values carrying the same letter on the same row are not statistically significant ($P \geq 0.05$)

was not significant ($P \geq 0.05$). However, the *b** value of the control muffins with egg was higher than that of the control eggless muffins. This result could be due to egg protein enhancing the Maillard reaction by providing amino acid which may have reacted with sugars to generate dark-brown substances, thereby reducing the lightness of the final product, as well as redness [21, 22]. However, high yellowness might be attributed to the yellow part of the egg which might have impaired the color of the muffin dough. Moreover, incorporating the *D. macrophylla* extract decreased the *L**, *a**, *b** and DE values of the muffins (crumb and crust). This might be due to the pigments and polyphenol interacting with other constituents of the dough to impart greenness, thereby darkening the muffin's color. These results were in line with those reported by Rashida *et al*. and Marina *et al*. who noticed a reduction in the *L**, *a**, and *b** values with increased amounts of wheatgrass powder and avocado puree in muffin dough, respectively [5, 24].

Since the eggless and egg-containing muffins with 0.5% DME were heterogeneous, they were not included in the color analyses.

The textural parameters of the muffins are presented in Table 6. We found that the eggless muffin (4.68) was firmer than the muffin with egg (3.65). This was expected because an egg is a good emulsifier that acts as a plasticizer by increasing dough viscoelasticity and thereby reducing muffin firmness.

We also noticed that muffin firmness showed an opposite trend to the specific volume. This was in line with Nazaninet and Mostafa who concluded that softness was improved by both a higher cake volume and the anti-firming effect of the emulsifiers [22]. Furthermore, we found that firmness decreased with the incorporation of *D. macrophylla* This result was consistent with Prerna *et al*. who reported a decrease in muffin hardness with an increase in capsicum pomace [12]. Chewiness corresponds to the amount of energy required to disintegrate food for swallowing. Chewiness and gumminess of muffins follow the same trend as hardness since both parameters are dependent on firmness [17]. Springiness is a desirable property indicative of muffin elasticity, since it measures the extent of recovery between the first and the second compression. In our study, the springiness values were generally higher (0.68–1.97) than those obtained by Shevkani and Singh who added different protein isolates to muffins (0.64–0.85) [21].

The higher springiness of the control muffin with egg (1.97), compared to the control eggless sample (1.27), might be due to egg protein aggregation that improved the quality of muffins. However, this textural parameter decreased with the incorporation of *D. macrophylla* Prerna *et al*. also reported a decrease in springiness with the incorporation of capsicum pomace [12].

Cohesiveness is the ability of a material to stick to itself. It measures the internal resistance of food

Table 6 Texture parameters of muffins under study

Texture parameters	Eggless muffins			Egg-containing muffins		
	Control	1% DME	0.5% DME	Control	1% DME	0.5% DME
Hardness	4.68 ± 1.57^{c}	2.96 ± 0.55^{a}	3.03 ± 0.14^{a}	3.65 ± 0.32^{b}	2.61 ± 0.20^{a}	3.01 ± 0.05^{a}
Adhesiveness, mJ	0.007 ±0.003^{a}	0.008 ± 0.002^{a}	0.006 ±0.002^{a}	0.022 ± 0.002^{b}	0.031 ± 0.004^{bc}	0.023 ± 0.005^{b}
Cohesiveness	0.17 ± 0.011^{a}	0.16 ± 0.01^{a}	0.17 ± 0.01^{a}	0.29 ± 0.04^{b}	0.23 ± 0.01^{ab}	0.24 ± 0.01^{ab}
Springiness, mm	1.27 ± 0.11^{ab}	0.68 ± 0.04^{a}	0.75 ± 0.24^{a}	1.97 ± 0.45^{b}	1.02 ± 0.14^{ab}	0.84 ± 0.14^{a}
Gumminess, N	1.35 ± 0.33^{b}	0.63 ± 0.06^{ab}	0.71 ± 0.06^{ab}	1.05 ± 0.21^{ab}	0.48 ± 0.09^{a}	0.52 ± 0.03^{a}
Chewiness, mJ	3.02 ± 1.14^{b}	0.54 ± 0.15^{a}	0.74 ± 0.15^{ab}	1.38 ± 0.40^{ab}	0.35 ± 0.01^{a}	0.40 ± 0.17^{a}

Values are mean ± standard deviation of triplicate experiments

Table 7 DPPH assay: Antiradical activity of muffins with *Dacryodes macrophylla* extract

Radical Scavenging Activity	Eggless muffins			Egg-containing muffins		
	Control	1% DME	0.5% DME	Control	1% DME	0.5% DME
DPPH, µM troloxeq/mg	3.90 ± 0.52^{a}	6.84 ± 0.93^{bc}	5.06 ± 0.19^{abc}	4.57 ± 0.26^{ab}	7.85 ± 0.96^{c}	6.22 ± 0.30^{abc}

Values are mean ± standard deviation of triplicate experiments

Table 8 Sensory indicators of muffins under study

Sample	Color	Odor	Texture	Taste	Overall acceptability
			Eggless muffins		
Control	7.9 ± 0.1^{cd}	7.7 ± 0.1^{c}	7.4 ± 0.1^{c}	7.7 ± 0.1^{cd}	7.7 ± 0.1^{c}
1% DME	6.6 ± 0.1^{b}	7.1 ± 0.1^{b}	6.6 ± 0.1^{a}	7.2 ± 0.1^{c}	6.9 ± 0.1^{b}
0.5% DME	7.1 ± 0.1^{c}	6.9 ± 1.0^{b}	6.8 ± 0.1^{ab}	6.4 ± 0.1^{b}	6.6 ± 0.1^{b}
			Egg-containing muffins		
Control	7.9 ± 0.1^{cd}	7.6 ± 0.1^{c}	8.2 ± 0.1^{d}	7.8 ± 0.1^{d}	7.9 ± 0.1^{c}
1% DME	6.1 ± 0.1^{a}	6.3 ± 0.1^{a}	7.1 ± 0.1^{bc}	4.6 ± 0.2^{a}	5.3 ± 0.1^{a}
0.5% DME	7.5 ± 0.1^{c}	7.6 ± 0.1^{c}	8.0 ± 0.1^{d}	7.8 ± 0.1^{d}	7.7 ± 0.1^{c}

Values are mean ± standard deviation of triplicate experiments. Values carrying the same letter in the same column are not statistically significant ($P \geq 0.05$)

structure under some compression. We found the cohesiveness value of the control muffin with egg to be significantly higher (0.29) than that of the control eggless muffin (0.17). This result might be attributed to the egg protein network along with starch gel that might have impacted the muffin crumb texture [21].

Nevertheless, there was no significant difference in cohesiveness and adhesiveness values in the muffins fortified with *D. macrophylla*. Our results were in agreement with those found by Maria *et al.* who reported no significant differences in cohesiveness values among fiber-enriched bake products (squash seed flour) [20].

Overall, hardness, chewiness, gumminess, and springiness decreased with the incorporation of *D. macrophylla*, whereas cohesiveness and adhesiveness did not show any significant difference. However, the muffins with egg had lower hardness, chewiness, and gumminess and higher springiness, cohesiveness, and adhesiveness compared to the eggless muffins.

The total phenolic content assay determines both bound and unbound phenolics, while the radical scavenging activity assay measures free antioxidants. Thus, the latter is more efficient at preventing the reactive oxygen species from attacking lipoproteins, polyunsaturated fatty acids, DNA, amino acids, and sugars because it describes the capacity of an antioxidant in both food and biological systems [25].

Therefore, we used DPPH, a stable free radical, to evaluate the antioxidant capacity of our fortified muffins (Table 7). We found that the DPPH inhibition values for both eggless muffins and those with eggs increased significantly with the incorporation of *D. macrophylla* fruit. This result may be attributed to antioxidant compounds in *D. macrophylla* fruit increasing the DPPH activity.

Our results were consistent with those found by other authors who reported better DPPH activity with higher levels of Jambolan fruit pulp in the gluten-free muffins [11, 16].

The results of sensory evaluation of the muffin samples are presented in Table 8. The overall acceptability ranged from 5.3 to 7.9, meaning that the muffins were considered slightly or moderately pleasant according to the 9-point scale, except for the sample scoring 5.3 (neither unpleasant, nor pleasant).

The egg-containing muffins with 1% of DME recorded the lowest score (5.3) and was considered not acceptable because its acceptance index (59%) was lower than 70% (Table 9). This low score resulted from the sample's taste, which also had the lowest score. Most panelists considered its taste unpleasant, indicating bitterness after swallowing.

In contrast, the control egg-containing muffins received the highest overall acceptability score (7.9) and the highest acceptance index (87.88%). However, we found no significant difference with the control eggless muffin or the egg-containing muffin with 0.5% DME.

Table 9 Acceptance index and acceptability among muffin samples

Sample	Acceptance index, %	Acceptability, %	
		Like	Dislike
	Eggless muffins		
Control	86.00	101 (100.0)	0 (0.0)
1% DME	77.11	89 (88.12)	12 (11.88)
0.5% DME	73.33	83 (82.18)	18 (17.82)
	Egg-containing muffins		
Control	87.88	101 (100.0)	0 (0.0)
1% DME	59.22	44 (43.56)	57 (56.44)
0.5% DME	85.55	98 (97.03)	3 (2.97)

A product is acceptable when its acceptance index is greater than 70%

Table 10 Ranking of muffin samples

Sample	Eggless muffins			Egg-containing muffins		
	Control	1% DME	0.5% DME	Control	1% DME	0.5% DME
Rank	2.4 ± 0.1^{b}	4.2 ± 0.1^{c}	4.6 ± 0.1^{d}	1.9 ± 0.1^{a}	5.8 ± 0.1^{e}	2.1 ± 0.1^{ab}

Values are mean ± standard deviation of triplicate experiments. Values carrying the same letter in the same row are not statistically significant ($P \geq 0.05$)

The highest score of the control egg-containing muffin might be attributed to its texture, which was rated highest (8.2). Its appreciation by the panelists was in agreement with its springiness and specific volume (1.97 and 2.36, respectively), also scored highest.

The incorporation of *D. macrophylla* fruits tended to lower the average acceptance scores both for the eggless muffins and for those with eggs. The same trends were observed by Abdessalem *et al.* who introduced date fiber concentrate in muffins [13]. In our work, the egg-containing muffins with 0.5% DME had the best rank among the samples and received the same rank as the controls (both with and without egg). This means that the panelists preferred the muffins with *D. macrophylla* extract to the eggless control muffins.

CONCLUSION

Our results revealed that the incorporation of *Dacryodes macrophylla* L. fruit decreased water activity, the *L**, *a**, and *b** values, as well as the firmness of the muffins, whereas no prominent difference was observed in their baking loss, height, moisture, fat, cohesiveness, springiness, gumminess, or chewiness.

In contrast, *D. macrophylla* increased specific gravity, changed rheology, and tended to increase adhesiveness, antioxidant activity, and mineral contents (particularly Na and K) of the muffins. Another interesting result was that the panelists statistically accepted the muffins with 0.5% of DME, scoring them in the same range as the control ones.

Therefore, *D. macrophylla* fruit is a good potential ingredient to develop new bakery products rich in minerals and antioxidants but further investigations need to be done to improve the color acceptance of muffins and to determine the optimal concentration of *D. macrophylla*.

CONTRIBUTION

Our results revealed that the incorporation of Dacryodes macrophylla L. fruit decreased water activity, the L*, a*, and b* values, as well as the firmness of the muffins, whereas no prominent difference was observed in their baking loss, height, moisture, fat, cohesiveness, springiness, gumminess, or chewiness.

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Therefore, D. macrophylla fruit is a good potential ingredient to develop new bakery products rich in minerals and antioxidants but further investigations need to be done to improve the color acceptance of muffins and to determine the optimal concentration of D. macrophylla.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Kumquat fruit and leaves extracted with different solvents: phenolic content and antioxidant activity

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Abstract:

Introduction. Kumquat is a good source of vitamin C, as well as phenolic and flavonoid substances. In this study, we used various solvents to obtain extracts from fresh and lyophilized dried fruits and leaves of kumquat plant, as well as six mutants, to compare their total phenolic and flavonoid contents and antioxidant activities.

Study objects and methods. The total phenolic and flavonoid content was determined by the Folin-Ciocalteu method and the colorimetric method, respectively. The antioxidant capacities of the extracts were determined by commonly used antioxidant tests, such as the DPPH radical scavenging activity, reducing power, and metal chelating activity.

Results and discussion. The total phenolic content of the extracts was in the range of 3705–86 329 mg GAE/g extract. The total amount of flavonoid substance ranged from 5556 to 632 222 mg QUE/g extract. The highest free radical scavenging activity was observed in the kumquat leaves. We also found that the activity of dried fruit was lower than that of fresh fruit. According to our results, the differences in the phenolic contents of the studied plants affected their antioxidant properties. We determined that the extracts with a high phenolic content showed high antioxidant activity. No significant difference was detected between the rootstock kumquat type and its mutants. Finally, we found no chelating activity in the extracts obtained from fresh and lyophilized dried fruits.

Conclusion. Kumquat fruit and its leaves can be considered as functional foods due to phenolic compounds, which are able to neutralize free radicals.

Keywords: Antioxidant activity, flavonoid substance, kumquat, phenolic content, extract, solvent

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INTRODUCTION

Constantly developing technology, environmental pollution, ultraviolet radiation, and many other factors cause us to be exposed to various toxic substances. This results in more diseases caused by external environmental effects, including more pronounced genetic diseases. Preventing these diseases should become our priority. Since most of them occur in people with a weak immune system, we must focus on strengthening it. For this, we should consume foods with high antioxidant capacity, especially fruits and green leafy vegetables that contain antioxidative phytochemicals [1, 2].

Phytochemicals, or "plant chemicals", are compounds of plant origin, mostly polyphenols, that are essential for human life. They work alongside macronutrients such as carbohydrates, fats, and proteins, as well as 13 essential vitamins and 17 minerals [3]. Antioxidant phytochemicals, especially in fruits and vegetables, combine with free radicals in the human body to protect cells from the attacks of harmful radicals [4]. Bioactive compounds in fruits contain ascorbic acid, organic and phenolic acids, flavonoids, anthocyanins, and carotenoid substances [5, 6].

Citrus fruits come in different types, varieties, and flavors and have positive effects on health and nutrition. Although they have been known as the best sources of vitamin C for a long time, studies on their use as an antioxidant substance have recently gained momentum, due to their richness in phenolic compounds [7]. These bioactive components are responsible for various health benefits of citrus fruits, such as prevention of various diseases or protective effects to lower the risk of various cancers [8–10].

Citrus is a fruit group belonging to the genus Citrus, which is a member of the *Aurantioideae* subfamily of the *Rutaceae* family. The most common citrus varieties are orange (*Citrus sinensis* L.), mandarin (*Citrus reticulata* L.), lemon (*Citrus limon* L.), golden ball (*Citrus paradisi* L.), bitter orange (*Citrus auranthium* L.), and bergamot (*Citrus bergami* L.) [11]. In addition to fresh table consumption, citrus fruits are used as jam, marmalade or fruit juice, as well as raw material in the cosmetics sector [11].

Citrus fruits grow in subtropical climate areas. While mainland China, Southeast Asia, and India are major producers of citrus fruits due to suitable ecological conditions, they are also cultivated in the Mediterranean and Aegean coastal regions and partly in the Eastern Black Sea region of Turkey [12, 13]. The distribution of species and varieties of citrus fruits has gained a regional identity. For example, Washington navel, as well as other navel oranges, and Jaffa are harvested in the Eastern Mediterranean region.

Orange is one of the most produced and consumed citrus fruits in Turkey due to its preference in the juice industry and its great potential in the oil industry [14]. Orange is followed by mandarin and lemon products, respectively. Apart from these species, kumquat, which is called the "little gem of the citrus family", has recently grown in popularity, as well as such species as Altıntop and citrus, which are lower in production but can be considered important [15].

Kumquat is also called "citrus fortunella", taking its name from the Scottish horticultural expert Robert Fortune (1812–1880). This species, referred to as "komquot" in some countries, is also called a "golden orange" [16]. It is like a tiny lemon in shape and orangish in color. However, while orange and lemon are consumed after they are peeled, kumquat is consumed with its peel. Its scent is reminiscent of bergamot. It tastes sweet and leaves a lasting scent when you hold it in your hand.

In addition to fresh consumption, kumquat can be used in products such as confectionery, marmalade, liquor, and wine [17, 18]. Essential oil and bioactive ingredients obtained from its peel are used in the perfumery, pharmaceutical, and food industries [19]. Kumquat is an excellent source of nutrients containing minerals, ascorbic acid, carotenoids, flavonoids, and essential oils [20]. It contains remarkable antioxidant properties due to its flavonoid content [18]. However, there are very few studies about kumquat grown in Turkey.

In this study, we aimed to determine the antioxidant capacity and the total phenolic and flavonoid contents of the extracts obtained from fresh and lyophilized dried fruits and leaves of kumquat and six mutants from the Mersin Alata Horticultural Research Institute Directorate.

STUDY OBJECTS AND METHODS

Plant materials. Kumquat leaf and fruit samples were obtained from the Mersin Alata Horticultural Research Institute in November 2017 and January 2018, respectively. We used EP (Old Parcel) with rootstock species; EP.4, EP.29, EP.31 and YP (New Parcel); YP.117, YP.141, YP.188 mutants. The leaf samples were dried in room conditions and in the shade, and stored in a dry and cool environment for analysis. The fruit samples were freeze-dried, or lyophilized.

Chemicals and equipment. We used chemicals and solvents of analytical purity produced by Sigma, Aldrich, and Riedel-de Haen.

The equipment used in the study included a lyophilizer (Christ Alpha 1-2 LC plus), a vortex (Fisons), a rotary evaporator (Laborota 4000-efficient Heidolph), a spectrophotometer (Shimadzu UV-1601), a shaking water bath (Clifton 100–400 rpm; with thermostat), an incubator (EnoLab MB-80), an analytical balance (Gec Avery), a centrifuge (Nüvefuge CN180), a pH-meter (WTW pH 330i), a heater and magnetic stirrer (Chiltern HS31), a disperser and micropipettes (Eppendorf).

Extraction process. Phenolic compounds were extracted from kumquat fruits and leaves with a Soxhlet extraction device, using 260 mL of 99, 80, 60, and 50% methanol and pure water as solvents. In addition, 1 and 0.5% acidified ethanol and hexane solvents were used for kumquat leaves.

For extraction, 20 g of the samples were weighed into the cartridge and then placed in the Soxhlet extractor. The solvent(s) was added to the glass flask and kept in the Soxhlet device for 8 h. The solvent used for extraction was concentrated from the obtained phenolic extracts using a laboratory scale rotary evaporator under vacuum. The remaining part was removed by standing in the open air. The extracts were weighed gravimetrically and stored in dark vials at +4°C in the refrigerator until analysis.

Determination of free radical capture capacity (DPPH method). We used 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical to determine the free radical capture capacity according to the Blois method [21]. This method is based on the ability of the extracts to donate a proton or electron and to decolorize the purple colored DPPH solution (from violet to yellow). A decrease in the absorbance of the reaction mixture is indicative of high free radical scavenging activity.

All the extracts, BHA and BHT standards, and α-tocopherol were dissolved in ethanol at 1 mg/mL. After taking the samples and standards into 5 different volumes of 50, 100, 150, 250, and 500 µL, ethanol was added to a total volume of 3 mL. 1000 µL of 0.1 mM DPPH was added to the tubes and vortexed. The absorbance of the mixture, which was incubated for 30 min in the dark at room temperature, was measured in the UV-visible spectrophotometer at 517 nm. Calculations were made using the following formula:

$$\% \text{ free-radical scavenging activity} = \frac{A_C - A_{S/S}}{A_C} \times 100 \quad (1)$$

where A_C is the absorbance of the control reaction; $A_{S/S}$ is the absorbance of the sample or standard.

Determination of reducing capacity. The Oyaizu method was used to determine the reduction capacity [22]. According to this method, the reducing agent in the medium reduces Fe^{3+} ions to Fe^{2+} ions and a complex is formed by adding $FeCl_3$. The absorbance of the resulting complex is measured in the UV-visible spectrophotometer at 700 nm. The increase in absorbance of the reaction mixture is directly proportional to the reducing power of the sample.

All the extracts, BHA and BHT standards, and α-tocopherol were dissolved in ethanol at 1 mg/mL. 100, 250, and 500 µL of the samples and standards were taken into test tubes in three different volumes, and 3400, 3250, and 3000 µL of pH 6.6 phosphate buffer was added to them, respectively, to a total volume of 3500 µL. Then, after adding 2500 µL of 1% K_3 [Fe $(CN)_6$] and vortexing, it was left to incubate for 20 min in a water bath at 50°C. After the incubation, 2500 µL of 10% trichloroacetic acid (TCA) was added to the test tubes and centrifuged at 3000 rpm for 10 min. 1250 µL of the resulting supernatant was taken into empty tubes and 1250 µL of distilled water and 500 µL of 0.1% $FeCl_3$ were added to them. The mixture was vortexed and its absorbance was measured at 700 nm in the UV-visible spectrophotometer.

Determination of iron (II) ions chelating activity. Antioxidants with metal chelating properties inactivate free iron by binding it and thus inhibit the formation of radicals such as hydroxyl and peroxide, which are formed as a result of Fenton reactions ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^-$) [23]. The Dinis method was used to determine the activity of chelating iron (II) ions [24]. All the extracts and EDTA used as control were dissolved in ethanol to 1 mg/mL. The samples and standards were taken into 50, 100, 150, 250, and 500 µL test tubes, and 3700, 3650, 3600, 3500, and 3250 µL of ethanol was added to them, respectively, to a total volume of 3750 µL. Then, 50 µL of 2mM $FeCl_2$ was added and vortexed to incubate at room temperature for 10 min. Then, 200 µL of 5mM ferrosine was added. The resulting purple color was measured in the UV-visible spectrophotometer at 562 nm after the mixture was kept at room temperature for 25 min.

Determination of total phenolic content. The Folin-Ciocalteu method was used to determine the total phenolic content [25]. The Folin-Ciocalteu reagent (FCR) used in this method is molybophosphotungstic heteropolyacid ($3H_2O{\cdot}P_2O_5{\cdot}13WO_3{\cdot}5MoO_3{\cdot}10H_2O$). This method is based on the transfer of electrons from phenolic compounds and other reducing compounds to molybdenum. Phenolic compounds only react with the FCR in basic conditions (pH ~ 10) [26].

$$Mo(VI) + e^- \text{ (antioxidant)} \rightarrow Mo(V)$$

Commercially available 2N Folin-Ciocalteu reagent was prepared daily by diluting it with purified water at a ratio of 1/1 (v/v). 500 µL of the extracts (1 mg/mL) was taken into test tubes and 500 µL of distilled water was added. After 250 µL of 1 N Folin reagent was added to the mixture, it was incubated for 5 min by vortexing. 1250 µL of 2% Na_2CO_3 solution was added to it, vortexed, and then kept at room temperature for 2 h. The absorbance of the resulting mixture was measured at 765 nm in the UV-visible spectrophotometer. The phenolic content of the extracts was given as mg gallic acid equivalent (GAE)/g extract.

Determination of total flavonoid content. The total flavonoid content was measured by an aluminum chloride colorimetric test according to Zhishen *et al.* [27]. All the extracts and a quercetin solution used as a standard were dissolved in 1 mg/mL ethanol. 500 µL was taken from the extracts prepared in the test tubes and pure water was added to a total volume of 5000 µL. To this mixture, 300 µL of 5% $NaNO_2$ solution was added and left to incubate at room temperature for 5 min, and then 300 µL of 10% $AlCl_3$ solution was added. After waiting for 6 min, 2 mL of 1.0 M NaOH solution was added and the volume was completed to 20 mL with distilled water. The absorbance of the solution was measured at 510 nm in the UV-visible spectrophotometer. The total flavonoid content of the extracts was given as mg quercetin equivalent (QUE)/g extract.

RESULTS AND DISCUSSION

The solubility and distribution of phenolic compounds in the solvent depend on the polarity of their structure, so the choice of solvent and method is one of the most important steps. In our study, for fresh and lyophilized dried fruits, we preferred methanol and its aqueous solutions, as well as pure water. For leaves, we preferred methanol and aqueous solutions, distilled water, and ethanol acidified with hexane.

Three different methods (DPPH radical scavenging activity, reducing capacity, and iron (II) ions chelating activity) were used to determine the antioxidant capacity. We thought that the extracts could show activity through different mechanisms depending on the diversity of phenolic substances. In addition, we determined the total phenolic content and flavonoid amounts in all the extracts in order to show that the antioxidant effect was proportional to the plant content.

Free radical scavenging activity. The DPPH method is commonly used to evaluate the antioxidant activity of natural products, as it is easy and highly sensitive. DPPH (2,2-diphenyl-1-picrylhydrazyl) is a commercially available stable organic nitrogen radical. The antioxidant effect is proportional to the removal of the DPPH radical. The DPPH radical (DPPH$^{\bullet}$) is purple in color due to the unpaired nitrogen atom. When the DPPH solution reacts with an oxygen atom of a substance (antioxidant chemical) that can give hydrogen atoms, the initial purple color disappears as the radical

reduces, turning yellow [28]. The reaction takes place stoichiometrically according to the number of hydrogen atoms absorbed. Therefore, the antioxidant effect was easily determined by following the decrease in UV absorbance at 517 nm until it stabilized.

We observed that the highest free radical scavenging activity was in kumquat leaves, and the activity of kumquat fruit decreased when dried (Table 1). There was no significant difference between the rootstock kumquat type and its mutants. The free radical scavenging activities of the extracts were slightly below the standards (BHA, BHT, and α-Tocopherol). The highest activity (81.66%) was seen in the YP.188 hybrid leaf extract using 80% methanol solvent. As for the fruits, the highest activity (61.37%) was in the EP.4 hybrid extract using a pure methanol solvent.

When we examined all the samples, we associated high phenolic content with high antioxidant activity. We found that the total phenolic content was higher in the samples with high antioxidant activity. As a matter of fact, the leaf extract with high antioxidant activity also had a high phenolic content (85.651 ± 0.030 mg GAE/g extract).

However, when we carefully examined the results, we saw that having a high amount of phenolic substances did not give high results in all antioxidant activity methods. For example, although the YP.188 Leaf 80% methanol and the YP.188 Leaf 50% methanol extracts contained almost the same amount of phenolic substances, the former had higher activity in the applied antioxidant activity methods. This could be explained by the differences between the phenolic substances they contained depending on the solvent used.

In fact, other studies have found that the antioxidant activity of methanol and ethanol extracts, which generally contained phenolic substances, was higher than in other solvent systems [29]. For example, Jayaprakasha *et al.* extracted powdered kumquat fruit in 5 different solvents and investigated the radical capture capacities of the extracts, their amounts in total phenolic matter, and their inhibitory properties for prostate cancer [30].

In this study, the extracts obtained from EtOAc and MeOH-water (4:1, v/v) solvents were found to have the highest and lowest total phenolics, respectively, according to the Folin-Ciocalteu method. It was also observed that the EtOAc and MeOH extracts exhibited the highest and lowest 1,1-diphenyl-2-picyrylhydrazyl (DPPH) radical scavenging activity, respectively [30].

Table 1 DPPH radical scavenging activity of kumquat fruit and leaf extracts, μg/mL (mean ± SD of triplicate)

Extracts and standards	12.5*	25.0*	37.5*	62.5*	125*
Rootstock fresh fruit pure methanol	7.22 ± 0.10	11.19 ± 0.2	12.64 ± 0.1	20.94 ± 0.1	30.32 ± 0.3
Rootstock fresh fruit 80% methanol	6.50 ± 0.10	7.94 ± 0.1	9.03 ± 0.2	12.64 ± 0.3	19.49 ± 0.1
Rootstock fresh fruit 60% methanol	4.69 ± 0.10	7.58 ± 0.1	8.66 ± 0.2	13.00 ± 0.3	21.30 ± 0.3
Rootstock fresh fruit 50% methanol	7.03 ± 0.10	9.03 ± 0.0	18.66 ± 0.1	22.02 ± 0.3	28.52 ± 0.1
Rootstock fresh fruit pure water	10.83 ± 0.0	14.08 ± 0.2	14.08 ± 0.2	22.02 ± 0.0	33.57 ± 0.3
Rootstock dry fruit pure methanol	3.09 ± 0.10	4.75 ± 0.2	7.56 ± 0.3	8.25 ± 0.3	9.97 ± 0.1
Rootstock dry fruit 80% methanol	5.15 ± 0.20	6.53 ± 0.0	8.25 ± 0.2	9.28 ± 0.3	12.37 ± 0.3
Rootstock dry fruit 60% methanol	4.81 ± 0.00	7.56 ± 0.1	8.25 ± 0.0	8.93 ± 0.0	9.62 ± 0.2
Rootstock dry fruit 50% methanol	3.78 ± 0.00	6.53 ± 0.1	7.22 ± 0.0	8.25 ± 0.0	10.31 ± 0.2
Rootstock dry fruit pure water	3.78 ± 0.20	4.47 ± 0.1	6.87 ± 0.0	7.22 ± 0.1	8.59 ± 0.2
Rootstock leaf pure methanol	12.46 ± 0.20	23.88 ± 0.3	32.87 ± 0.1	41.87 ± 0.1	57.09 ± 0.1
Rootstock leaf 80% methanol	21.45 ± 0.10	29.76 ± 0.2	37.72 ± 0.2	50.52 ± 0.1	65.74 ± 0.5
Rootstock leaf 60% methanol	13.49 ± 0.20	18.15 ± 0.1	33.91 ± 0.2	46.71 ± 0.0	65.40 ± 0.3
Rootstock leaf 50% methanol	20.76 ± 0.30	31.49 ± 0.0	39.10 ± 0.1	50.87 ± 0.2	66.44 ± 0.3
Rootstock leaf pure water	12.11 ± 0.10	21.11 ± 0.1	30.10 ± 0.3	36.33 ± 0.2	52.25 ± 0.2
Rootstock leaf 0.5% acidified ethanol	3.46 ± 0.10	8.30 ± 0.2	13.84 ± 0.3	20.42 ± 0.1	34.26 ± 0.4
Rootstock leaf 1% acidified ethanol	5.19 ± 0.10	13.15 ± 0.2	15.22 ± 0.1	25.61 ± 0.2	40.83 ± 0.1
Rootstock leaf hexane	n.d.	n.d.	2.42 ± 0.2	11.07 ± 0.1	12.04 ± 0.1
EP.4 fresh fruit pure methanol	16.97 ± 0.1	20.22 ± 0.3	35.02 ± 0.2	42.60 ± 0.1	61.37 ± 0.3
EP.4 fresh fruit 80% methanol	15.88 ± 0.1	16.61 ± 0.2	21.66 ± 0.2	28.52 ± 0.3	42.96 ± 0.5
EP.4 fresh fruit 60% methanol	13.36 ± 0.1	15.88 ± 0.1	15.88 ± 0.2	22.74 ± 0.3	31.05 ± 0.1
EP.4 fresh fruit 50% methanol	15.88 ± 0.2	18.05 ± 0.1	19.86 ± 0.1	23.10 ± 0.3	33.57 ± 0.2
EP.4 fresh fruit pure water	15.88 ± 0.2	22.38 ± 0.3	28.05 ± 0.1	34.55 ± 0.3	35.38 ± 0.2
EP.4 dry fruit pure methanol	2.06 ± 0.1	2.75 ± 0.3	10.31 ± 0.1	11.37 ± 0.1	14.43 ± 0.1
EP.4 dry fruit 80% methanol	6.25 ± 0.2	7.56 ± 0.0	8.25 ± 0.2	10.31 ± 0.1	14.43 ± 0.2
EP.4 dry fruit 60% methanol	6.53 ± 0.1	8.25 ± 0.1	9.97 ± 0.3	10.97 ± 0.4	13.06 ± 0.1
EP.4 dry fruit 50% methanol	7.56 ± 0.1	8.93 ± 0.1	9.08 ± 0.2	9.97 ± 0.4	13.06 ± 0.3
EP.4 dry fruit pure water	5.15 ± 0.2	8.93 ± 0.2	10.65 ± 0.3	11.68 ± 0.0	15.12 ± 0.3

Continuation of Table 1

Extracts and standards	12.5*	25.0*	37.5*	62.5*	125*
EP.4 leaf pure methanol	14.88 ± 0.1	23.53 ± 0.0	28.03 ± 0.1	36.33 ± 0.2	54.67 ± 0.7
EP.4 leaf 80% methanol	17.65 ± 0.1	32.53 ± 0.3	39.45 ± 0.2	47.06 ± 0.1	71.63 ± 0.5
EP.4 leaf 60% methanol	16.65 ± 0.1	25.61 ± 0.3	34.95 ± 0.2	44.64 ± 0.1	63.67 ± 0.3
EP.4 leaf 50% methanol	16.61 ± 0.2	26.99 ± 0.3	36.33 ± 0.1	46.02 ± 0.1	65.05 ± 0.1
EP.4 leaf pure water	18.34 ± 0.2	27.68 ± 0.2	34.26 ± 0.2	47.40 ± 0.3	55.36 ± 0.3
EP.4 leaf 0.5% acidified ethanol	1.73 ± 0.0	7.61 ± 0.1	9.69 ± 0.3	17.99	29.41 ± 0.5
EP.4 leaf 1% acidified ethanol	6.92 ± 0.1	12.80 ± 0.2	18.34 ± 0.3	24.57 ± 0.2	35.64 ± 0.3
EP.4 leaf hexane	n.d.	n.d.	n.d.	n.d.	7.96 ± 0.5
EP.29 fresh fruit pure methanol	9.42 ± 0.2	15.16 ± 0.2	22.38 ± 0.1	28.88 ± 0.1	37.91 ± 0.1
EP.29 fresh fruit 80% methanol	9.75 ± 0.0	14.08 ± 0.2	17.69 ± 0.1	22.02 ± 0.3	31.77 ± 0.5
EP.29 fresh fruit 60% methanol	12.64 ± 0.1	17.33 ± 0.2	21.30 ± 0.3	25.63 ± 0.3	36.10 ± 0.2
EP.29 fresh fruit 50% methanol	13.72 ± 0.1	17.69 ± 0.1	19.49 ± 0.0	26.71 ± 0.2	36.10 ± 0.4
EP.29 fresh fruit pure water	14.44 ± 0.1	15.16 ± 0.1	17.69 ± 0.1	20.94 ± 0.3	33.21 ± 0.4
EP.29 dry fruit pure methanol	7.90 ± 0.1	9.28 ± 0.1	10.31 ± 0.2	13.06 ± 0.1	15.81 ± 0.5
EP.29 dry fruit 80% methanol	7.56 ± 0.1	11.68 ± 0.1	14.09 ± 0.2	15.43 ± 0.1	19.59 ± 0.2
EP.29 dry fruit 60% methanol	7.90 ± 0.1	10.31 ± 0.1	12.65 ± 0.1	14.43 ± 0.2	19.93 ± 0.4
EP.29 dry fruit 50% methanol	6.80 ± 0.1	9.28 ± 0.1	10.31 ± 0.2	11.68 ± 0.3	14.09 ± 0.5
EP.29 dry fruit pure water	4.81 ± 0.1	5.84 ± 0.1	7.56 ± 0.1	9.28 ± 0.3	12.03 ± 0.1
EP.29 leaf pure methanol	15.57 ± 0.2	26.99 ± 0.1	29.76 ± 0.1	36.33 ± 0.0	52.25 ± 0.3
EP.29 leaf 80% methanol	9.00 ± 0.1	22.15 ± 0.1	31.49 ± 0.1	41.87 ± 0.4	59.86 ± 0.7
EP.29 leaf 60% methanol	12.46 ± 0.2	26.99 ± 0.2	32.53 ± 0.3	46.71 ± 0.1	63.32 ± 0.4
EP.29 leaf 50% methanol	16.96 ± 0.2	28.37 ± 0.1	33.22 ± 0.2	45.67 ± 0.2	60.55 ± 0.4
EP.29 leaf pure water	10.73 ± 0.1	20.7 ± 0.1	26.99 ± 0.1	35.99 ± 0.2	51.21 ± 0.2
EP.29 leaf 0.5% acidified ethanol	3.11 ± 0.1	8.65 ± 0.2	13.84 ± 0.1	20.42 ± 0.2	34.26 ± 0.5
EP.29 leaf 1% acidified ethanol	6.57 ± 0.2	11.07 ± 0.2	15.57 ± 0.1	24.91 ± 0.3	39.79 ± 0.2
EP.29 leaf hexane	n.d.	n.d.	n.d.	n.d.	5.54 ± 0.1
EP.31 fresh fruit pure methanol	2.89 ± 0.1	17.69 ± 0.0	22.74 ± 0.3	29.24 ± 0.2	40.7 ± 0.4
EP.31 fresh fruit 80% methanol	12.27 ± 0.2	16.97 ± 0.1	23.10 ± 0.1	33.94 ± 0.2	51.62 ± 0.5
EP.31 fresh fruit 60% methanol	11.91 ± 0.1	22.02 ± 0.1	25.63 ± 0.2	40.43 ± 0.3	54.51 ± 0.1
EP.31 fresh fruit 50% methanol	14.80 ± 0.1	15.52 ± 0.3	21.66 ± 0.1	28.16 ± 0.5	42.96 ± 0.1
EP.31 fresh fruit pure water	8.30 ± 0.2	13.00 ± 0.3	13.72 ± 0.0	18.41 ± 0.1	23.83 ± 0.1
EP.31 dry fruit pure methanol	7.38 ± 0.2	8.72 ± 0.1	30.20 ± 0.2	39.73 ± 0.1	43.42 ± 0.1
EP.31 dry fruit 80% methanol	7.05 ± 0.2	8.39 ± 0.2	8.39 ± 0.1	10.74 ± 0.3	14.43 ± 0.2
EP.31 dry fruit 60% methanol	3.69 ± 0.1	6.38 ± 0.1	8.72 ± 0.2	9.73 ± 0.1	11.07 ± 0.2
EP.31 dry fruit 50% methanol	3.36 ± 0.0	6.04 ± 0.2	6.38 ± 0.1	8.72 ± 0.0	11.41 ± 0.1
EP.31 dry fruit pure water	6.04 ± 0.1	8.39 ± 0.1	3.36 ± 0.1	3.02 ± 0.3	4.36 ± 0.1
EP.31 leaf pure methanol	13.49 ± 0.1	22.15 ± 0.2	28.37 ± 0.1	37.37 ± 0.2	53.98 ± 0.3
EP.31 leaf 80% methanol	20.42 ± 0.1	31.14 ± 0.2	39.45 ± 0.2	50.52 ± 0.3	68.17 ± 0.4
EP.31 leaf 60% methanol	17.99 ± 0.1	30.80 ± 0.1	39.10 ± 0.1	49.83 ± 0.5	65.05 ± 0.4
EP.31 leaf 50% methanol	19.72 ± 0.1	30.45 ± 0.1	33.22 ± 0.2	49.13 ± 0.1	63.67 ± 0.1
EP.31 leaf pure water	12.11 ± 0.1	21.11 ± 0.1	24.91 ± 0.2	36.33 ± 0.3	53.98 ± 0.5
EP.31 leaf 0.5% acidified ethanol	8.30 ± 0.1	17.30 ± 0.2	24.57 ± 0.2	33.56 ± 0.3	51.56 ± 0.5
EP.31 leaf 1% acidified ethanol	10.3 ± 0.2	16.96 ± 0.1	21.45 ± 0.0	32.18 ± 0.3	47.06 ± 0.3
EP.31 leaf hexane	n.d.	n.d.	n.d.	n.d.	8.65 ± 0.3
YP.117 fresh fruit pure methanol	10.83 ± 0.2	18.41 ± 0.2	21.66 ± 0.4	33.94 ± 0.1	46.93 ± 0.4
YP.117 fresh fruit 80% methanol	10.11 ± 0.2	15.75 ± 0.1	20.58 ± 0.2	27.08 ± 0.5	41.88 ± 0.4
YP.117 fresh fruit 60% methanol	12.27 ± 0.1	15.88 ± 0.1	18.41 ± 0.3	27.08 ± 0.1	40.7 ± 0.3
YP.117 fresh fruit 50% methanol	12.64 ± 0.1	16.61 ± 0.2	22.38 ± 0.1	30.69 ± 0.1	48.38 ± 0.4
YP.117 fresh fruit pure water	15.88 ± 0.1	13.36 ± 0.3	20.94 ± 0.2	28.16 ± 0.1	41.52 ± 0.4
YP.117 dry fruit pure methanol	2.68 ± 0.1	3.45 ± 0.1	4.68 ± 0.1	6.71 ± 0.0	10.40 ± 0.3
YP.117 dry fruit 80% methanol	3.69 ± 0.1	6.38 ± 0.2	7.05 ± 0.1	8.05 ± 0.1	8.39 ± 0.2
YP.117 dry fruit 60% methanol	5.03 ± 0.1	7.72 ± 0.1	8.71 ± 0.3	8.92 ± 0.1	11.74 ± 0.1
YP.117 dry fruit 50% methanol	4.70 ± 0.1	5.09 ± 0.2	6.38 ± 0.3	6.38 ± 0.1	6.38 ± 0.3
YP.117 dry fruit pure water	5.03 ± 0.3	5.18 ± 0.3	6.04 ± 0.3	7.05 ± 0.2	11.41 ± 0.2
YP.117 leaf pure methanol	13.84 ± 0.2	22.84 ± 0.1	30.45 ± 0.1	42.91 ± 0.5	60.55 ± 0.5
YP.117 leaf 80% methanol	20.70 ± 0.3	26.99 ± 0.2	33.56 ± 0.1	47.40 ± 0.2	67.47 ± 0.7
YP.117 leaf 60% methanol	14.53 ± 0.2	21.80 ± 0.3	39.45 ± 0.2	50.87 ± 0.2	65.40 ± 0.1

Continuation of Table 1

Extracts and standards	12.5*	25.0*	37.5*	62.5*	125*
YP.117 leaf 50% methanol	19.03 ± 0.3	33.56 ± 0.2	39.10 ± 0.2	52.25 ± 0.1	65.74 ± 0.1
YP.117 leaf pure water	14.53 ± 0.4	20.76 ± 0.2	32.18 ± 0.1	40.83 ± 0.3	57.44 ± 0.4
YP.117 leaf 0.5% acidified ethanol	7.22 ± 0.1	13.15 ± 0.2	19.72 ± 0.1	28.72 ± 0.1	45.67 ± 0.3
YP.117 leaf 1% acidified ethanol	7.96 ± 0.1	15.22 ± 0.4	17.99 ± 0.1	28.72 ± 0.1	46.37 ± 0.3
YP.117 leaf hexane	n.d.	n.d.	n.d.	2.69 ± 0.1	14.19 ± 0.2
YP.141 fresh fruit pure methanol	9.03 ± 0.1	11.91 ± 0.1	14.80 ± 0.2	23.47 ± 0.2	35.38 ± 0.2
YP.141 fresh fruit 80% methanol	9.39 ± 0.1	10.83 ± 0.2	16.61 ± 0.3	25.27 ± 0.1	35.74 ± 0.3
YP.141 fresh fruit 60% methanol	11.91 ± 0.1	16.08 ± 0.2	19.86 ± 0.2	26.35 ± 0.2	37.91 ± 0.5
YP.141 fresh fruit 50% methanol	5.05 ± 0.1	8.66 ± 0.1	14.08 ± 0.2	24.55 ± 0.3	41.88 ± 0.2
YP.141 fresh fruit pure water	9.39 ± 0.1	10.11 ± 0.1	15.16 ± 0.2	21.30 ± 0.0	31.05 ± 0.2
YP.141 dry fruit pure methanol	5.03 ± 0.1	5.70 ± 0.1	7.72 ± 0.1	10.40 ± 0.2	12.42 ± 0.2
YP.141 dry fruit 80% methanol	7.72 ± 0.1	9.40 ± 0.0	14.43 ± 0.2	10.40 ± 0.0	11.74 ± 0.4
YP.141 dry fruit 60% methanol	8.05 ± 0.1	8.72 ± 0.1	9.73 ± 0.2	30.87 ± 0.3	32.35 ± 0.1
YP.141 dry fruit 50% methanol	1.01 ± 0.1	6.71 ± 0.1	7.38 ± 0.3	10.40 ± 0.2	12.42 ± 0.5
YP.141 dry fruit pure water	7.05 ± 0.2	16.78 ± 0.1	15.7 ± 0.2	15.37 ± 0.1	16.7 ± 0.2
YP.141 leaf pure methanol	18.34 ± 0.2	28.03 ± 0.2	33.56 ± 0.1	48.79 ± 0.3	64.71 ± 0.1
YP.141 leaf 80% methanol	17.65 ± 0.0	33.56 ± 0.1	43.94 ± 0.2	57.09 ± 0.3	72.66 ± 0.4
YP.141 leaf 60% methanol	18.69 ± 0.2	32.53 ± 0.2	39.79 ± 0.1	53.63 ± 0.6	67.82 ± 0.4
YP.141 leaf 50% methanol	17.65 ± 0.3	31.49 ± 0.2	39.79 ± 0.1	51.90 ± 0.3	63.32 ± 0.5
YP.141 leaf pure water	16.61 ± 0.4	28.03 ± 0.2	32.87 ± 0.2	45.67 ± 0.7	61.59 ± 0.3
YP.141 leaf 0.5% acidified ethanol	7.61 ± 0.1	15.57 ± 0.1	21.45 ± 0.3	32.87 ± 0.2	50.52 ± 0.4
YP.141 leaf 1% acidified ethanol	8.30 ± 0.1	14.88 ± 0.1	19.03 ± 0.3	32.18 ± 0.2	48.79 ± 0.4
YP.141 leaf hexane	n.d.	n.d.	1.38 ± 0.1	5.88 ± 0.1	15.92 ± 0.1
YP.188 fresh fruit pure methanol	5.42 ± 0.2	10.83 ± 0.1	13.72 ± 0.2	21.66 ± 0.1	36.10 ± 0.6
YP.188 fresh fruit 80% methanol	5.39 ± 0.2	9.42 ± 0.1	11.91 ± 0.3	22.74 ± 0.1	33.57 ± 0.2
YP.188 fresh fruit 60% methanol	9.39 ± 0.2	12.27 ± 0.2	14.08 ± 0.2	23.10 ± 0.1	33.94 ± 0.2
YP.188 fresh fruit 50% methanol	11.05 ± 0.2	11.19 ± 0.2	15.88 ± 0.5	22.74 ± 0.3	32.85 ± 0.4
YP.188 fresh fruit pure water	13.00 ± 0.2	13.72 ± 0.1	22.38 ± 0.3	33.57 ± 0.3	46.93 ± 0.3
YP.188 dry fruit pure methanol	3.09 ± 0.2	4.75 ± 0.2	7.56 ± 0.2	8.25 ± 0.1	9.97 ± 0.1
YP.188 dry fruit 80% methanol	5.15 ± 0.0	6.53 ± 0.2	8.25 ± 0.2	9.28 ± 0.1	12.37 ± 0.1
YP.188 dry fruit 60% methanol	4.81 ± 0.2	7.56 ± 0.2	8.25 ± 0.1	8.93 ± 0.1	9.62 ± 0.2
YP.188 dry fruit 50% methanol	3.78 ± 0.2	6.53 ± 0.1	7.22 ± 0.3	8.25 ± 0.2	10.31 ± 0.5
YP.188 dry fruit pure water	3.78 ± 0.2	4.47 ± 0.2	6.87 ± 0.3	7.22 ± 0.1	8.59 ± 0.1
YP.188 leaf pure methanol	21.11 ± 0.1	31.14 ± 0.4	35.99 ± 0.2	53.98 ± 0.2	73.70 ± 0.1
YP.188 leaf 80% methanol	25.26 ± 0.2	41.18 ± 0.1	47.06 ± 0.3	66.09 ± 0.3	81.66 ± 0.4
YP.188 leaf 60% methanol	29.07 ± 0.0	46.02 ± 0.5	52.25 ± 0.2	66.78 ± 0.4	80.97 ± 0.4
YP.188 leaf 50% methanol	20.42 ± 0.2	33.56 ± 0.1	45.67 ± 0.1	57.09 ± 0.0	73.70 ± 0.1
YP.188 leaf pure water	13.15 ± 0.2	18.69 ± 0.2	21.45 ± 0.1	48.79 ± 0.4	67.82 ± 0.7
YP.188 leaf 0.5% acidified ethanol	7.27 ± 0.1	15.22 ± 0.3	22.49 ± 0.2	37.02 ± 0.2	57.44 ± 0.2
YP.188 leaf 1% acidified ethanol	10.38 ± 0.1	16.96 ± 0.0	21.11 ± 0.2	32.18 ± 0.3	50.17 ± 0.2
YP.188 leaf hexane	n.d.	n.d.	n.d.	4.50 ± 0.2	17.30 ± 0.2
BHA	73.36 ± 0.2	79.58 ± 0.2	80.62 ± 0.1	83.39 ± 0.3	84.43 ± 0.2
BHT	65.74 ± 0.0	72.32 ± 0.1	73.01 ± 0.2	73.36 ± 0.1	72.32 ± 0.0
α-tocopherol	76.12 ± 0.2	76.12 ± 0.1	81.66 ± 0.2	84.78 ± 0.2	84.43 ± 0.0

*It represents the concentrations of the solutions prepared by taking 50, 100, 150, 250, and 500 µL of standard and extract stock solutions prepared as 1 mg/mL and completing the total volume of 3 mL

n.d.: not detected

The chelating activity of iron (II) ions. Antioxidants with metal chelating properties inactivate it by binding free iron and thus inhibit the formation of radicals such as hydroxyl and peroxide, which are formed as a result of Fenton reactions. Therefore, metal chelating plays an important role in determining antioxidant activity [31].

We evaluated the metal ion chelating activity according to the competition between plant extracts with ferrosine in order to bind Fe^{2+} ions in the solution. We observed no chelating activity in the extracts obtained from moist and lyophilized dried fruits (Table 2). The pure methanol extracts showed weak activity in kumquat leaves, while the extracts obtained from aqueous solvents showed no activity at all.

Table 2 Metal chelating capacities of kumquat fruit and leaf extract, µg/mL (mean ± SD of triplicate)

Extracts and standards	12.5*	25.0*	37.5*	62.5*	125*
Rootstock leaf pure methanol	10.70 ± 0.20	14.95 ± 0.1	20.44 ± 0.1	20.71 ± 0.3	5.76 ± 0.1
Rootstock leaf 0.5% acidified ethanol	4.39 ± 0.10	5.12 ± 0.0	4.39 ± 0.1	5.95 ± 0.1	6.73 ± 0.1
Rootstock leaf 1% acidified ethanol	3.51 ± 0.10	10.10 ± 0.1	11.86 ± 0.2	13.47 ± 0.1	18.59 ± 0.3
Rootstock leaf hexane	2.99 ± 0.0	8.52 ± 0.1	15.10 ± 0.2	18.30 ± 0.1	17.19 ± 0.4
EP.4 leaf pure methanol	10.56 ± 0.10	21.26 ± 0.1	23.32 ± 0.2	28.94 ± 0.1	16.74 ± 0.2
EP.4 leaf 0.5% acidified ethanol	3.51 ± 0.1	10.10 ± 0.2	11.86 ± 0.1	13.47 ± 0.1	18.59 ± 0.3
EP.4 leaf 1% acidified ethanol	4.10 ± 0.1	3.07 ± 0.2	5.42 ± 0.1	6.59 ± 0.3	6.83 ± 0.2
EP.4 leaf hexane	9.87 ± 0.2	14.20 ± 0.1	24.22 ± 0.2	34.08 ± 0.3	25.41 ± 0.3
EP.29 leaf pure methanol	13.03 ± 0.1	25.24 ± 0.1	32.24 ± 0.2	36.90 ± 0.3	19.48 ± 0.1
EP.29 leaf 0.5% acidified ethanol	4.93 ± 0.0	16.29 ± 0.1	23.47 ± 0.1	37.07 ± 0.1	24.96 ± 0.3
EP.29 leaf 1% acidified ethanol	5.38 ± 0.0	10.91 ± 0.2	17.32 ± 0.1	30.19 ± 0.2	31.24 ± 0.1
EP.29 leaf hexane	1.35 ± 0.1	2.54 ± 0.1	6.13 ± 0.1	12.26 ± 0.2	8.97 ± 0.2
EP.31 leaf pure methanol	27.36 ± 0.2	43.84 ± 0.1	44.64 ± 0.1	42.06 ± 0.3	31.20 ± 0.4
EP.31 leaf 0.5% acidified ethanol	2.54 ± 0.2	5.38 ± 0.2	10.46 ± 0.1	15.40 ± 0.3	15.99 ± 0.1
EP.31 leaf 1% acidified ethanol	2.69 ± 0.0	6.43 ± 0.1	9.72 ± 0.2	10.27 ± 0.1	7.92 ± 0.1
EP.31 leaf hexane	8.37 ± 0.2	9.57 ± 0.1	18.22 ± 0.1	20.33 ± 0.2	20.78 ± 0.3
YP.117 leaf pure methanol	11.17 ± 0.2	16.48 ± 0.3	22.35 ± 0.3	24.21 ± 0.2	24.58 ± 0.1
YP.117 leaf 0.5% acidified ethanol	3.44 ± 0.1	9.87 ± 0.0	11.36 ± 0.2	24.66 ± 0.0	19.28 ± 0.3
YP.117 leaf 1% acidified ethanol	5.23 ± 0.2	5.48 ± 0.1	14.20 ± 0.2	14.35 ± 0.2	14.05 ± 0.2
YP.117 leaf hexane	8.67 ± 0.1	20.63 ± 0.4	30.64 ± 0.3	36.32 ± 0.2	38.57 ± 0.3
YP.141 leaf pure methanol	14.79 ± 0.2	30.1 ± 0.2	35.43 ± 0.2	38.53 ± 0.3	35.56 ± 0.1
YP.141 leaf 0.5% acidified ethanol	2.09 ± 0.1	2.64 ± 0.1	6.43 ± 0.2	8.37 ± 0.2	8.74 ± 0.1
YP.141 leaf 1% acidified ethanol	1.20 ± 0.1	5.53 ± 0.1	10.31 ± 0.1	11.96 ± 0.0	14.80 ± 0.2
YP.141 leaf hexane	6.13 ± 0.1	11.36 ± 0.2	13.49 ± 0.1	17.04 ± 0.3	19.73 ± 0.1
YP.188 leaf pure methanol	16.84 ± 0.1	27.38 ± 0.2	31.63 ± 0.3	37.67 ± 0.3	44.39 ± 0.2
YP.188 leaf 0.5% acidified ethanol	2.54 ± 0.0	6.28 ± 0.1	8.07 ± 0.2	11.96 ± 0.0	17.32 ± 0.3
YP.188 leaf 1% acidified ethanol	2.69 ± 0.1	6.88 ± 0.1	10.91 ± 0.1	16.89 ± 0.1	16.35 ± 0.3
YP.188 leaf hexane	13.15 ± 0.1	28.10 ± 0.2	42.75 ± 0.2	50.37 ± 0.1	42.75 ± 0.3
EDTA	3.30 ± 0.0	25.93 ± 0.1	64.18 ± 0.2	91.40 ± 0.1	92.26 ± 0.1

*It represents the concentrations of the solutions prepared by taking 50, 100, 150, 250, and 500 µL of standard and extract stock solutions prepared as 1 mg/mL and completing the total volume of 3 mL

In addition, weak chelating activity was detected in the 0.5 and 1% acidified ethanol extracts of kumquat leaves and the hexane solvent extracts. The highest activity (50.37%) was found in 62.5 µg/mL concentration of the extract obtained from kumquat leaves with a hexane solvent. We determined no correlation between the chelating activity of the extracts and their concentration. No significant difference was found between the rootstock kumquat type and its hybrids.

When we evaluated all the activities, we concluded that the extracts obtained from kumquat fruits and leaves were not good at chelating iron (II) ions. The most important feature that affects the metal chelating activity depends on the functional groups in the structure of phenolic compounds and the position and amount of these functional groups. For this reason, the difference in the chelating activity of the samples can be explained by different amounts of phenolic substances, as well as phenolic substance groups in different structures and positions [32].

The reducing capacity of the extracts. The reducing agent in the environment reduces Fe^{3+} ions to Fe^{2+} ions depending on its antioxidant capacity. The absorbance of the Prussian blue complex ($Fe_4[Fe(CN)_6]$) formed by adding $FeCl_3$ to the reduced product is measured at 700 nm [22]. The increase in absorbance of the reaction mixture is directly proportional to the reducing power of the sample.

We found that the capacity of kumquat leaves to reduce Fe^{3+} ions was higher than that of lyophilized and wet kumquat fruits (Table 3). We observed that lyophilizing and drying of kumquat fruits did not cause a significant change in their reducing capacity. The reducing capacity of the fruit and leaf extracts was lower than the standards (BHA, BHT and α-tocopherol).

Table 3 The reducing power of extracts and standards, μg/mL (mean ± SD of triplicate)

Extracts and standards	5.88*	14.7*	29.41*
Rootstock fresh fruit pure methanol	0.104 ± 0.001	0.115 ± 0.003	0.138 ± 0.002
Rootstock fresh fruit 80% methanol	0.105 ± 0.002	0.106 ± 0.001	0.124 ± 0.001
Rootstock fresh fruit 60% methanol	0.120 ± 0.001	0.133 ± 0.001	0.140 ± 0.003
Rootstock fresh fruit 50% methanol	0.096 ± 0.001	0.100 ± 0.002	0.104 ± 0.001
Rootstock fresh fruit pure water	0.082 ± 0.002	0.098 ± 0.003	0.115 ± 0.001
Rootstock dry fruit pure methanol	0.075 ± 0.001	0.082 ± 0.003	0.094 ± 0.001
Rootstock dry fruit 80% methanol	0.074 ± 0.002	0.087 ± 0.006	0.097 ± 0.005
Rootstock dry fruit 60% methanol	0.076 ± 0.001	0.081 ± 0.001	0.089 ± 0.001
Rootstock dry fruit 50% methanol	0.076 ± 0.002	0.082 ± 0.001	0.089 ± 0.003
Rootstock dry fruit pure water	0.078 ± 0.003	0.081 ± 0.001	0.089 ± 0.001
Rootstock leaf pure methanol	0.103 ± 0.002	0.145 ± 0.001	0.241 ± 0.004
Rootstock leaf 80% methanol	0.098 ± 0.001	0.149 ± 0.001	0.227 ± 0.003
Rootstock leaf 60% methanol	0.093 ± 0.001	0.136 ± 0.005	0.218 ± 0.003
Rootstock leaf 50% methanol	0.097 ± 0.002	0.148 ± 0.001	0.240 ± 0.003
Rootstock leaf pure water	0.089 ± 0.001	0.143 ± 0.003	0.209 ± 0.005
Rootstock leaf 0.5% acidified ethanol	0.074 ± 0.001	0.096 ± 0.002	0.128 ± 0.001
Rootstock leaf 1% acidified ethanol	0.076 ± 0.001	0.098 ± 0.003	0.129 ± 0.001
Rootstock leaf hexane	0.091 ± 0.002	0.125 ± 0.003	0.179 ± 0.002
EP.4 fresh fruit pure methanol	0.111 ± 0.002	0.144 ± 0.001	0.199 ± 0.001
EP.4 fresh fruit 80% methanol	0.108 ± 0.001	0.110 ± 0.003	0.100 ± 0.001
EP.4 fresh fruit 60% methanol	0.104 ± 0.002	0.095 ± 0.003	0.112 ± 0.001
EP.4 fresh fruit 50% methanol	0.099 ± 0.003	0.092 ± 0.001	0.143 ± 0.001
EP.4 fresh fruit pure water	0.086 ± 0.001	0.093 ± 0.001	0.115 ± 0.002
EP.4 dry fruit pure methanol	0.070 ± 0.001	0.077 ± 0.001	0.091 ± 0.001
EP.4 dry fruit 80% methanol	0.071 ± 0.002	0.078 ± 0.001	0.089 ± 0.003
EP.4 dry fruit 60% methanol	0.074 ± 0.001	0.076 ± 0.001	0.087 ± 0.003
EP.4 dry fruit 50% methanol	0.071 ± 0.001	0.075 ± 0.003	0.085 ± 0.001
EP.4 dry fruit pure water	0.070 ± 0.002	0.072 ± 0.001	0.081 ± 0.001
EP.4 leaf pure methanol	0.087 ± 0.002	0.134 ± 0.004	0.201 ± 0.001
EP.4 leaf 80% methanol	0.097 ± 0.001	0.145 ± 0.003	0.245 ± 0.004
EP.4 leaf 60% methanol	0.093 ± 0.003	0.139 ± 0.001	0.211 ± 0.003
EP.4 leaf 50% methanol	0.091 ± 0.002	0.149 ± 0.003	0.227 ± 0.005
EP.4 leaf pure water	0.116 ± 0.001	0.193 ± 0.003	0.307 ± 0.001
EP.4 leaf 0.5% acidified ethanol	0.075 ± 0.002	0.093 ± 0.001	0.125 ± 0.001
EP.4 leaf 1% acidified ethanol	0.079 ± 0.001	0.102 ± 0.002	0.133 ± 0.006
EP.4 leaf hexane	0.091 ± 0.003	0.125 ± 0.001	0.179 ± 0.001
EP.29 fresh fruit pure methanol	0.107 ± 0.004	0.118 ± 0.004	0.135 ± 0.006
EP.29 fresh fruit 80% methanol	0.107 ± 0.001	0.114 ± 0.002	0.108 ± 0.002
EP.29 fresh fruit 60% methanol	0.109 ± 0.000	0.109 ± 0.000	0.138 ± 0.000
EP.29 fresh fruit 50% methanol	0.113 ± 0.000	0.117 ± 0.001	0.133 ± 0.000
EP.29 fresh fruit pure water	0.086 ± 0.001	0.092 ± 0.000	0.100 ± 0.001
EP.29 dry fruit pure methanol	0.072 ± 0.000	0.081 ± 0.001	0.098 ± 0.000
EP.29 dry fruit 80% methanol	0.073 ± 0.000	0.080 ± 0.001	0.093 ± 0.000
EP.29 dry fruit 60% methanol	0.072 ± 0.001	0.077 ± 0.001	0.090 ± 0.001
EP.29 dry fruit 50% methanol	0.071 ± 0.001	0.078 ± 0.000	0.088 ± 0.000
EP.29 dry fruit pure water	0.073 ± 0.000	0.076 ± 0.001	0.090 ± 0.000
EP.29 leaf pure methanol	0.090 ± 0.000	0.125 ± 0.001	0.206 ± 0.002
EP.29 leaf 80% methanol	0.093 ± 0.000	0.145 ± 0.001	0.236 ± 0.000
EP.29 leaf 60% methanol	0.106 ± 0.001	0.158 ± 0.000	0.260 ± 0.000
EP.29 leaf 50% methanol	0.103 ± 0.000	0.163 ± 0.000	0.281 ± 0.000
EP.29 leaf pure water	0.101 ± 0.000	0.158 ± 0.001	0.244 ± 0.000
EP.29 leaf 0.5% acidified ethanol	0.086 ± 0.000	0.103 ± 0.001	0.135 ± 0.000
EP.29 leaf 1% acidified ethanol	0.077 ± 0.001	0.094 ± 0.001	0.119 ± 0.001
EP.29 leaf hexane	0.088 ± 0.000	0.136 ± 0.000	0.193 ± 0.000
EP.31 fresh fruit pure methanol	0.091 ± 0.001	0.098 ± 0.001	0.109 ± 0.001
EP.31 fresh fruit 80% methanol	0.087 ± 0.000	0.095 ± 0.000	0.117 ± 0.000
EP.31 fresh fruit 60% methanol	0.081 ± 0.000	0.103 ± 0.001	0.129 ± 0.001

Continuation of Table 3

Extracts and standards	5.88*	14.7*	29.41*
EP.31 fresh fruit 50% methanol	0.089 ± 0.000	0.115 ± 0.001	0.104 ± 0.000
EP.31 fresh fruit pure water	0.088 ± 0.001	0.094 ± 0.000	0.105 ± 0.001
EP.31 dry fruit pure methanol	0.093 ± 0.000	0.099 ± 0.000	0.125 ± 0.000
EP.31 dry fruit 80% methanol	0.095 ± 0.000	0.102 ± 0.000	0.099 ± 0.000
EP.31 dry fruit 60% methanol	0.099 ± 0.000	0.085 ± 0.001	0.096 ± 0.001
EP.31 dry fruit 50% methanol	0.099 ± 0.000	0.092 ± 0.001	0.097 ± 0.000
EP.31 dry fruit pure water	0.107 ± 0.000	0.100 ± 0.001	0.111 ± 0.001
EP.31 leaf pure methanol	0.089 ± 0.001	0.119 ± 0.001	0.176 ± 0.001
EP.31 leaf 80% methanol	0.093 ± 0.000	0.133 ± 0.001	0.200 ± 0.000
EP.31 leaf 60% methanol	0.101 ± 0.001	0.148 ± 0.001	0.214 ± 0.000
EP.31 leaf 50% methanol	0.100 ± 0.001	0.142 ± 0.000	0.212 ± 0.001
EP.31 leaf pure water	0.094 ± 0.001	0.133 ± 0.000	0.206 ± 0.001
EP.31 leaf 0.5% acidified ethanol	0.089 ± 0.000	0.127 ± 0.000	0.184 ± 0.000
EP.31 leaf 1% acidified ethanol	0.088 ± 0.000	0.113 ± 0.001	0.155 ± 0.000
EP.31 leaf hexane	0.098 ± 0.001	0.119 ± 0.000	0.202 ± 0.001
YP.117 fresh fruit pure methanol	0.099 ± 0.001	0.117 ± 0.000	0.153 ± 0.000
YP.117 fresh fruit 80% methanol	0.096 ± 0.000	0.099 ± 0.000	0.117 ± 0.000
YP.117 fresh fruit 60% methanol	0.100 ± 0.000	0.100 ± 0.001	0.114 ± 0.000
YP.117 fresh fruit 50% methanol	0.107 ± 0.000	0.116 ± 0.001	0.142 ± 0.000
YP.117 fresh fruit pure water	0.088 ± 0.000	0.094 ± 0.000	0.114 ± 0.000
YP.117 dry fruit pure methanol	0.077 ± 0.000	0.082 ± 0.000	0.108 ± 0.001
YP.117 dry fruit 80% methanol	0.074 ± 0.000	0.079 ± 0.001	0.085 ± 0.000
YP.117 dry fruit 60% methanol	0.081 ± 0.000	0.088 ± 0.001	0.093 ± 0.000
YP.117 dry fruit 50% methanol	0.085 ± 0.001	0.080 ± 0.000	0.087 ± 0.000
YP.117 dry fruit pure water	0.079 ± 0.000	0.083 ± 0.000	0.089 ± 0.000
YP.117 leaf pure methanol	0.092 ± 0.001	0.141 ± 0.001	0.206 ± 0.000
YP.117 leaf 80% methanol	0.093 ± 0.000	0.133 ± 0.001	0.201 ± 0.000
YP.117 leaf 60% methanol	0.101 ± 0.001	0.157 ± 0.000	0.235 ± 0.001
YP.117 leaf 50% methanol	0.109 ± 0.001	0.159 ± 0.001	0.262 ± 0.001
YP.117 leaf pure water	0.105 ± 0.000	0.152 ± 0.000	0.242 ± 0.000
YP.117 leaf 0.5% acidified ethanol	0.091 ± 0.000	0.116 ± 0.001	0.165 ± 0.000
YP.117 leaf 1% acidified ethanol	0.087 ± 0.001	0.113 ± 0.001	0.163 ± 0.001
YP.117 leaf hexane	0.072 ± 0.000	0.091 ± 0.000	0.154 ± 0.000
YP.141 fresh fruit pure methanol	0.096 ± 0.001	0.104 ± 0.000	0.124 ± 0.000
YP.141 fresh fruit 80% methanol	0.091 ± 0.000	0.091 ± 0.001	0.105 ± 0.000
YP.141 fresh fruit 60% methanol	0.146 ± 0.000	0.138 ± 0.001	0.139 ± 0.000
YP.141 fresh fruit 50% methanol	0.092 ± 0.000	0.103 ± 0.001	0.142 ± 0.000
YP.141 fresh fruit pure water	0.091 ± 0.000	0.099 ± 0.000	0.117 ± 0.001
YP.141 dry fruit pure methanol	0.092 ± 0.001	0.091 ± 0.001	0.102 ± 0.000
YP.141 dry fruit 80% methanol	0.102 ± 0.000	0.105 ± 0.001	0.120 ± 0.000
YP.141 dry fruit 60% methanol	0.093 ± 0.000	0.090 ± 0.001	0.097 ± 0.000
YP.141 dry fruit 50% methanol	0.097 ± 0.001	0.088 ± 0.001	0.095 ± 0.000
YP.141 dry fruit pure water	0.094 ± 0.001	0.087 ± 0.000	0.098 ± 0.000
YP.141 leaf pure methanol	0.105 ± 0.000	0.155 ± 0.000	0.241 ± 0.001
YP.141 leaf 80% methanol	0.108 ± 0.000	0.165 ± 0.001	0.254 ± 0.000
YP.141 leaf 60% methanol	0.100 ± 0.000	0.154 ± 0.001	0.250 ± 0.000
YP.141 leaf 50% methanol	0.106 ± 0.001	0.162 ± 0.000	0.252 ± 0.002
YP.141 leaf pure water	0.101 ± 0.000	0.141 ± 0.000	0.247 ± 0.001
YP.141 leaf 0.5% acidified ethanol	0.088 ± 0.000	0.123 ± 0.001	0.186 ± 0.001
YP.141 leaf 1% acidified ethanol	0.082 ± 0.000	0.108 ± 0.000	0.148 ± 0.000
YP.141 leaf hexane	0.070 ± 0.001	0.102 ± 0.000	0.162 ± 0.000
YP.188 fresh fruit pure methanol	0.092 ± 0.001	0.111 ± 0.000	0.146 ± 0.000
YP.188 fresh fruit 80% methanol	0.094 ± 0.000	0.107 ± 0.001	0.136 ± 0.001
YP.188 fresh fruit 60% methanol	0.090 ± 0.000	0.104 ± 0.001	0.123 ± 0.000
YP.188 fresh fruit 50% methanol	0.095 ± 0.000	0.096 ± 0.001	0.112 ± 0.000
YP.188 fresh fruit pure water	0.099 ± 0.000	0.103 ± 0.000	0.126 ± 0.000
YP.188 dry fruit pure methanol	0.090 ± 0.001	0.086 ± 0.000	0.110 ± 0.000

Continuation of Table 3

Extracts and standards	5.88*	14.7*	29.41*
YP.188 dry fruit 80% methanol	0.091 ± 0.000	0.088 ± 0.000	0.094 ± 0.001
YP.188 dry fruit 60% methanol	0.089 ± 0.001	0.087 ± 0.000	0.098 ± 0.000
YP.188 dry fruit 50% methanol	0.092 ± 0.000	0.094 ± 0.001	0.099 ± 0.000
YP.188 dry fruit pure water	0.093 ± 0.000	0.087 ± 0.001	0.100 ± 0.000
YP.188 leaf pure methanol	0.102 ± 0.000	0.182 ± 0.001	0.252 ± 0.001
YP.188 leaf 80% methanol	0.115 ± 0.001	0.164 ± 0.000	0.263 ± 0.000
YP.188 leaf 60% methanol	0.116 ± 0.000	0.176 ± 0.000	0.279 ± 0.000
YP.188 leaf 50% methanol	0.109 ± 0.001	0.159 ± 0.000	0.253 ± 0.001
YP.188 leaf pure water	0.111 ± 0.000	0.169 ± 0.000	0.271 ± 0.000
YP.188 leaf 0.5% acidified ethanol	0.098 ± 0.000	0.157 ± 0.001	0.218 ± 0.0001
YP.188 leaf 1% acidified ethanol	0.088 ± 0.000	0.110 ± 0.001	0.147 ± 0.000
YP.188 leaf hexane	0.076 ± 0.001	0.102 ± 0.001	0.167 ± 0.001
BHA	0.690 ± 0.001	1.346 ± 0.000	1.984 ± 0.000
BHT	0.504 ± 0.000	0.939 ± 0.000	1.290 ± 0.002
α-tokeferol	0.234 ± 0.000	0.477 ± 0.001	0.872 ± 0.000

*It represents the concentrations of the solutions prepared by taking 100, 250, and 500 µL of standard and extract stock solutions prepared as 1 mg/mL and completing the total volume of 3.750 µmL

The highest reducing capacity (0.307 ± 0.001) was observed at a concentration of 29.41 µg/mL of the EP.4 mutant leaf extract obtained with pure water. Among the fruits, the highest reducing capacity (0.199 ± 0.001) was found at a concentration of 29.41 µg/mL of the EP.4 hybrid wet fruit extract obtained with pure methanol. The reducing capacities of the standards were 1.984 ± 0.001, 1.290 ± 0.002, 0.872 ± 0.001 for BHA, BHT, and α-toceferol, respectively, at the highest concentration of 29.41 µg/mL.

No significant difference was observed between the rootstock kumquat plant and its mutants. Although the reducing power is an important factor of antioxidant activity, in our study, the reducing power was lower in the extracts with high antioxidant activity. Other studies also show that extracts with high antioxidant activity may have low reducing power [33, 34]. This is because in the systems where free iron ions are present in trace amounts, the net oxidation rate increases with the Fenton reaction. Substances with high reducing power may cause further acceleration of oxidation by reducing Fe(III) to Fe(II). The presence of trace levels of iron ions in kumquat materials may have caused its low reducing power and ncreased antioxidant activity [35].

Phenolic and flavonoid content. Since phenolic and flavonoid compounds contain hydroxyl groups in their structures and can easily give a hydrogen radical in hydroxyl groups, they have free radical quenching properties. Therefore, it is important to know the total phenolic and flavonoid contents of the samples to determine their contribution to the antioxidant activity, including radical scavenging activity tests. For this, we used the Folin-Ciocalteu method, a standard method in antioxidant studies. The basis of the method is that phenolic compounds dissolved in water and other organic solvents form a colored complex with a Folin reagent in an alkaline medium. The total phenolic content of the extracts obtained by Soxhlet extraction with different solvents was calculated using the regression equation (y = 0.0292x + 0.0749 and

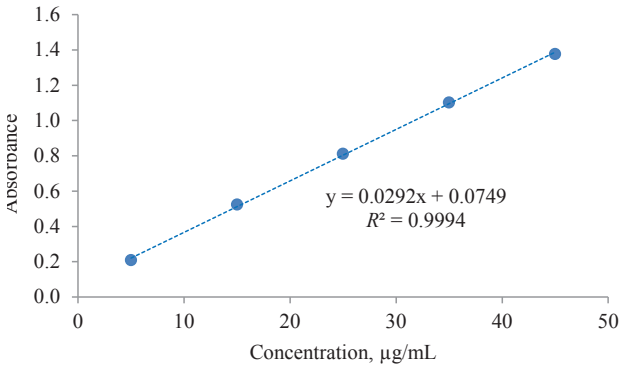

Figure 1 Standard calibration curve of gallic acid to determine total phenolic content

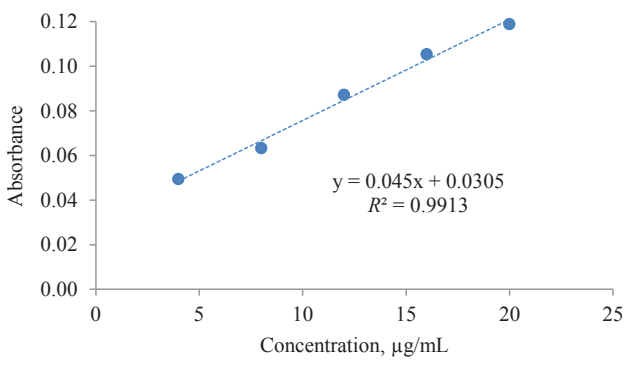

Figure 2 Calibration curve of standard quercetin to determine total flavonoid content

Table 4 Total phenolic and total flavonoid contents in kumquat fruit and leaf extracts

Extracts	Total Phenolic Substance, mg GAE/g extract	Total Flavonoid Substance, mg QUE/g extract
Rootstock fresh fruit pure methanol	16.096 ± 0.045	42.222 ± 0.018
Rootstock fresh fruit 80% methanol	8.432 ± 0.024	24.444 ± 0.014
Rootstock fresh fruit 60% methanol	5.808 ± 0.012	22.222 ± 0.012
Rootstock fresh fruit 50% methanol	7.089 ± 0.018	26.667 ± 0.018
Rootstock fresh fruit pure water	13.747 ± 0.011	41.111 ± 0.020
Rootstock dry fruit pure methanol	8.959 ± 0.038	46.667 ± 0.016
Rootstock dry fruit 80% methanol	9.856 ± 0.033	10.022 ± 0.010
Rootstock dry fruit 60% methanol	5.829 ± 0.011	10.100 ± 0.012
Rootstock dry fruit 50% methanol	5.425 ± 0.010	5.556 ± 0.011
Rootstock dry fruit pure water	3.705 ± 0.011	14.444 ± 0.016
Rootstock leaf pure methanol	66.356 ± 0.034	454.444 ± 0.046
Rootstock leaf 80% methanol	72.548 ± 0.021	258.889 ± 0.024
Rootstock leaf 60% methanol	68.979 ± 0.023	213.333 ± 0.034
Rootstock leaf 50% methanol	67.096 ± 0.018	248.889 ± 0.032
Rootstock leaf pure water	54.062 ± 0.023	174.444 ± 0.024
Rootstock leaf 0.5% acidified ethanol	31.925 ± 0.030	314.444 ± 0.042
Rootstock leaf 1% acidified ethanol	31.062 ± 0.018	308.889 ± 0.014
Rootstock leaf hexane	n.d.	n.d.
EP.4 fresh fruit pure methanol	20.281 ± 0.013	67.778 ± 0.026
EP.4 fresh fruit 80% methanol	8.678 ± 0.025	32.222 ± 0.024
EP.4 fresh fruit 60% methanol	5.479 ± 0.012	26.667 ± 0.018
EP.4 fresh fruit 50% methanol	7.760 ± 0.021	35.556 ± 0.012
EP.4 fresh fruit pure water	7.534 ± 0.011	25.556 ± 0.010
EP.4 dry fruit pure methanol	11.247 ± 0.013	25.556 ± 0.014
EP.4 dry fruit 80% methanol	11.315 ± 0.022	16.667 ± 0.016
EP.4 dry fruit 60% methanol	14.288 ± 0.023	27.778 ± 0.022
EP.4 dry fruit 50% methanol	9.137 ± 0.014	30.000 ± 0.023
EP.4 dry fruit pure water	7.521 ± 0.021	23.333 ± 0.024
EP.4 leaf pure methanol	63.438 ± 0.015	410.000 ± 0.032
EP.4 leaf 80% methanol	64.797 ± 0.017	271.111 ± 0.023
EP.4 leaf 60% methanol	64.685 ± 0.010	231.111 ± 0.023
EP.4 leaf 50% methanol	65.568 ± 0.022	248.889 ± 0.023
EP.4 leaf pure water	73.034 ± 0.015	255.556 ± 0.023
EP.4 leaf 0.5% acidified ethanol	33.068 ± 0.032	315.556 ± 0.023
EP.4 leaf 1% acidified ethanol	33.952 ± 0.014	355.556 ± 0.023
EP.4 leaf hexane	n.d.	n.d.
EP.29 fresh fruit pure methanol	14.596 ± 0.011	42.222 ± 0.023
EP.29 fresh fruit 80% methanol	8.884 ± 0.021	31.111 ± 0.023
EP.29 fresh fruit 60% methanol	8.842 ± 0.021	20.000 ± 0.023
EP.29 fresh fruit 50% methanol	11.534 ± 0.018	30.000 ± 0.023
EP.29 fresh fruit pure water	13.404 ± 0.016	21.111 ± 0.023
EP.29 dry fruit pure methanol	12.404 ± 0.012	65.556 ± 0.023
EP.29 dry fruit 80% methanol	12.918 ± 0.012	16.667 ± 0.023
EP.29 dry fruit 60% methanol	9.623 ± 0.018	26.667 ± 0.023
EP.29 dry fruit 50% methanol	9.747 ± 0.017	21.111 ± 0.023
EP.29 dry fruit pure water	7.205 ± 0.013	13.333 ± 0.023
EP.29 leaf pure methanol	60.836 ± 0.022	438.889 ± 0.023
EP.29 leaf 80% methanol	67.589 ± 0.032	223.333 ± 0.023
EP.29 leaf 60% methanol	70.226 ± 0.043	256.667 ± 0.023
EP.29 leaf 50% methanol	64.822 ± 0.023	268.889 ± 0.023
EP.29 leaf pure water	50.390 ± 0.013	184.444 ± 0.023
EP.29 leaf 0.5% acidified ethanol	41.158 ± 0.011	486.667 ± 0.023
EP.29 leaf 1% acidified ethanol	25.856 ± 0.033	242.222 ± 0.023
EP.29 leaf hexane	n.d.	n.d.
EP.31 fresh fruit pure methanol	6.384 ± 0.014	38.889 ± 0.023
EP.31 fresh fruit 80% methanol	9.952 ± 0.012	20.000 ± 0.023

Continuation of Table 4

Extracts	Total Phenolic Substance, mg GAE/g extract	Total Flavonoid Substance, mg QUE/g extract
EP.31 fresh fruit 60% methanol	17.500 ± 0.023	42.222 ± 0.023
EP.31 fresh fruit 50% methanol	5.822 ± 0.023	14.444 ± 0.023
EP.31 fresh fruit pure water	8.164 ± 0.013	27.778 ± 0.023
EP.31 dry fruit pure methanol	12.212 ± 0.015	105.556 ± 0.023
EP.31 dry fruit 80% methanol	7.452 ± 0.028	25.556 ± 0.023
EP.31 dry fruit 60% methanol	7.767 ± 0.026	23.333 ± 0.023
EP.31 dry fruit 50% methanol	7.486 ± 0.024	26.667 ± 0.023
EP.31 dry fruit pure water	6.568 ± 0.022	13.333 ± 0.023
EP.31 leaf pure methanol	61.973 ± 0.022	450.000 ± 0.023
EP.31 leaf 80% methanol	64.739 ± 0.018	284.444 ± 0.023
EP.31 leaf 60% methanol	74.082 ± 0.020	260.000 ± 0.023
EP.31 leaf 50% methanol	72.363 ± 0.014	281.111 ± 0.023
EP.31 leaf pure water	50.274 ± 0.024	180.000 ± 0.023
EP.31 leaf 0.5% acidified ethanol	47.699 ± 0.010	454.444 ± 0.023
EP.31 leaf 1% acidified ethanol	43.603 ± 0.018	632.222 ± 0.033
EP.31 leaf hexane	n.d.	n.d.
YP.117 fresh fruit pure methanol	13.322 ± 0.022	36.667 ± 0.023
YP.117 fresh fruit 80% methanol	8.527 ± 0.012	16.667 ± 0.023
YP.117 fresh fruit 60% methanol	8.486 ± 0.014	17.778 ± 0.023
YP.117 fresh fruit 50% methanol	7.349 ± 0.022	158.889 ± 0.023
YP.117 fresh fruit pure water	8.308 ± 0.018	112.222 ± 0.023
YP.117 dry fruit pure methanol	9.445 ± 0.012	36.667 ± 0.023
YP.117 dry fruit 80% methanol	8.822 ± 0.010	16.667 ± 0.023
YP.117 dry fruit 60% methanol	7.705 ± 0.016	17.778 ± 0.023
YP.117 dry fruit 50% methanol	6.986 ± 0.020	158.889 ± 0.023
YP.117 dry fruit pure water	5.740 ± 0.018	112.222 ± 0.023
YP.117 leaf pure methanol	65.356 ± 0.016	458.889 ± 0.023
YP.117 leaf 80% methanol	70.205 ± 0.014	194.444 ± 0.023
YP.117 leaf 60% methanol	68.514 ± 0.023	298.889 ± 0.023
YP.117 leaf 50% methanol	65.616 ± 0.022	285.556 ± 0.023
YP.117 leaf pure water	55.425 ± 0.020	248.889 ± 0.023
YP.117 leaf 0.5% acidified ethanol	43.603 ± 0.016	312.222 ± 0.023
YP.117 leaf 1% acidified ethanol	41.205 ± 0.022	381.111 ± 0.023
YP.117 leaf hexane	n.d.	n.d.
YP.141 fresh fruit pure methanol	9.342 ± 0.022	313.333 ± 0.023
YP.141 fresh fruit 80% methanol	7.630 ± 0.020	40.000 ± 0.023
YP.141 fresh fruit 60% methanol	10.740 ± 0.014	40.000 ± 0.023
YP.141 fresh fruit 50% methanol	9.164 ± 0.018	31.111 ± 0.023
YP.141 fresh fruit pure water	8.432 ± 0.012	27.778 ± 0.023
YP.141 dry fruit pure methanol	15.637 ± 0.020	97.778 ± 0.023
YP.141 dry fruit 80% methanol	9.089 ± 0.022	26.667 ± 0.023
YP.141 dry fruit 60% methanol	10.918 ± 0.018	50.000 ± 0.023
YP.141 dry fruit 50% methanol	8.295 ± 0.014	55.556 ± 0.023
YP.141 dry fruit pure water	6.144 ± 0.022	26.667 ± 0.023
YP.141 leaf pure methanol	72.342 ± 0.023	564.444 ± 0.023
YP.141 leaf 80% methanol	76.658 ± 0.010	387.778 ± 0.023
YP.141 leaf 60% methanol	64.322 ± 0.022	354.444 ± 0.023
YP.141 leaf 50% methanol	63.767 ± 0.016	357.778 ± 0.023
YP.141 leaf pure water	60.082 ± 0.014	305.556 ± 0.023
YP.141 leaf 0.5% acidified ethanol	51.048 ± 0.012	470.000 ± 0.023
YP.141 leaf 1% acidified ethanol	32.329 ± 0.012	300.000 ± 0.023
YP.141 leaf hexane	n.d.	n.d.
YP.188 fresh fruit pure methanol	11.336 ± 0.010	111.111 ± 0.023
YP.188 fresh fruit 80% methanol	8.993 ± 0.012	87.778 ± 0.023
YP.188 fresh fruit 60% methanol	9.986 ± 0.008	86.667 ± 0.023
YP.188 fresh fruit 50% methanol	8.979 ± 0.016	104.444 ± 0.023
YP.188 fresh fruit pure water	20.144 ± 0.022	102.222 ± 0.023

Continuation of Table 4

Extracts	Total Phenolic Substance, mg GAE/g extract	Total Flavonoid Substance, mg QUE/g extract
YP.188 dry fruit pure methanol	9.151 ± 0.014	15.556 ± 0.023
YP.188 dry fruit 80% methanol	8.212 ± 0.028	16.667 ± 0.023
YP.188 dry fruit 60% methanol	7.048 ± 0.014	21.111 ± 0.023
YP.188 dry fruit 50% methanol	7.021 ± 0.012	38.889 ± 0.023
YP.188 dry fruit pure water	5.418 ± 0.008	26.667 ± 0.023
YP.188 leaf pure methanol	72.637 ± 0.010	446.667 ± 0.023
YP.188 leaf 80% methanol	85.651 ± 0.030	330.000 ± 0.023
YP.188 leaf 60% methanol	86.329 ± 0.022	345.556 ± 0.023
YP.188 leaf 50% methanol	75.418 ± 0.022	300.000 ± 0.023
YP.188 leaf pure water	70.849 ± 0.018	313.333 ± 0.023
YP.188 leaf 0.5% acidified ethanol	62.890 ± 0.020	582.222 ± 0.023
YP.188 leaf 1% acidified ethanol	33.226 ± 0.018	275.556 ± 0.023
YP.188 leaf hexane	n.d.	n.d.

n.d.: not detected

R^2 = 0.9994) of the calibration line of the standard gallic acid solution prepared in the concentration range of 5–50 µg/mL and expressed as gallic acid equivalent (mg GAE/g extract). The gallic acid standard curve is shown in Fig. 1. We found that the kumquat leaf extracts had the highest total phenolic content (Table 4). In particular, the highest total phenolic content (86.329 ± 0.022 mg GAE/g extract) was in the YP.188 mutant extract obtained with 60% methanol. In the fruit samples, the highest total phenolic content (20.281 mg GAE/g extract) was found in the EP.4 mutant extract obtained with pure methanol. There was no significant difference in total phenolic contents between the fresh and dried fruit samples.

Lou *et al.* compared total phenolic contents in fresh and dried kumquat fruits [36]. The scientists investigated changes in total phenolic matter by changing the drying degree and time. They found that the total amount of phenolic substances increased with drying, amounting to 15–17 mg GAE/g extract and 48–50 mg GAE/g extract in fresh and dried fruit (130°C), respectively [36].

In another study, Özcan *et al.* dried kumquat fruit in hot air, under vacuum, and in a microwave oven [27]. The authors found that the total phenolic content of hot air-dried fruit was approximately 5 mg GAE/g extract, but with other drying methods, it varied in the range of 25–30 mg GAE/g extract [37].

Yıldız Turgut *et al.* studied the functional quality parameters of the powder obtained from Fortunella margarita kumquat varieties grown in Turkey. They reported the total phenolic content of kumquat between 2.62 ± 0.051 – 6.97 ± 0.053 mg GAE/g depending on the type of drying method [38].

Having determined the total phenolic content, we measured the total flavonoid content of the samples. Total flavonoid concentration was determined colorimetrically using a UV spectrophotometer according to the method applied by Zhishen *et al.* [27].

In our study, quercetin was used as a standard and the results were calculated as quercetin equivalent (mg QUE/g extract) from the quercetin standard calibration chart (y = 0.0185x – 0.0019 and R^2 = 0.9666) (Fig. 2). The highest amount of total flavonoid substance was seen in kumquat leaves (Table 4). In particular, the highest flavonoid content was found in the EP.31 mutant extract (632.222 ± 0.033 mg QUE/g extract) obtained with 1% acidified ethanol.

Among the fruit samples, the highest amount (313.333 ± 0.023 mg QUE/g extract) was found in the YP.141 mutant extract obtained with pure methanol. There were no significant differences between the total flavonoid amounts in the fresh and dried fruits.

Lou *et al.* reported that the total amount of flavonoid substance in kumquat varied between 58.23–91.42 mg/g depending on the drying temperature [36]. In another study, Lou *et al.* found that the total phenolic and flavonoid contents were higher in the extracts from kumquat and calamondin peel compared to fruit pulp, and that they were higher in the extracts from unripe kumquat compared to those from ripe kumquat [39, 40].

CONCLUSION

In antioxidant activity studies, it is common to use a different polarity solvent system in order to determine which compound types have the highest activity. There may be a relationship between phenolic or flavonoid amounts and antioxidant capacity determination methods. In particular, a relationship between methods such as the DPPH, which is based on radical capture, and total phenolic and flavonoid amounts may be important in some plant structures. Phenolic acids and flavonoids are soluble in polar solvents and show strong activity in polar systems.

In this study, we investigated the effect of different solvents and their concentrations on the bioactivity of kumquat fruit and leaf extracts. We found that the solvent type was extremely important for the extracts' bioactivity. In particular, the extraction performed with

pure methanol in the fruits and 60 or 80% methanol in the leaves showed the highest total phenolic and flavonoid contents, the highest extraction efficiency (50.18–59.95%), and the highest antioxidant capacity.

We found no statistically significant difference between the total amount of phenolic/flavonoid substances and % inhibition value in the extraction performed with 60 and 80% methanol solutions. This shows that the amount of phenolic substances was affected by the polarity of the solvent, depending on the difference in phenolic compounds found in kumquat fruit and leaves. We concluded that phenolic components in the structure of a kumquat fruit could be extracted with a single solvent type, whereas those in the structure of a kumquat leaf could be extracted better with an aqueous solution of the relevant solvent, rather than a single solvent type.

We also observed that the aqueous solutions gave better results than the pure solutions in the production of phenolics from kumquat leaves, maximizing at certain water ratios and showing different distributions according to the solvents. These results can be explained by the fact that water increases diffusion by causing swelling in the leaf structure. In this context, methanol was the most effective solvent for bioactive component extraction from the kumquat fruits, whereas methanol + water was most effective for the leaves.

Having examined the effect of a solvent amount, we concluded that the extraction with 260 mL solvent ensured the highest total phenolic content, extraction efficiency, and antioxidant capacity. In addition, since methanol is a toxic solvent, it must be removed so that the obtained extract can be used in foods or consumed as a food supplement.

Plants are complex systems by nature and have multiple reaction characteristics and dissolution properties in different phases. Thus, it is not possible for a single method to reveal all of their radical sources or antioxidants [41–43]. For these reasons, we used a combination of methods, namely the DPPH, metal chelation, and iron reduction. In addition, we used the Folin-Ciocalteu method and the aluminum chloride method to determine the total phenol and flavonoid contents, respectively. The results clearly showed that the differences in the phenolic contents affected the plants' antioxidant properties.

We found that having a high phenolic content or high radical scavenging activity did not yield high results in all antioxidant activity studies. Thus, we concluded that determining the antioxidant activity with a single method was not the right approach and that it would be more accurate to simulate biochemical events in living systems by using a variety of methods. In summary, antioxidant structures can demonstrate their antioxidant activities by different mechanisms such as binding transition metal ions, breaking down peroxides, preventing hydrogen absorption, and removing radicals.

Our study revealed that the kumquat leaf extracts had a higher DPPH radical scavenging power than the fruit extracts. However, both the fruit and leaf extracts showed high levels of free radical scavenging activity with high antioxidant activity at a 125 μg/mL concentration. Due to high antioxidant activity, kumquat leaves can be recommended to be used as food, just as kumquat fruit, against many diseases – from gastrointestinal to infertility, from cardiovascular to respiratory and excretory disorders, especially to prevent cell damage caused by free radicals in human and animal bodies.

CONTRIBUTION

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

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this manuscript.

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Black mahlab (*Monechma ciliatum* L.) seeds: processing effects on chemical composition and nutritional value

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Abstract:

Introduction. *Monechma ciliatum* L. seeds are rich in proteins, carbohydrates, oils and mineral contents. Researchers have focused on new production development but there is no available data on the impact of processing techniques on the quality of the seeds. Our study aimed to investigate the impact of boiling, roasting, and germination on the composition and nutritional value of *Monechma ciliatum* (black mahlab) seeds.

Study objects and methods. We analyzed 7 kg of black mahlab seeds purchased from the local market. We applied standard methods used in boiling, roasting, and germination techniques. Proximate analyses were performed using the methods of the Association of Official Analytical Chemists. Minerals were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS), and fatty acids were determined by gas chromatography. Tocopherols and amino acids in processed seeds were determined by high-performance liquid chromatography.

Results and discussion. The results showed that the proximate compositions of untreated, boiled, roasted, and germinated mahlab seeds were affected by boiling, roasting, and germination techniques. Most of the nutritional values were enhanced by all the treatments. In particular, all the processing techniques increased the protein content. Boiling and roasting increased the fat content, while boiling and germination increased the fiber content. Tocopherols were higher only in the germinated samples. Amino acids were increased by all the techniques. Minerals were affected by all the techniques, except for Na, which was higher in the germinated sample.

Conclusion. Boiling, roasting, and germination enhanced significantly the chemical composition of *Monechma ciliatum* seeds, which make them a value ingredient to develop new food products.

Keywords: *Monechma ciliatum*, boiling, roasting, germination, tocopherols, fatty acids, amino acids, minerals

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INTRODUCTION

Plants are generally considered an important source of nutrients and food supplements since they are rich in nutritive components essential for humans and animals. Growing scientific evidence that supports their health benefits has led to an increase in plant-based foods and diets [1]. Seeds, which are key components of several plant-based diets, are recognized as having a wide range of potential health benefits. Replacing energy-dense foods with high protein seeds has been shown to have beneficial effects on the prevention and management of obesity and related disorders, such as cardiovascular disease, diabetes and the metabolic syndrome. A great number of people in the world depend on conventional plants to obtain remedies as pharmaceuticals. Medicinal plants are not only used as an alternative to traditional treatment if it does not exist, but they also provide an excellent source of bioactive natural products [2].

Acanthaceae is a tropical and subtropical family of dicotyledonous flowering plants rich in nutritional and medicinal components. It includes 346 genera and around 4300 species distributed across temperate regions, mostly in Indonesia, Malaysia, Africa, Brazil, and Central America. Some species have colorful flower petals and are used as a source of natural dyes. Chemically, *Acanthaceae* plants

contain important secondary metabolites such as glycosides, flavonoids, alkaloids, triterpenoids, fatty acid methyl esters, and fatty acids. These compounds play an important role in many biological reactions and work against many lethal diseases [3]. Most of the *Acanthaceae* species have high therapeutic applications due to their alkaloid contents [4]. Their leaves and seeds are used to treat bronchial diseases, flu, and ulcers, as well as to relieve poisonous insect and snake bites, dry cough, and diarrhea [5].

Monechma Hochst., closely related to *Justicia* L., is an *Acanthaceae* genus that contains about 60 species mostly found in tropical and sub-tropical regions, particularly in South Africa. *Monechma* plants are well adapted to harsh environments. As reported by Darbyshire and Goyder, twelve species are recorded in Angola, two of which have recently been added to *Monechma* [6]. Although these two species are morphologically similar, especially in flower and fruit morphology, there is some morphological evidence to support their separation. In particular, there are notable differences in inflorescence form.

Monechma ciliatum is a species with unique biochemicals and phytochemicals that make it traditionally useful for many African communities, especially in rural areas. It shows significant antibacterial activity against *Bacillus subtilis*, *Staphylococcus aereus*, *Escherichia coli*, and *Pseudomonas aeraginosa*, compared to well-known antibiotics, as well as antifungal activity against *Cladosporium cucumerinum* and *Candida albicans* [7]. Studies on their seed extracts, seedcakes, and leaves reveal great contents of nutrients with antioxidant, antimicrobial and medicinal properties [8].

M. ciliatum mainly grows in tropical regions. It is found in the west and southwest of Sudan where it is well known and traditionally used. Owing to its small brownish black seeds, this species is referred to as black mahlab, or El-Mahlab El-Aswad, in Sudan. In one of our earlier works, we reported its richness in fat and other essential nutrients, as well as many benefits in traditional treatments and cosmetic uses [9]. According to that study, the protein content of the *M. ciliatum* seed was 21%, with 783.3 mg/g N as total amino acid. The main fatty acids in *M. ciliatum* fat were oleic (47.3%), linoleic (31.4%), stearic (16.0%), and palmitic (4.5%). The content of tocopherols was 45.2 mg/100 g. Boiling, roasting, and germination are traditional methods generally used to improve the nutritional properties of seeds. Studies of the impact of cooking on the nutrient contents in several seeds revealed changes in their chemical composition and nutritional components.

In another work, we studied the effect of introducing mahlab seed flour as a vegetarian food supplement on kisra (Sudanese bread made of sorghum flour) [10]. Mahlab seeds were subjected to three industrial treatments, namely boiling, roasting, and germination. The processed mahlab seed flour was added to sorghum flour and after the necessary fermentation, four samples of supplemented kisra were made. We performed proximal chemical analysis and evaluated the sensory parameters of the samples against those of conventional sorghum kisra. The results showed that the use of *M. ciliatum* seed flour as a supplement to sorghum kisra significantly improved its nutritional value. We also found that all the panelists gave 10% higher scores to sorghum kisra supplemented with roasted *M. ciliatum* seed flour, compared to the other samples.

Mbah *et al*. reported an increase in protein, fiber, iron, and zinc contents in Morenga seeds as a result of boiling and roasting. In another study, processing techniques such as boiling, roasting, soaking, and blanching significantly ($P < 0.05$) reduced tyrosine and cystine contents in black gram (*Vigna mungo*), but increased histidine [11, 12]. We also reported that boiling and roasting increased fat and protein contents and decreased moisture, carbohydrate, and fiber contents in safflower seeds [13]. We found that these processing methods had an insignificant effect on fatty acids, while Ghazzawi and Ismail showed that roasting and frying of nuts had a positive effect on the fatty acids profile and antioxidant activity [14].

Roasted watermelon seeds have fewer benefits and a lower nutritional value compared to raw seeds. They are heated at about 160 degrees Celsius for only 15 min in order to give them a delicious roasting flavor without causing them to burn and lose their nutritional value [15]. A study published in 2014 indicated that roasting sesame seeds and their subsequent fermentation enhanced their nutrient content after they were ground to a fine powder [16]. Sesame roasting and peeling decreased the content of phytates and oxalate, the compounds that affect digestion and reduce protein absorption in the intestine. Therefore, it is preferable to eat peeled and roasted sesame.

Muangrat *et al*. studied the effect of heat and time of roasting and microwave treatment on the contents of acids, free fatty acids, and iodine, as well as the saponification and peroxide number of black sesame seed oil [17]. They found that the microwave-roasted oil samples showed higher antioxidant activity due to a greater content of total phenols, sesamol, and sesamolin. This indicates that both roasting and microwave treatment are suitable methods to achieve better quality for black sesame oil products [17].

Germination is an effective technique used to improve the nutritional content of legume seeds. It decreases their fat content and increases minerals and fatty acids, thus producing healthy nutrients with bioactive components [18]. Ren *et al*. found that germination provided brown rice with considerable amounts of beneficial nutrients and bioactive compounds [19]. Due to the high cost of animal protein, researchers conduct studies on plants as an affordable source of protein. Including plant protein in the daily diet can prevent malnutrition among poor people, especially in developing countries. Sranacharoenpong *et al*. reported increasing numbers of stunted children

in Africa. Stunting, or impaired growth, is caused by poor nutrition [20]. Since *M. ciliatum* seeds are rich in protein, fat, minerals, and other nutrients, they could be used to prevent this condition. These seeds are very hard to mill so they are traditionally soaked in water before milling. We aimed to study the impact of boiling, roasting, and germination processes on the composition and nutritional contents of *M. ciliatum* seeds.

STUDY OBJECTS AND METHODS

Monechma ciliatum L. seeds (7 kg) were purchased from a local market. The seeds were hand-sorted to remove broken seeds and foreign materials. Then, they were well cleaned with running tap water twice and stored in white/clear reclosable self-seal zip lock polyethylene bags (2.36"×3.94", 4 Mil thick) at 25°C.

Boiling of *M. ciliatum* seeds. 600 g of *M. ciliatum* seeds was put into three 1.0 L beakers, 200 g in each. Water was added at a ratio of 1:4 and boiled to 100°C for 40 min on a magnetic stirrer hot plate until the seeds were cooked. The seeds were drained and dried in a 50°C vacuum oven and then ground to 0.5–0.8 mm particles in a grinder (Moulinex, Japan). Finally, they were put into white/clear reclosable self-seal zip lock polyethylene bags (2.36"×3.94", 4 Mil thick) and stored in a refrigerator at 0–5°C at a relative humidity of 55–65% for analysis.

Roasting of *M. ciliatum* seeds. 500 g of washed and dried *M. ciliatum* seeds were arranged in 3 aluminum foil dishes and then put in an electric air oven, as described by Chirinos *et al.* with some modifications [21]. The seeds were roasted at 180°C for 20 min. The roasted seeds were left to cool to 25°C and then were ground to 0.5–0.8 mm particles in an electric grinder (Moulinex, Japan). They were stored in a refrigerator at 0–5°C at a relative humidity of 55–65% for analysis.

Germination of *M. ciliatum* seeds. In line with the method described by de Jesus *et al.*, 500 g of *M. ciliatum* seeds were soaked in 2500 mL of 0.7 g/L sodium hypochlorite solution for 30 min at 25°C [22]. Then, the seeds were well washed with running tap water twice, drained, and soaked in deionized water for 5 h. After that, they were kept between two layers of cotton cloth for 72 h at room temperature (25°C). The germinated seeds were dried in an air oven at 60°C till constant weight. Then, they were ground to 0.5–0.8 mm particles in an electric grinder (Moulinex, Japan) and stored in a refrigerator at 0–5°C at a relative humidity of 55–65% for further use. The control samples were only ground to 0.5–0.8 mm particles in an electric grinder (Moulinex, Japan) and stored in the same conditions.

Proximate chemical analysis. Moisture, crude fat, crude fiber, and ash were analyzed using the methods of the Association of Official Analytical Chemists [23]. Total nitrogen was analyzed by the micro-Kjeldahl method, with nitrogen converted to protein using the factor of 6.25. The carbohydrate content was calculated by subtracting the sum of fat, protein moisture, fiber, and ash from 100.

Mineral determination. 0.03 Ag ground sample was put in a microwave vessel containing 5.0 mL of HNO_3 and 2 mL of H_2O_2 (Suprapur, Merck). Then, it was heated to 205°C for 15 min to obtain a finely digested mixture. The mixture was left to cool to 25°C and a colorless solution was obtained. The solution was analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) according to the method described by Ngigi and Muraguri with some modifications [24].

Fatty acid composition. Test seeds (15.0 g) were ground and their oil was separated in a Soxhlet extractor (Gerhardt), as indicated by the American Oil Chemists Society [25]. The removed oil was methylated and changed over to fatty acid methyl esters. Then, it was analyzed on a Shimadzu GC-2010 gas chromatograph with a DB-23 column (60 m×0.25 mm ID, 0.25 µm film thickness). The injector, column, and indicator temperatures were 230, 190, and 240°C, respectively. The split proportion was 80:1. Helium (1.0 mL/min) was used as a transporter gas.

Determination of tocopherols. A solution of 250 mg of black mahlab seeds oil in 25 mL n-heptane was used for the high performance liquid chromatography (HPLC). The HPLC analysis was conducted using a low-pressure gradient system fitted with an L-6000 pump, an F-1000 fluorescence spectrophotometer (detector wavelengths of 295 nm and 330 nm for excitation and emission, respectively), and a D-2500 integration system (Merck-Hitachi). 20 µL samples were injected by a 655-A40 autosampler onto a 25 cm×4.6 mm ID Diol phase HPLC column (Merck, Darmstadt, Germany) at a flow rate of 1.3 mL/min. The mobile phase was n-heptane/tert, butyl methyl ether (99+1, v/v) [26].

Amino acid composition. A 200 mg sample was digested with 5.0 mL 6N HCL in a hydrolysis tube. The solution was incubated at 11°C for 24 h and filtered through filter paper. Then, 200 mL of the filtered solution was evaporated at 140°C for about an hour and 1.0 mL of a diluted buffer was added to the dried sample. The amino acid composition of the hydrolyzed sample was determined on an S 433 automatic amino acid analyzer (Sykam, Germany) [9].

Statistical analysis. The analyses were performed in triplicate. The mean values and standard deviation (mean ± SD) were determined by Duncan's test ($P < 0.05$). The measurable analysis of variance (ANOVA) was applied on all the values using a Statgrafics® Statistical Graphics System (version 18.1.12).

RESULTS AND DISCUSSION

The weight of a hundred or thousand seeds is an important characteristic of the seeds' fullness and maturity. It also indicates the amount of flour from the seeds [27]. In our study, the average length of *Monechma ciliatum* seeds was about 4.0 mm and 100 seeds weighed about 3.0 g.

Proximate chemical composition of boiled, roasted, and germinated *M. ciliatum* seeds. The

seeds' chemical composition is very important because they contain many nutrients and growth materials that affect germination. Seeds are considered a basic source of food. For example, Chia (*Salvia hispanica*) seeds are used as ingredients or supplements in many foodstuffs such as baked products, muesli, dairy drinks, fruit smoothies, or salads. They are also used as thickeners in soups and sauces [28]. The proximate compositions of untreated black mahlab boiled black mahlab, roasted black mahlab, and germinated black mahlab seeds are presented in Table l.

Moisture content. The seed's moisture, which is usually expressed as a percentage on a wet weight basis, is an important indicator that affects the seed's quality and shelf-life. In our study, the moisture content of untreated black mahlab seeds was 9.43%, while boiling, roasting, and germination decreased it to 6.91, 6.41, and 9.41%, respectively. This finding was consistent with the one we made in our earlier study, namely a decrease in the moisture of crude safflower seeds after roasting and boiling [29]. Hatamian *et al*., who studied chia seeds, also found a diminished moisture content after roasting [30].

Fat content. The fat contents of untreated and treated black mahlab seeds are shown in Table 1. The untreated black mahlab seed flour had a fat content of 11.65%, which was lower than 14.66% for untreated black mahlab and 12.39% for boiled black mahlab, but higher than 11.30% for germinated black mahlab. As we can see, boiling and roasting increased the fat content, while germination had an insignificant effect on this indicator. This result disagreed with Onyeike and Oguike, who showed that crude fat was highest in raw groundnut seeds and lowest in boiled groundnut seeds [31].

Protein content. Proteins are essential nutrients for the human body. They are the basic units of body tissue and can also serve as an energy source. Proteins provide as much energy density as sugars. Most importantly for nutrition is that protein contains amino acids [32]. The protein contents of untreated and treated black mahlab seeds are shown in Table 1. We found that all the processing methods increased the protein content. Amounting to 22.29% in untreated black mahlab, it increased to 23.89, 22.90, and 24.34% in boiled, roasted, and germinated black mahlab, respectively. Thus, boiling and germination contributed to a higher protein content, unlike roasting. This finding was consistent with that in our earlier study, where the germination of black cumin increased both the oil and the protein contents, while other constituents decreased [29]. In a study by Olanipekun *et al*., the flour from processed kidney bean seeds had a significantly higher protein content than that of the raw seeds [33]. Cargo-Froom *et al*. reported that boiling and roasting enhanced the pulses' protein content, availability, and digestibility, as well as the content of essential amino acids [34]. Similarly, Mbah *et al*. showed that boiling and roasting increased the protein content in Moringa seeds [11]. The higher protein content in the processed seeds might be due to the increase of proteolytic enzymes activity which hydrolyzed proteins to their amino acids during processing.

Fiber content. Dietary fiber includes parts of plant food that the body cannot digest or absorb. Unlike other food components (fats, proteins, or carbohydrates), which the body breaks down and absorbs, fiber passes relatively well through the stomach, small intestine, and colon, and then out of the body [35]. The fiber content in untreated and treated black mahlab seeds is shown in Table 1. As we can see, it reached 9.2, 10.1, 9.0, and 9.9% in untreated, boiled, roasted, and germinated black mahlab, respectively. It was slightly affected by roasting and germination and increased by boiling. This result agreed with Mbah *et al*. who reported that boiling and roasting increased the fiber content in Moringa seeds [11]. However, it was opposed to our earlier finding that roasting and boiling decreased the fiber content in safflower [13].

Carbohydrate content. Carbohydrates are a group of organic compounds that include sugars, starches, and fibers that provide the body with energy. During digestion, carbohydrates are converted into glucose sugar. The pancreas secretes insulin to help glucose sugar enter the cells in the brain and muscles and provide them with the energy needed to perform various functions. The excess of glucose sugar is stored in the liver in the form of glycogen to be used when needed [36]. The carbohydrate contents of untreated and treated black mahlab seeds are shown in Table 1. As we can see, the total available carbohydrates in untreated, boiled, roasted, and germinated black mahlab amounted to 43.60, 40.89, 45.62, and 41.39%, respectively. The carbohydrate content was the highest in roasted black mahlab followed by untreated, germinated, and boiled black mahlab. Our findings agreed with those of Onyeike and Oguike, who reported that boiling and

Table 1 Approximate chemical analysis of raw, boiled, roasted, and germinated *Monechma ciliatuim* seeds, %

Sample	Moisture	Fat, %	Carbohydrate, %	Protein, %	Fiber, %	Ash, %
Untreated mahlab	9.43 ± 0.03^{b}	11.56 ± 0.37^{a}	43.60 ± 0.70^{a}	22.29 ± 0.23^{a}	9.20 ± 0.22^{a}	3.92 ± 0.10^{a}
Boiled mahlab	6.91 ± 0.01^{a}	14.66 ± 0.31^{c}	40.89 ± 0.50^{b}	23.89 ± 0.29^{b}	10.11 ± 0.14^{b}	3.54 ± 0.10^{a}
Roasted mahlab	6.14 ± 0.11^{a}	12.39 ± 0.25^{b}	45.62 ± 0.70^{c}	22.90 ± 0.13^{a}	9.00 ± 0.23^{a}	3.95 ± 0.20^{a}
Germinated mahlab	9.41 ± 0.30^{b}	11.30 ± 0.08^{a}	41.39 ± 0.20^{d}	24.34 ± 0.17^{b}	9.90 ± 0.29^{a}	3.66 ± 0.10^{a}

Values are means of triplicate determinations. ^{a,b,c,d} Means in the same column followed by the same superscript are not significantly different at $P < 0.05$

frying increased the total carbohydrate content in groundnuts [31]. This indicates that *M. ciliatuim* flour is a good source of energy for consumers.

Ash content. The ash contents in untreated and treated black mahlab seeds are shown in Table 1. The ash content in untreated black mahlab was 3.92%. Insignificantly affected by all processing techniques, it amounted to 3.95, 3.66, and 3.45% in roasted, germinated, and boiled black mahlab, respectively.

Mineral content in boiled, roasted, and germinated *M. ciliatum* seeds. The mineral compositions of untreated, boiled, roasted, and germinated black mahlab seeds are presented in Table 2. The concentrations of major and trace elements in the untreated seeds were significantly ($P < 0.05$) higher than in the processed seeds. Table 2 shows how boiling, roasting, and germination affected sodium, calcium, potassium, copper, iron, zinc, magnesium, manganese, selenium, and phosphorus contents in *M. ciliatum* seeds. As we can see, the three processing treatments varied in their effects on the mineral contents.

The sodium (Na) content in untreated black mahlab was 264.1 mg/kg. This value insignificantly decreased to 251.6 mg/kg in untreated black mahlab, significantly decreased to 227.4 mg/kg in roasted black mahlab, and insignificantly increased to 270.7 mg/kg in germinated black mahlab.

The calcium (Ca) content in untreated black mahlab was 4911 mg/kg. After treatment, it significantly decreased to 4158.5, 4666.3, and 3880.3 mg/kg in boiled, roasted, and germinated black mahlab, respectively. The roasted sample had the highest content of calcium.

The potassium (K) content in untreated black mahlab was 7812.7 mg/kg. After treatment, it decreased significantly to 4787.6 mg/kg in boiled black mahlab and insignificantly to 7702.6 and 7140.0 mg/kg in roasted and germinated black mahlab, respectively. The roasted *M. ciliatum* seeds had the highest content of potassium.

The copper (Cu) content in untreated black mahlab was 12.40 mg/kg. It did not change significantly after the treatments, amounting to 12.11, 11.53, and 11.73 mg/kg in boiled, roasted, and germinated black mahlab, respectively.

The iron (Fe) content in untreated black mahlab was 166.5 mg/kg. After treatment, it significantly decreased to 59.2 and 89.6 mg/kg in boiled and roasted black mahlab, respectively, and insignificantly decreased to 162.2 mg/kg in germinated black mahlab.

The zinc (Zn) content in untreated black mahlab was 23.66 mg/kg. After treatment, it significantly decreased to 19.67 and 21.36 mg/kg in boiled and roasted black mahlab, respectively, and insignificantly decreased to 22.88 mg/kg in germinated black mahlab. The germinated sample was the richest in zinc.

The magnesium (Mg) content in untreated black mahlab was 4747.2 mg/kg. After treatment, it significantly decreased to 4387.6 and 4367.3 mg/kg in boiled and germinated black mahlab, respectively. However, roasting had no significant effect on the magnesium content, which amounted to 4747.6 mg/kg in roasted black mahlab.

The manganese (Mn) content in untreated black mahlab was 93.19 mg/kg. This value significantly decreased after treatment, amounting to 66.02, 84.79, and 67.36 mg/kg in boiled, roasted, and germinated black mahlab, respectively. The roasted sample was the richest in manganese.

The selenium (Se) content in untreated black mahlab was 0.56 mg/kg. It did not change significantly after boiling, amounting to 0.54 mg/kg in boiled black mahlab. However, it significantly decreased to 0.26 and 0.41 mg/kg in roasted and germinated black mahlab, respectively.

The phosphorus (P) content in untreated black mahlab was 3059.5 mg/kg. It did not change significantly after germination, amounting to 3002.7 mg/kg in germinated black mahlab. However, it significantly increased to 3118.8 and 3205.8 mg/kg in boiled and roasted black mahlab, respectively. The roasted sample had the highest content of phosphorus.

Thus, the three processing treatments generally decreased the contents of minerals in the raw seeds. Sodium was decreased by boiling and roasting, but increased by germination. Iron and zinc were insignificantly affected by boiling and roasting. Magnesium was not affected by roasting but it was decreased by boiling and germination. Selenium slightly diminished with boiling but significantly diminished after roasting and germination. However, the roasting technique contributed most to the minerals retention, followed by germination and then boiling. This might be due to the fact that minerals leached from the seeds into distilled water at different rates during cooking. This result agreed with Kinge *et al.*, who reported that boiling and roasting of Djansang (*Ricinondron*

Table 2 Effects of boiling, roasting, and germination on the mineral contents (g/kg) in *Monechma ciliatum* seeds

Sample	Na	Ca	K	Cu	Fe	Zn	Mg	Mn	Se	P
Untreated mahlab	264.1^{a}	4911.2^{a}	7812.7^{a}	12.40^{a}	166.5^{a}	23.66^{a}	4747.2^{a}	93.19^{a}	0.56^{a}	3059.5^{a}
Boiled mahlab	251.6^{b}	4158.5^{b}	4787.6^{b}	12.11^{a}	59.2^{d}	19.67^{b}	4387.6^{b}	66.02^{b}	0.54^{a}	3118.9^{b}
Roasted mahlab	227.4^{c}	4666.3^{c}	7702.6^{c}	11.53^{b}	89.6^{c}	21.36^{a}	4747.6^{a}	84.79^{c}	0.26^{b}	3205.8^{c}
Germinated mahlab	270.7^{d}	3880.3^{d}	7140.0^{d}	11.73^{b}	162.5^{b}	22.88^{a}	4367.2^{b}	67.36^{d}	0.41^{c}	3002.7^{d}

Values are means of triplicate determinations ± S.D. ^{a,b,c,d} Means in the same column followed by the same superscript are not significantly different at $P < 0.05$

heudelotii L.) seeds significantly increased the amount of phosphorous, iron, calcium, and magnesium [37]. Boiling retained those minerals better than roasting. However, the amounts of potassium and sodium were significantly lower in the boiled samples compared to the roasted ones. Their study concluded that the roasting process preserved minerals better than boiling. Our findings were also consistent with the ones we made earlier, namely that the contents of major elements in raw safflower seeds were higher than in the roasted and boiled seeds [13]. According to Table 2, only sodium and phosphorus were significantly increased by germination and roasting, respectively.

Fatty acid composition of the oil from boiled, roasted, and germinated *M. ciliatum* seeds. The human body uses essential fatty acids (EFAs) to produce healthy cell membranes and benefit from their multiple biological roles. In particular, they influence the inflammatory cascade, reduce the oxidative stress, and provide neural and cardiovascular protection. A significant factor in various illnesses, fatty acid levels are used to distinguish potential biomarkers for a few pathologies, for example, polycystic ovary condition [38]. Some treatments, such as progressive heating, can influence the arrangement of fatty acids in food [39].

The compositions of fatty acids in untreated and treated *M. ciliatum* seed oils (untreated, boiled, roasted, and germinated black mahlab) determined by gas chromatography are presented in Table 3. As we can see, oleic and linoleic were the major fatty acids. The untreated sample had 68.15% of unsaturated fatty acids and 31.40% of saturated fatty acids. Boiling slightly changed the composition of unsaturated fatty acids and decreased the content of saturated fatty acids to 22.82%. Palmitic acid increased from 6.11% in untreated black mahlab to 31.80 and 21.80% in roasted and germinated black mahlab, respectively. Myristic acid increased from 0.14% in the untreated seeds to 4.46 and 8.43% in roasted and germinated black mahlab, respectively. Oleic and linoleic acids decreased from 44.87 and 16.84% in the untreated samples to 39.21 and 10.26% in roasted black mahlab and to 29.40 and 9.16% in germinated black mahlab, respectively. It was clear that roasting and germination increased the content of saturated fatty acids and decreased that of unsaturated fatty acids. These results disagreed with our previous study, where fatty acids of black cumin seeds did not change with roasting and boiling [29]. Ali *et al.* found that the relative content of polyunsaturated fatty acids decreased while that of saturated fatty acids increased in groundnut seed oil exposed to microwave heating [35]. However, the roasting process slowed down the oxidative deterioration of polyunsaturated fatty acids.

Tocopherol composition of the oil from boiled, roasted, and germinated *M. ciliatum* seeds. Tocopherols are fat-soluble compounds with vitamin E. This is a term for eight different molecules, namely *α*-, *β*-, *γ*-, *δ*-tocopherol, and the corresponding tocotrienols. The activity of vitamin E in humans is related to its antioxidant properties. It is synthesized only in photosynthetic organisms and acts as a protective component. Tocopherol has also been found to be crucial for seed storage and germination [40]. The nutritional benefits of vitamin E (α-tocopherol) and its importance in the daily diet have been well documented. The contents of total tocopherols in treated and untreated *M. ciliatum* seeds oil are shown in Table 3. As we can see, the total tocopherol concentration decreased during boiling and roasting as a result of heating. However, it was significantly increased by germination. In particular, the content of tocopherols in the untreated oil was 0.11 mg/100 g. This amount was affected equally by boiling and roasting,

Table 3 Boiling, roasting, and germination effects on fatty acids (%) and tocopherols (mg/100 g) in *Monechma ciliatum* seed oil

Fatty acids		Untreated mahlab	Boiled mahlab	Roasted mahlab	Germinated mahlab
C12	Lauric	0.859^{a}	1.807^{b}	2.258^{c}	20.427^{d}
C14	Myristic	0.140^{a}	0.124^{a}	4.462^{b}	8.433^{c}
C16	Palmitic	6.116^{a}	6.495^{a}	31.804^{b}	21.806^{c}
C18	Stearic	3.238^{a}	3.236^{a}	6.135^{b}	3.806^{a}
C20	Archidic	9.183^{a}	9.020^{a}	–	–
C22	Behenic	0.839^{a}	0.829^{a}	–	–
C23	Tricosanoic	7.033^{a}	6.626^{b}	–	–
C24	Lignoceric	3.994^{a}	3.709^{a}	–	–
C16:1	Pamitoleic	0.251^{a}	0.270^{a}	4.083^{b}	2.177^{c}
C18:1	Oleic	44.878^{a}	44.420^{a}	39.216^{b}	29.408^{c}
C18:2	Linoleic	16.480^{a}	16.734^{a}	10.264^{b}	9.159^{c}
C20:1	Eicosenoic	6.545^{a}	6.453^{a}	1.378^{b}	0.73^{c}
Saturated		31.402^{a}	22.826^{b}	45.059^{c}	54.203^{d}
Unsaturated		68.154^{a}	67.881^{b}	54.941^{c}	41.475^{d}
Tocopherol		0.11^{a}	0.10^{a}	0.10^{a}	0.18^{b}

^{a,b,c,d} Means in the same row followed by the same superscript are not significantly different at $P < 0.05$

Table 4 Boiling, roasting, and germination effects on amino acids in *Monechma ciliatum* seeds, g/100 g

Amino acid	Untreated mahlab	Boiled mahlab	Roasted mahlab	Germinated mahlab
Aspartic acid	2.294	2.349	2.285	2.514
Serine	1.555	1.665	1.576	1.676
Glumatic acid	2.485	3.679	3.386	3.824
Glycine	1.165	1.224	1.243	1.229
Histidine	0.525	0.559	0.586	0.525
Arginine	2.532	2.930	2.889	2.889
Therionine	1.014	1.111	1.092	1.096
Alanine	1.130	1.207	1.170	1.213
Proline	1.157	1.276	1.198	1.306
Threonine	0.695	0.744	0.743	0.750
Valine	1.215	1.318	1.262	1.354
Methionine	0.300	0.000	0.023	0.000
Lysine	1.386	1.453	1.246	1.462
Isoleucine	1.009	1.123	1.061	1.124
Leucine	1.912	2.130	2.003	2.112
Phenylalanine	1.017	1.124	1.134	1.164
Total	22.291	23.894	22.899	24.336

Values are means ± SD

decreasing to 0.10 mg/100 g in both boiled and roasted black mahlab. Germination, however, increased it to 0.18 mg/100 g. Our results agreed with Junmin *et al.*, who reported that the roasting of sesame seeds at 160°C for 30 min led to a steady decrease in total tocopherols and sesamolin [41].

Amino acid composition in boiled, roasted, and germinated *M. ciliatum* seeds. Table 4 shows the amino acid composition in the treated and untreated *M. ciliatum* seeds. Generally, amino acids increased with boiling, roasting, and germination, except for methionine acid which was decreased by all the treatments. Aspartic acid and lysine were decreased by roasting. Total amino acids in the untreated black mahlab seeds amounted to 22.291 g/100 g. They increased to 23.894, 22.899, and 24.336 g/100 g in the boiled, roasted, and germinated samples, respectively. The roasted sample had the lowest content of total amino acids due to the decrease in aspartic acid and lysine. These results were in agreement with those in our earlier work, where we observed extremely high contents of amino acids in the boiled and roasted safflower seeds, compared to the fresh samples [13]. This finding was also consistent with that of EL-Suhaibani *et al.*, who found that germination and cooking of goat pea (*Securigera securidaca* L.) seeds increased the proportion of essential amino acids [42]. They also reported that soaking and cooking processes increased valine, phenylalanine, isoleucine, and leucine, but reduced methionine and lysine. Our results disagreed with those reported by Nwosu *et al.*, who found that boiling black gram (*Vigna mungo* L.) seeds for 120 min generally decreased the concentration of leucine, lysine, and arginine [12]. Blanching and soaking improved the concentrations of lysine, isoleucine, and histidine, compared to the control samples.

CONCLUSION

Generally, most of the nutritional factors were enhanced by processing treatments. All the treatments increased protein and amino acids. Boiling and roasting increased the fat content, while boiling and germination increased the fiber content. Saturated fatty acids were higher and unsaturated fatty acids were lower in the roasted and germinated samples. Minerals were decreased by all the treatments, except for sodium which increased in the germinated sample. Our results can be applied in large-scale research experiments with *Monechma ciliatum* L. seeds used as a food product, supplement, or ingredient in new products.

CONTRIBUTION

Abdalbasit Adam Mariod conceived and designed the analysis, contributed data and analysis tools, and wrote the paper. Eshraga Mustafa Abdalrahman Mustafa collected the data, performed the analysis, and wrote the paper. Mahdi Abbas Shakak contributed data and analysis tools and wrote the paper.

CONFLICT OF INTEREST

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

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Egg-free low-fat mayonnaise from virgin coconut oil

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Abstract:

Introduction. Mayonnaise is a widely consumed product all over the world. Nowadays, the number of vegetarians, egg allergy cases, and heart diseases are increasing. This makes manufacturers develop alternatives. The research objective was to select the optimal concentration of emulsifiers for egg-free mayonnaise made from virgin coconut oil.

Study objects and methods. We produced 20 egg-free mayonnaise samples with different amounts of emulsifiers. We also determined physicochemical properties of the samples, as well as performed proximate and statistical analyses.

Results and discussion. The response surface methodology made it possible to define such parameters as viscosity, stability, and firmness as affected by the following concentrations: cashew nut protein isolates – 5–15%, xanthan gum – 0–1%, and modified starch – 0–0.5%. The optimal values of emulsifiers were obtained as follows: cashew nut protein isolates – 13 g, xanthan gum – 1.0 g, and modified starch – 0.4 g. The optimized mayonnaise had the following parameters: viscosity – 120.2 mPa·s, stability – 98.7%, and firmness – 25 g. The study revealed no significant differences ($P > 0.05$) between the actual and predicted data, which confirmed the efficiency of the suggested models.

Conclusion. The obtained low-fat egg-free mayonnaise was relatively similar to the traditional commercial products. However, virgin coconut oil should be emulsified with a combination of cashew nut protein isolates, modified starch, and xanthan gum.

Keywords: Mayonnaise, emulsion, egg yolk, emulsifier, protein isolates, cashew nut, virgin coconut oil

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INTRODUCTION

Mayonnaise is an emulsion of oil in water. Therefore, dietary mayonnaise has a smaller dispersed step and larger water content [1–3]. Mayonnaise consists of 60–80% fat [4]. Conventionally, it contains egg yolk, oil, lemon juice or vinegar, and seasonings, e.g. salt, mustard, paprika, sweeteners, etc. Three main components in mayonnaise perform as different phases in the formulation: oil is the dispersed phase, water is the continuous phase, and egg yolk is the emulsifier [5, 6]. Mayonnaise is fat-free if its fat level is at least 50% lower than that of standard mayonnaise; mayonnaise is considered light if its fat level is 25% lower than standard [7].

Eggs are a common mayonnaise emulsifier because their emulsifying properties are perfect for mayonnaise production. However, the growing rates of vegetarianism, egg allergy, heart diseases, and production costs make producers look for egg-free formulation variants.

Furthermore, plant-based diets have gained popularity not only due to the health benefits they promise but as a way to reduce environmental footprint [8]. Therefore, new egg substitutes and egg-free products are of great importance in vegetarian food supplies [9]. In general, protein acts as a surfactant to reduce the surface tension between hydrophilic and lipophilic materials in food systems and stabilize emulsions. Cashew nut protein isolates can serve as an egg alternative and a fat replacer agent due to their excellent emulsifying property [10]. However, cashew nuts are a much less popular plant protein, despite their excellent sensory and nutritional benefits [11].

Several studies have evaluated plant-based emulsifiers as potential substitutes for eggs. Chetana *et al.* reported egg-free mayonnaise of rice bran oil and sesame oil produced by replacing egg with xanthan gum [14]. Gaikwad *et al.* managed to replace egg yolk with skim milk powder [15]. In another study, wheat germ protein isolate and xanthan gum substituted egg yolk to produce low-cholesterol mayonnaise with acceptable characteristics [16]. Modified starch can also serve as an alternative to fat and eggs in low-fat mayonnaise [17]. Among vegetable oils, coconut oil obtained from coconut kernel (*Cocos nucifera* L.) was reported to have antibacterial and antioxidant biological activities [18]. Virgin coconut oil is widely used in other vegetable oils since it has many health benefits. Virgin coconut oil decreases total cholesterol, triglycerides, phospholipids and low-density lipoprotein (LDL) cholesterol, while increasing high-density lipoprotein (HDL) cholesterol in the blood [19].

Although many studies reported this or that kind of egg-free mayonnaise produced from various oils and emulsifiers, none of them featured the combination of cashew nut protein isolates, xanthan gum, and modified starch. Consequently, the current study aims at selecting the optimal concentration of emulsifiers of cashew nut protein isolates, xanthan gum, and modified starch to produce egg-free virgin coconut oil mayonnaise and compare its properties with commercial mayonnaise products.

STUDY OBJECTS AND METHODS

Materials. Table 1 shows the ingredients of egg-free Lady's Choice mayonnaise (Bangi, Selangor) used as a reference sample. Xanthan gum, cashew nut protein isolates, and modified starch (maize) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Experimental design. The methods of response surface methodology and central composite design were used with three independent variables of emulsifiers, namely cashew nut protein isolates (5–15%) (X_c), xanthan gum (0–1%) (X_x), and modified starch (0–0.5%) (X_m) (Table 2). Viscosity (Y_1), stability (Y_2), and firmness (Y_3) served as response variables.

Preparation of egg-free virgin coconut oil mayonnaise. The low-fat and egg-free mayonnaise-like emulsion gel was prepared according to Mozafari *et al.* with some modifications [20]. Briefly, a fixed amount of distilled water, lemon juice, mustard, sugar, acetic acid, and salt (Table 1) were mixed in a blender 8010S (Waring Commercial Torrington, USA), at medium speed for 3 min to achieve a smooth and creamy coarse-phase emulsion. Virgin coconut oil was then gradually added to the coarse-phase emulsion, followed by emulsifiers, i.e. cashew nut protein isolates, modified starch, and xanthan gum (Table 2). The mix (500 mL) was further homogenized at high speed for 2 min until smooth and creamy. All mayonnaise samples were transferred into 500-mL sterilized glass jars, capped, tightly sealed, and kept at room temperature (25 ± 2°C) prior further analysis.

Physicochemical properties. Physicochemical properties are given for the optimized formulation only.

Viscosity. The viscosity measurement followed the method developed by Makeri *et al.* [21]. It involved a rheometer HAAKE RheoStress RS600 (Thermo Electron Corporation, Karlsruhe, Germany) and a parallel stainless-steel plate with a 25-mm diameter at a 1-mm distance at 25°C. A sample of 10 mL was loaded onto the plate with extreme carefulness to prevent emulsion softening. The excess sample was carefully trimmed from the sensor edge with a thin blade [22]. The flow characteristics were determined at a temperature of 25°C and a shear rate of 1–100 s^{-1}. Each viscosity measurement was performed in triplicate, and mean ± SD values were plotted.

Texture. The texture of the egg-free virgin coconut oil mayonnaise was determined using a texture analyser (XT2i, Surrey, UK) following the method described in [23] with slight modifications. A total of 100 mg for each sample was placed in round plastic containers at a depth of 30 mm. The texture was determined using a P/35-cylinder probe (Stable Micro System, Surrey, UK). The force was measured in compression mode at fixed 75% strain at room temperature (25 ± 2°C). The test conditions included 10 mm penetration, 1 mm/s pre-test speed, as well as 1 and 10 mm/s test speed. The tests were performed in triplicate, and the mean values were tabulated.

Stability. The mayonnaise emulsion stability test was based on the amount of oil removed from the emulsion after centrifugation [24]. Briefly, 1.5 g of the sample was placed in a 25-mL centrifuge tube (Refrigerated centrifuge SIGMA 3-18K, Goettingen, Germany) and weighed (initial weight, F_0). The sample was heated for 30 min at 80°C in a shaking water bath at 120 rpm to form emulsion. After heating, the emulsions were centrifuged in a Thermo Sorvall Legend Micro 17 micro-centrifuge (Thermo Science, Waltham, MA) for

Table 1 Formulation for egg-free virgin coconut oil mayonnaise

Ingredients	Amount
Distilled water, mL	32.20
Virgin coconut oil, mL	32.20
Lemon juice, mL	16.10
Mustard, g	3.35
Sugar, g	2.68
Acetic acid, mL	2.68
Salt, g	0.13 g
Cashew nut protein isolates, X_c, %*	5–15
Xanthan gum, X_x, %*	0–0.1
Modified starch, X_m, %*	0–0.5

* % varies according to formulations generated using response surface methodology experimental design

Table 2 Response surface methodology experimental design of the three independent variables in egg-free mayonnaise formulations

Run order	Block	Cashew nut protein isolate	Xanthan gum	Modified starch
		(X_c)	(X_x)	(X_m)
1(c)	3	10(0)	0.5(0)	0.25(0)
2(c)	3	10(0)	0.5(0)	0.25(0)
3	3	15(1)	0.5(0)	0.25(0)
4	3	15(1)	1(1)	0.5(1)
5	3	5(–1)	0(–1)	0(–1)
6(c)	3	10(0)	0.5(0)	0.25(0)
7	3	5(–1)	0(–1)	0.5(1)
8	3	15(1)	1(1)	0(–1)
9(c)	1	10(0)	0.5(0)	0.25(0)
10	1	10(0)	0.5(0)	0.5(1)
11	1	10(0)	0.5(0)	0(–1)
12(c)	1	10(0)	0.5(0)	0.25(0)
13	1	5(–1)	1(1)	0.5(1)
14	1	15(1)	0(–1)	0.5(1)
15	2	10(0)	0(–1)	0.25(0)
16(c)	2	10(0)	0.5(0)	0.25(0)
17	2	5(–1)	1(1)	0(–1)
18	2	10(0	1(1)	0.25(0)
19	2	5(–1)	0.5(0)	0.25(0)
20	2	15 (1)	0(–1)	0(–1)

c is center point

5 min at 5000 rpm, and the top oil layer was extracted with a long-needle syringe. The precipitated fraction (F_1) was weighed, and the stability of the emulsions was estimated using the equation below:

$$\text{Percentage of emulsion stability (\%)} = \frac{F1}{F0} \times 100 \quad (1)$$

where F_0 is the Initial weight; F_1 is the weight of the precipitated fraction.

Water activity. The water activity test followed the calibration procedure. The sample cup was filled halfway with 3 g of mayonnaise sample using an AquaLab water activity meter (Model 3TE, Decagon Devices, USA). The sample chamber lid was sealed to reach vapor equilibrium. The dew point/temperature was later translated into water activity (A_w) reading.

pH measurement. The pH values were assessed by using a pH meter (S210 Seven compact, Mettler-Toledo Instrument Co., Ltd., Shanghai, China) at 25°C. The pH meter was adjusted at pH 7.01, 4.01, and 10.01 buffer solutions. The pH values were presented as a mean of three readings for one sample.

Proximate analysis. ***Moisture content.*** The moisture content was determined using the method developed by the Association of Official Analytical Chemists (AOAC) [25]. A sample of 5 g was put into a covered crucible and placed into a Memmert 800 oven (Schwabach, Germany). There it stayed for at least 7 h at 105°C; the temperature of the oven was constant. The crucible and its cover were set on the balance and weighed quickly and accurately. The weighing process was repeated to obtain constant weight. The moisture content was calculated based on the percentage of wet-weight:

$$\text{Wet} - \text{weight percentage (\%)} = \frac{(A-B)}{A} \times 100 \quad (2)$$

where A is the weight of sample before oven drying, g; B is the weight of dried sample after oven drying, g.

Protein content. This crude protein analysis method was designed by AOAC: it is based on the nitrogen (N) determination according to the Kjeldahl method in a Kjeltec 2100 Distillation Unit (Foss Tecator, Hoganas, Sweden) [25]. The protein content was calculated using the following formula:

$$\text{Protein content} \left(\frac{\text{g}}{100\text{g}}\right) = \text{Nitrogen content} \times F \quad (3)$$

where F is the protein factor (6.25, depends on the sample).

Fat content. The fat content was measured according to another AOAC method by petroleum ether extraction using a Soxtec System (2055 Soxtec Avanti; Foss Tecator, Höganäs, Sweden) [25]. The fat content was calculated by using the following formula:

$$\text{Fat content} \left(\frac{\text{g}}{100\text{g}}\right) = \frac{W_1 - W_2}{W_1} \times 100 \quad (4)$$

where W_1 is the sample weight, g; W_2 is the plain aluminum weight, g; W_3 is the aluminum with sample weight, g.

Ash content. The ash content was measured according to AOAC method: 10 g of the sample were placed into the crucible [25]. After recording the weight, it was put into a muffle furnace at 550°C. The sample burned for at least 2 h to obtain permanent weight, until no black particles. Next, the crucible and ash were cooled in the desiccators. Finally, the crucible was weighed together with the ash.

$$\text{Ash percentage (\%)} = \frac{(\text{a} + \text{b}) - \text{b}}{\text{c}} \times 100 \quad (5)$$

where a is the weight of ash; b is the weight of crucible; c is the weight of sample.

Carbohydrates content. The carbohydrate content was determined by extracting the protein, fat, moisture, and ash amount from 100%.

Statistical analysis. Minitab 17.0 (Minitab, Inc, State College Pennsylvania, USA) was used for optimization. The software programmed a face-centered composite design with three independent variables, namely cashew nut protein isolates (X_c), xanthan gum (X_x), and modified starch (X_m) at three coded levels (–1, 0, +1). The experiment involved six replicates at the center stage, with a total design of 20 experimental runs per sample. As a result, the effect of the two independent variables on the response surface was obtained as 3-D graphs of response. The polynomial regression model equation was used to define the performance of the response surface. The generalized response surface model looked as follows:

Table 3 Viscosity, stability, and firmness of egg-free virgin coconut oil mayonnaise produced with different percentages of cashew nut protein isolate, xanthan gum, and modified starch

Run order	Cashew nut protein isolate (X_c)	Xanthan gum (X_x)	Modified starch (X_m)	Viscosity, mPa·s (Y_v)	Stability, % (Y_s)	Firmness, g (Y_f)
1	10	0.5	0.3	104.2 ± 11.4	95.2 ± 2.2	24.6 ± 3.7
2	10	0.5	0.3	98.2 ± 4.9	89.3 ± 0.6	25.8 ± 1.6
3	15	0.5	0.3	101.6 ± 6.9	92.9 ± 1.0	22.3 ± 3.0
4	15	1	0.5	120.3 ± 22.8	100.0 ± 0.0	21.1 ± 2.0
5	5	0	0	47.8 ± 5.7	81.8 ± 0.7	9.2 ± 2.2
6	10	0.5	0.3	100.3 ± 15.6	93.9 ± 1.4	30.0 ± 2.0
7	5	0	0.5	88.2 ± 3.9	93.4 ± 1.8	17.3 ± 1.5
8	15	1	0	92.1 ± 3.6	96.4 ± 0.1	10.8 ± 1.4
9	10	0.5	0.3	106.8 ± 6.5	94.3 ± 0.9	22.3 ± 2.2
10	10	0.5	0.5	127.8 ± 19.4	95.8 ± 0.2	30.8 ± 3.1
11	10	0.5	0	84.0 ± 6.8	93.1 ± 1.6	17.5 ± 1.6
12	10	0.5	0.3	107.9 ± 9.0	93.9 ± 0.4	21.1 ± 1.8
13	5	1	0.5	122.8 ± 14.3	100.0 ± 0.0	16.6 ± 1.7
14	15	0	0.5	97.8 ± 4.8	94.2 ± 2.0	19.3 ± 5.1
15	10	0	0.3	72.8 ± 7.3	87.2 ± 1.3	19.4 ± 2.0
16	10	0.5	0.3	103.4 ± 14.4	94.9 ± 1.6	27.6 ± 2.7
17	5	1	0	93.4 ± 3.6	97.4 ± 1.2	6.7 ± 1.2
18	10	1	0.3	123.0 ± 6.5	95.2 ± 0.2	27.7 ± 1.8
19	5	0.5	0.3	98.1 ± 7.6	89.8 ± 3.1	18.0 ± 1.5
20	15	0	0	44.1 ± 3.1	79.2 ± 0.9	13.1 ± 2.1

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 \quad (6)$$

where y is the response calculated by the model; β_0 is the constant regression; β_i, β_{ii}, β_{1j} are the linear, squared, and interaction coefficients, respectively; x_1, x_2 are the independent variables.

The responses were evaluated by multiple regressions and the square least method. A t-test was performed to compare the properties of both mayonnaise samples.

To validate the model, the experimental data were compared to the predicted values using the t-test at P-value = 1 and F-ratio = 0 for each response. Therefore, the model was declared suitable when no statistically significant difference was observed between the experimental and predicted values.

RESULTS AND DISCUSSION

Response surface methodology. The goal of the optimization was to obtain target values for responses, viscosity, and firmness, as well as to maximize the stability of the emulsion. The initial step was to decide on the experimental ranges for the independent variables. The levels of variation were selected according to a preliminary study. A uniform precision type central-composite design consisted of three variables, namely cashew nut protein isolates, xanthan gum, and modified starch. It had a three-level pattern with 20 runs and was prepared using the response surface methodology. The experimental design contained six cube center points, where six out of twenty runs were replications of the central points of all the factors. Twenty samples of egg-free virgin coconut oil mayonnaise were prepared based on the emulsifier quantity proposed in the experimental design. Other ingredients remained constant.

All twenty samples were measured for viscosity, stability, and firmness. Table 3 displays the variables, levels, and results obtained for all the responses. The analysis of variance was used to determine the significance of the linear, quadratic, and interaction effects, as well as the lack of fit value against the responses in the variables. The models fit well for all the response variables because they had acceptable levels of R^2 of more than 80%.

Table 4 illustrates the summary of R^2, %, P-value, and multiple regression equation of response for reduced regression equation model in the decoded units. The best model was the one with the highest R^2, lowest P-value (model), and the highest number of significant factors. The emulsifiers were optimized by identifying the desired response. The anticipated responses were designated based on the viscosity, stability, and firmness of commercial mayonnaise. These properties are known to be accepted by consumers. The reference mayonnaise underwent an analysis to obtain the desired response. The lack-of-fit in all the models had a P-value ≥ 0.05, i.e. the models were acceptable. The next step involved the P-value of individual factors of the quadratic and interaction effect against response. The factors with insignificant effects were removed to obtain a fitted reduced model equation.

In this study, X_c, X_x, and X_m were coded values for independent variables in the experiment, i.e. cashew

Table 4 Summary of reduced model equation for all responses

Response	R^2, %	*P*-value	Reduced model equation
Viscosity, mPa·s	97.5	0.00	$Y_v = 22.76 + 5.64\,X_c + 84.9\,X_x + 96.48\,X_m - 0.2760\,X_c \cdot X_c - 35.56\,X_x \cdot X_x - 36.5\,X_g \cdot X_m$
Stability, %	88.7	0.00	$Y_s = 81.89 - 0.033\,X_c + 15.74\,X_x + 24.42\,X_m - 20.40\,X_x \cdot X_m$
Firmness, g	80.9	0.00	$Y_f = -21.18 + 7.74\,X_c + 0.97\,X_x + 19.10\,X_m - 0.3683\,X_c \cdot X_c$

nut protein isolates, xanthan gum and modified starch, respectively. Likewise, Y_v, Y_s, and Y_f were coded values for viscosity, stability, and firmness dependent variables. All the initial and reduced model multiple regression equations used the code above.

Effect of independent variables on viscosity (Y_v). Viscosity measurement is essential to characterize the structure and stability of the food emulsion products, such as mayonnaise. Figure 1a shows that both the linear and square effects were significant for viscosity, while the overall model *P*-value was < 0.05 for both. The interaction effect of cashew nut protein isolates with xanthan gum and modified starch was not significant, with a *P*-value of 0.472 and 0.372, respectively.

The analysis of regression coefficient showed that viscosity experienced significant impact ($P < 0.05$) from the linear effect of cashew nut protein isolates (X_c), xanthan gum (X_x), modified starch (X_m), quadratic effect cashew nut – cashew nut ($X_c \cdot X_c$), xanthan gum – xanthan gum ($X_x \cdot X_x$), and interaction effect xanthan gum – modified starch ($X_x \cdot X_m$). The increased amount of these variables resulted in increased viscosity. The reduced model equation for viscosity was predicted as below:

$$Y_v = 22.76 + 5.64\,X_c + 84.9\,X_x + 96.48\,X_m - 0.2760\,X_c \cdot X_c - 35.56\,X_x \cdot X_x - 36.5\,X_x \cdot X_m \quad (7)$$

The equation above was fitted using a second-degree polynomial model for independent variable effects of cashew nut protein isolates, xanthan gum, and modified starch on apparent viscosity response. The modified value ($R^2 = 97.5$) proved that more than 97% of the experimental points were adequate independent variables.

The highest viscosity reading obtained was 127.8 ± 19.4 mPa·s, and the lowest was 44.1 ± 3.1 mPa·s. Among all three factors, xanthan gum had the most significant effect on viscosity. This finding was similar to the results obtained by Mozafari *et al.*, who found that xanthan affected the viscosity of low-fat low-cholesterol mayonnaise [20].

Also, Kumar *et al.* illustrated that xanthan gum significantly impacted the viscosity of egg-free

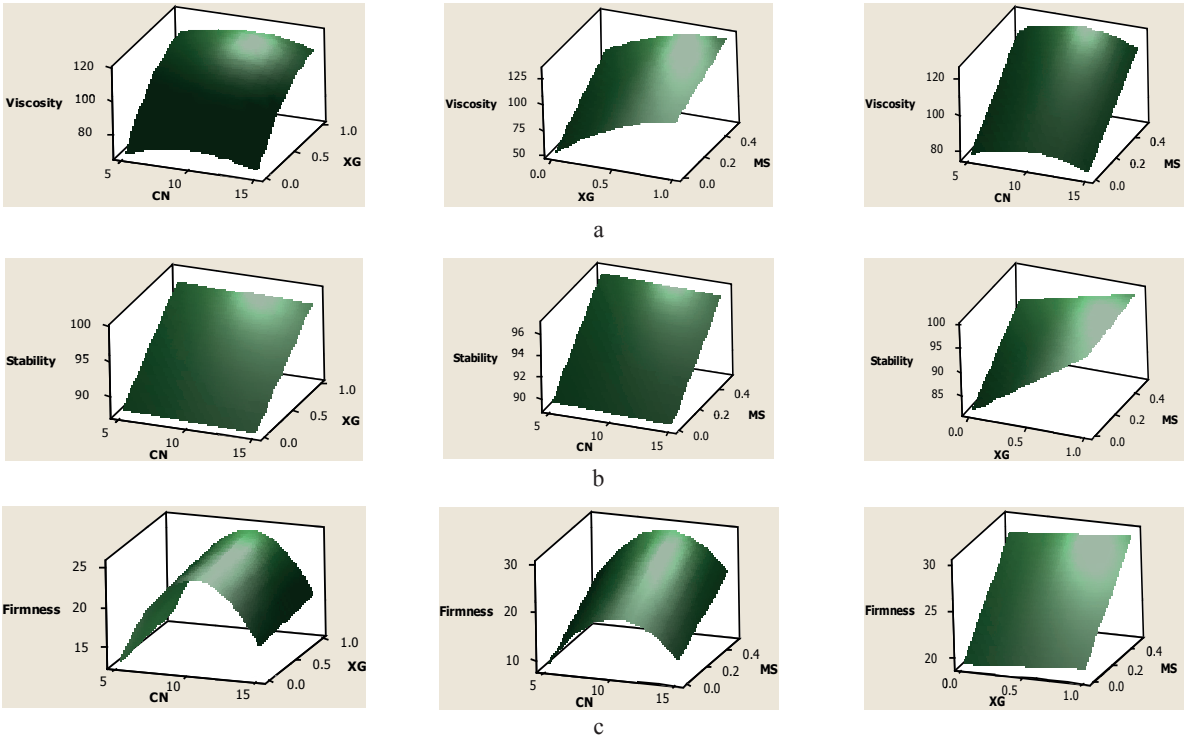

Figure 1 Surface plots of viscosity (a), solubility (b), and firmness (c) changes in low-fat egg-free mayonnaise by formulation parameters

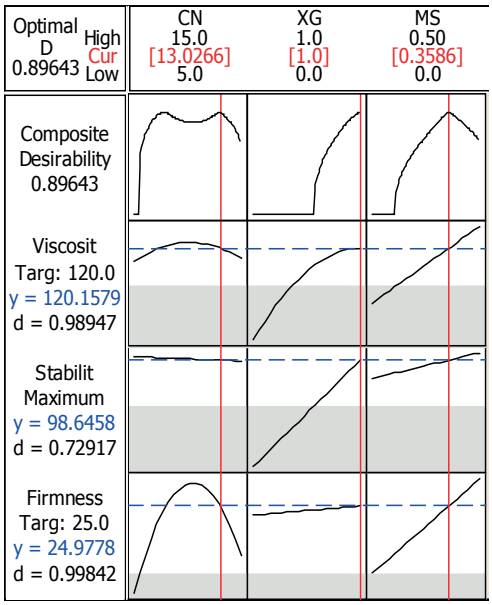

Figure 2 Response optimization

mayonnaise produced by ultrasonication [26]. The experimental outcomes and contour plots showed that a larger amount of xanthan gum followed by modified starch improved the viscosity of mayonnaise samples. For standard oil, the viscosity and flow behavior in water emulsion was captivated by the dispersed phase and controlled by the hydrophilic additives, such as sugar, salt, and polymeric thickener [27].

Effect of independent variables on stability (Y_s). Egg-free virgin coconut oil mayonnaise samples proved moderate to high stability, depending on the emulsifier used in the formulation. The linear effect of cashew nut protein isolates (X_c), xanthan gum (X_x), modified starch (X_m), and a combination of xanthan gum and modified starch ($X_x \cdot X_m$) had significant effects on the stability of egg-free virgin coconut oil mayonnaise. The remaining factors proved insignificant (P-value > 0.05) and were removed. A reduced model equation for stability was predicted as below:

$$Y_S = 81.89 - 0.033\,X_c + 15.74\,X_x + \\ + 24.42\,X_m - 20.40\,X_x \cdot X_m \quad (8)$$

The stability of samples was 100% for formulations 4 and 13, which had the maximal amount of emulsifier. This result was similar to the findings obtained by Mozafari *et al.*, who achieved a good stability of low-fat low-cholesterol mayonnaise with the maximal amount of xanthan gum and Zodo gum as emulsifiers [20]. In this study, the formulations of egg-free virgin coconut oil mayonnaise with xanthan gum and modified starch had higher emulsion stability than the control samples. Lee *et al.* reported similar findings in their study of low-fat mayonnaise with gelatinized rice starch and xanthan gum [24].

Table 5 Optimal values of emulsifiers (factors) derived through response surface methodology

Factor	Optimized value, g	Percentage in formulation, %
Cashew nut protein isolates	13.0	12.6
Xanthan gum	1.0	1.0
Modified starch	0.4	0.4

Two formulations demonstrated a much lower stability, namely formulation 5 (cashew nut protein isolates (X_c) – 5, Xanthan gum (X_x) – 0, Modified starch (X_m) – 0) and formulation 20 (cashew nut protein isolates (X_c) – 15, Xanthan gum (X_x) – 0, Modified starch (X_m) – 0). The stability was 81.8 ± 0.7 and 79.2 ± 0.9%, respectively.

Therefore, cashew nut protein isolates had an almost negligible effect as natural emulsifiers on the stability of the emulsion. In addition, the percentage of virgin coconut oil used in this formulation was approximately only 30–31%. This indicates that emulsion stability was affected by the biopolymers used in the system. According to Lee *et al.*, a lower amount of oil resulted in a significant decline in mayonnaise stability [24]. Therefore, such biopolymers as starches and gums have to be combined with such fat-reduced formulation products as stabilizers.

Effect of independent variables on firmness (Y_f). According to Khor *et al.*, firmness is the product's ability to resist deformation or breaking and increases with the force required for penetration [28]. Higher firmness of emulsion makes it difficult for the mouth to break the sample and swallow. The interactions between proteins and oils in a network structure are known to increase mayonnaise firmness [29].

Based on the P-value, all linear (X_c, X_x, and X_m) and quadratic effects of cashew nut protein isolates ($X_c \cdot X_c$) had a significant impact ($P < 0.05$) on the firmness. The best reduced model equation for predicting firmness was as follows:

$$Y_f = -21.18 + 7.74\,X_c + 0.97\,X_x + \\ + 19.10\,X_m - 0.3683\,X_c \cdot X_c \quad (9)$$

In this study, fat content in egg-free virgin coconut oil mayonnaise was 30%, which was lower than that in whole fat mayonnaise (70%). Such reduction of fat content caused a lower droplet density, which affected the emulsion stability by weakening the interactions between droplets. However, such lower oil content increased the aqueous phase and decreased the dispersed phase, which reduced the firmness and viscosity of the emulsion [30]. Singla *et al.* reported similar findings: a higher amount of xanthan gum with maltodextrin as thickener increased firmness and stickiness values [31].

Response optimization and model validation. A graphical optimization (Fig. 2) was performed using Minitab 16 package to optimize the percentage of

Table 6 Predicted optimal value and experimental values of response

Response	Experimental value	Predicted value	*P*-value
Viscosity, mPa·s	102.4	120.2	0.1
Stability, %	99.5	98.7	0.1
Firmness, g	21.8	25.0	0.2

* *P*-values < 0.05 are significant differences using Tukey Method test between experimental value and predicted value

emulsifier. The optimal values of emulsifiers were 13.0 g for cashew nut protein isolates, 1.0 g for xanthan gum, and 0.36 g for modified starch (Table 5). The desired response required the highest amount of xanthan gum.

Table 6 illustrates the predicted optimal and experimental values of response, viscosity, stability, and firmness. Based on the two-sample t-test, the *P*-value for all responses was > 0.05. Statistically, there was no significant difference between the experimental and predicted values. Thus, the model and the reduced model equations were validated and accepted.

Proximate analysis and physicochemical properties. Table 7 shows the proximate analysis and physicochemical properties of the optimal formulation of egg-free virgin coconut oil mayonnaise and reference samples. They demonstrated a significant difference ($P < 0.05$) in fat content, protein content, water activity, and consistency. In the egg-free virgin coconut oil mayonnaise, fat content, water activity, and consistency were significantly lower, whereas the protein content was higher compared to the reference product. However, the comparative analysis showed no significant difference in terms of viscosity, stability, firmness, cohesiveness, pH, moisture content, ash content, and carbohydrate content.

Singla *et al.* compared the firmness of the standard and the egg-free mayonnaise samples, and the egg-free mayonnaise showed a higher firmness [31]. However, the high-fat content in the standard mayonnaise caused an increment in textural firmness and stickiness by keeping the neighboring oil droplets flocculated to form a thin gel network.

In this study, thickeners enhanced the firmness and stickiness values in the egg-free virgin coconut oil mayonnaise compared with the egg-containing sample. Generally, the texture of mayonnaise depends on the ingredient selection and the effect of thickening agents used in the system.

The pH of the egg-free virgin coconut oil mayonnaise was acidic, and pH 4 was similar to that of the reference mayonnaise. The acidic emulsion is formed when adding lemon juice or vinegar. Acidic state extends the shelf life of the product and ensures its microbiological stability [28].

Based on [32, 33], mayonnaise producers favor higher acidity because it improves the microbial stability, emulsion stability, and viscoelasticity

Table 7 Proximate analysis and physicochemical properties of optimal formulation of egg-free virgin coconut oil mayonnaise and reference sample

Analysis	Egg-free virgin coconut oil mayonnaise	Reference mayonnaise	*P*-value
Viscosity, mPa·s	102.4 ± 4.1^a	121.1 ± 16.0^b	0.2
Stability, %	99.5 ± 0.3^a	99.7 ± 0.2^a	0.2
Firmness, g	21.8 ± 1.5^a	25.3 ± 5.1^a	0.3
Water activity	1.0 ± 0.0^a	1.0 ± 0.0^a	0.0
pH	4.0 ± 0.0^a	4.0 ± 0.0^b	0.2
Moisture content, %	34.7 ± 2.9^a	35.8 ± 4.3^a	1.0
Ash content, %	3.3 ± 0.5^a	3.6 ± 1.1^b	1.0
Protein content, g/100 g	2.6 ± 0.2	1.4*	0.0
Carbohydrate content, g/100 g	14.0 ± 3.7	9.2*	0.1
Fat content, g/100 g	27.5± 3.6	66.2*	0.0

*Values obtained from product nutritional information

properties. Moisture content is a significant factor as it affects stability and shelf life. The moisture content in the sample produced by applying the optimal conditions was 34.7 ± 2.9%, while for the commercial sample it was 35.8 ± 4.3%, which indicated no significant differences. This result could be due to the similar content of solid materials used to formulate the egg-free virgin coconut oil mayonnaise. The water activity of the egg-free virgin coconut oil mayonnaise was significantly lower compared to reference sample. Even though the percentage of water was higher in this formulation, a higher amount of emulsifier was expected to bind all the molecules to obtain properties similar to standard mayonnaise.

Ash content was 3.3 ± 0.5% for the egg-free virgin coconut oil mayonnaise and 3.6 ± 1.1% for the commercial sample. The differences between these results might be due to the different ingredients applied for the production.

The protein content of the egg-free virgin coconut oil mayonnaise was 2.6 ± 0.2 g, which was higher than the labeled value of commercial sample (1.4 g). This is primarily because of the protein-based emulsifiers used in the formulation. The carbohydrate content of the egg-free virgin coconut oil mayonnaise was 14.0 ± 3.7 g, which was higher than in the labeled value of commercial sample (9.2 g). This result also could be due to the differences in the formulations.

The fat content of the egg-free virgin coconut oil mayonnaise was 27.5 ± 3.6 g/100 g, whereas for the reference mayonnaise it was 66.2 g/100 g. This result was expected because the experimental low-fat eggless mayonnaise contained 30% of fat, while the commercial sample was a whole-fat mayonnaise. Standard mayonnaise formulation includes 60–80% of fat, depending on the composition and type of oil [33, 34].

Therefore, a lower amount of oil in the formulation resulted in a lower fat content.

CONCLUSION

The research objective was to improve the application of egg replacers in low-fat virgin coconut oil mayonnaise using response surface methodology. The optimal combination of three independent variables was as follows: cashew nut protein isolates – 12.6%, xanthan gum – 1.0%, and modified starch – 0.3%. We produced a high-quality egg-free virgin coconut oil mayonnaise with optimal viscosity, stability, and firmness. The predicted response values under the defined optimal levels were generally in accordance with the model. The proximate analysis and physicochemical properties of the egg-free virgin coconut oil mayonnaise had a lower fat content, water activity, and consistency, as well as a higher protein content compared to the reference sample.

Therefore, a mix of cashew nut protein isolates, xanthan gum, and modified starch at optimal levels could be used as a plant-based substitute to improve the viscosity, texture characteristics, and stability of mayonnaise. More investigations are required to assess the sensory properties and storage stability of the egg-free virgin coconut oil mayonnaise, which could be a good product for vegan consumers.

CONTRIBUTION

Nameer Khairullah Mohammed performed the experiments, drafted the manuscript, and proofread the article. Hemala Ragavan developed the research concept, performed the formal analysis, worked with the software, and drafted the article. Nurul Hawa Ahmad performed the data validation, wrote the review, and edited the manuscript. Anis Shobirin Meor Hussin supervised the project, developed the methodology, and acquired the funding. The manuscript was checked and approved by all the authors. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

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

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^{137}Cs and ^{40}K activity concentrations in edible wild mushrooms from China regions during the 2014–2016 period

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Abstract:

Introduction. Contamination by radiocaesium of edible wild mushrooms after major nuclear accidents is a long-lasting process in some regions of the world. Following greater awareness of radioactive pollution in Asia, particularly after the Fukushima accident, this study investigated the radioactivity of ^{137}Cs and ^{40}K contamination in edible wild mushrooms in China.

Study objects and methods. The objects of the research were edible wild mushrooms collected during 2014 to 2016, from the Inner Mongolian and Yunnan regions of China. To obtain an insight into any environmental impacts to distant regions of mainland Asia, the mushrooms were analyzed for ^{137}Cs activity. In parallel, the natural activity of ^{40}K was also determined and used to estimate the content of total K. The topsoil underneath the mushrooms was also investigated from a few sites in Bayanhushu in Inner Mongolia in 2015.

Results and discussion. The results showed that in 4 to 6 mushrooming seasons after the accident, mushrooms from both regions were only slightly contaminated with ^{137}Cs, which implied negligible consequences. The activity concentrations of ^{137}Cs in dried caps and whole mushrooms in 63 of 70 lots from 26 locations were well below 20 Bq kg^{-1} dry weight. Two species (*Lactarius hygrophoroides* L. and *Lactarius volemus* L.), from Jiulongchi in Yuxi prefecture showed higher ^{137}Cs activities, from 130 ± 5 to 210 ± 13 Bq kg^{-1} dw in the caps. ^{40}K activities of mushrooms were around two- to three-fold higher. A composite sample of topsoil (0–10 cm layer) from the Bayanhushu site (altitude 920 m a.s.l.) in Inner Mongolia showed ^{137}Cs activity concentration at a low level of 6.8 ± 0.7 Bq kg^{-1} dw, but it was relatively rich in potassium (^{40}K of 595 ± 41 Bq kg^{-1} and total K of 17000 ± 1000 mg kg^{-1} dw).

Conclusion. Wild mushrooms from the Yunnan and Inner Mongolia lands only slightly affected with radioactivity from artificial ^{137}Cs. Lack of ^{134}Cs showed negligible impact from Fukushima fallout. Ionizing radiation dose from ^{137}Cs in potential meals was a fraction of ^{40}K radioactivity. The associated dietary exposure to ionizing irradiation from ^{137}Cs and ^{40}K contained in mushrooms from the regions studied was considered negligible and low, respectively. Mushroom species examined in this study are a potentially good source of dietary potassium.

Keywords: Asia, forest, fungi, pollution, soil, radioactivity, radiocaesium, wild food

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INTRODUCTION

Radiocaesium ($^{134/137}$Cs), if not mention the short-lived radioactive ^{131}I ($t_{0.5}$ = 8.02 days), is the main mass and a long-term source of the toxic radiation, polluting the Earth in the past from the nuclear weapon explosions and nuclear power plant accidents [1, 2].

Macromycetes (fungi) can accumulate various elements in their fruiting bodies, including radioactive isotopes (^{134}Cs, ^{137}Cs, ^{40}K, ^{210}Po, ^{210}Pb, ^{238}Pu, $^{239+240}$Pu, ^{90}Sr, ^{230}Th, ^{232}Th, ^{234}U, ^{238}U) emitting radiation of various toxicities [3–9]. Many wild fungi are effective accumulators of artificial radioactive cesium, which circulates in forest ecosystems for years in contaminated areas and can cause a potential health hazard from ingestion of the mushrooms [2, 10–14].

Radiocaesium (^{137}Cs) is an artificial and long-lived ($t_{0.5}$ = 30.1 years) nuclide, which appeared in mushrooms after global fallout from nuclear weapons detonations in

the atmosphere. High levels of radioactivity reappeared following the collapse of the Chernobyl nuclear power plant in 1986, including massive levels of ^{134}Cs and ^{137}Cs emissions [15]. The consequent radioactive fallout caused a long-lasting and substantial contamination with ^{137}Cs of forest ecosystems including mushrooms in regions surrounding the collapsed plant, especially in the Ukraine, Belarus and Russia, as well as elsewhere in Europe [16–23].

As in Chernobyl, a similar accident occurred in Japan in March 2011, where, following a major earthquake, a 15-meter tsunami disabled the power supply and cooling systems of three Fukushima Daiichi nuclear power plant reactors. All three cores largely melted in the first three days, caused radioactive contamination of the environment on a large scale, including high ^{137}Cs pollution of fungi growing in the region [24–26].

The nuclear accidents caused long-term psychosocial consequences on exposed individuals. One of the consequences was that big game and domesticated ruminants that eat contaminated mushrooms could be also heavily loaded with ^{137}Cs [27–29]. In humans, mushrooms can be also the most important exposure route to ^{137}Cs when there is elevated consumption of wild species [30]. As mentioned, contamination by ^{137}Cs after the Chernobyl accident as well as atomic weapon testing is a long-lasting process in some mushroom species even collected relatively away from this source [12–14].

The contribution of the ^{137}Cs fallout from the Chernobyl accident to ecosystems in distant places like the Japanese islands was considered small compared to the previous global fallout [31]. The Chernobyl fallout had also some impacts on continental Asia. In China, soils (layer 0–10 cm) sampled from 56 sites in the Inner Mongolia province in 1982–1987 showed ^{137}Cs mean activity concentration of 13.6 ± 6.6 Bq kg^{-1} dry weight (dw) (from 5.8 ± 4.4 to 23.4 ± 13.4 Bq kg^{-1} dw) [32]. Soil from Yunnan province was also contaminated, showing activity of 6.2 ± 5.4 Bq kg^{-1} dw (from 1.9 ± 0.3 to 31.6 ± 0.8 Bq kg^{-1} dw) in 1982–1987 [33].

The accident in the Fukushima nuclear power plant caused a high alert on a direct and indirect radioactive pollution consequences regarding to exposed staff and local residents. It affected public health and foods safety in Japan, as well as continental Asia from serious accidental discharge and included studies on the consequence to various types of environmental media including soils, vegetation and wild growing mushrooms [25, 34–46].

Edible mushrooms collected from the wild are common foodstuffs in Yunnan, a land diverse in climate, soil, forest types and landscape topography and with a high biodiversity of mushroom species [47, 48]. Certain species are conditionally edible or medicinal mushrooms, e.g. *Caloboletus calopus* (Pers.) Vizzini or *Tricholoma sejunctum* (Fr. ex Sow.) Quél. Inner Mongolia has an area of 1 183 000 km^2 (457 000 sq mi) with a landscape made up largely of meadows with an abundance of saprobic mushrooms. This region is poor in ectomycorrhizal mushrooms, a result of the limited wooded areas, apart from the thickets along the Huang He River [49].

To get greater awareness of radioactive pollution in Asia, particularly after the Fukushima accident, this study investigated the radioactivity contamination with ^{137}Cs and ^{40}K of edible wild mushrooms from the Inner Mongolian and Yunnan provinces of China. The activity concentrations of ^{137}Cs and ^{40}K were studied for the first time in wild mushrooms (five species) from Inner Mongolia and also in more than 26 species, including taxa without previous data on ^{137}Cs, from Yunnan, collected during 2014–2016.

STUDY OBJECTS AND METHODS

Mushroom and topsoil samples. Mushrooms were collected from the Inner Mongolia province (approximate distance from Fukushima Daiichi power plant site is 2500 km). They all represented saprobic species and included *Agaricus arvensis* Schaeff, *Calocybe gambosa* (Fr.) Donk, *Calvatia gigantea* (Batsch) Lloyd, *Macrolepiota excoriata* (Schaeff.) Wasser and *Lepista personata* (Fr.:Fr) Sing. The 26 species collected from Yunnan province (distance from Fukushima is in the range of 3500 to 4500 km) included *Auricularia delicata* (Fr.) Henn, *Baorangia bicolor* (Kuntze), *Boletus bainiugan* Dentinger, *Boletus ferrugineus* Schaeff., *Hemileccinum impolitum* (Fr.) Šutara, *Boletus reticulatus* Schaeff., *Butyriboletus roseoflavus*, *Boletus tomentipes* Earle, *Caloboletus calopus* (previous name *Boletus calopus* Fr.), *Neoboletus brunneissimus* (W.F. Chiu), *Retiboletus griseus* (Frost), *Rubroboletus sinicus* (W.F. Chiu), *Sutorius magnificus* (W.F. Chiu), *Sutorius obscureumbrinus* (Hongo), *Laccaria vinaceoavellanea* Hongo, *Lactarius deliciosus* (L.:Fr.) Gray, *Lactarius hatsudake* Tanaka, *Lactarius hygrophoroides* Berk. & M.A. Curtis, *Lactarius volemus* Fr., *Lentinula edodes* (Berk.) Pegler, *Leccinum rugosiceps* (Peck) Singer, *Morchella esculenta* Pers., *Russula compacta* Frost and *Tricholoma sejunctum*. The *L. edodes* samples were taken from cultivars from the Wuding in Chuxiong and Longyang in Baoshan from Yunnan, while solely composite samples were from Baise in Guangxi province and the Northeast of China.

Soil samples were collected in parallel as two pooled samples of topsoil (0–10 cm layer) beneath the fruiting bodies of *A. arvensis* from grassy stands in the Bayanhushu site in Inner Mongolia. Details of the geographical locations of the sampling sites from which mushrooms and topsoil were collected are given in Fig. 1 and Table 1.

Preparation of materials. To examine the distribution of ^{137}Cs and ^{40}K and total K between the morphological parts, individual fruiting bodies were rinsed and separated into caps (with skin) and stipes, but some were examined as whole (Table 1). Before drying, the fungal materials were sliced into pieces using a ceramic knife and pooled to create composite

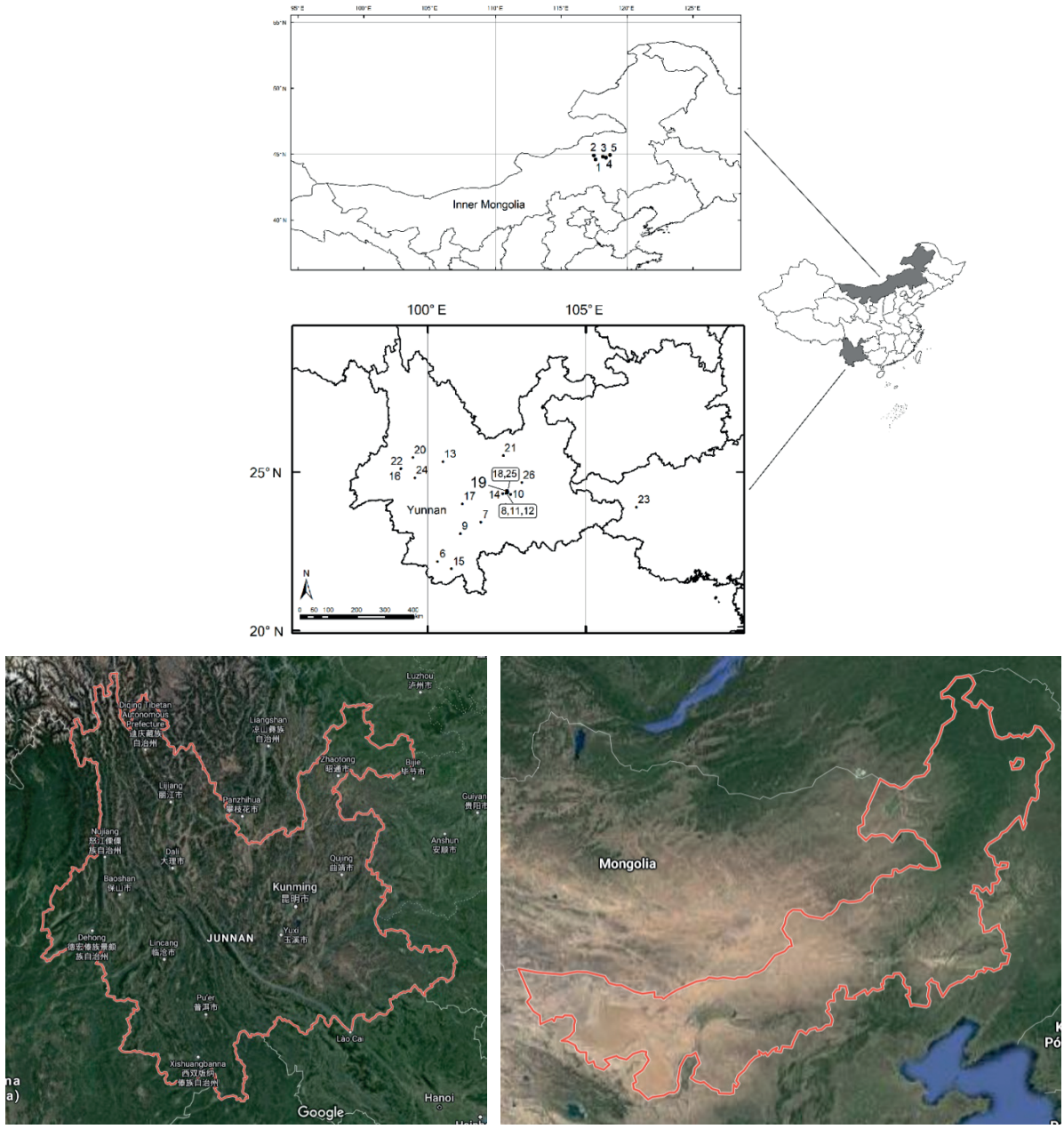

Figure 1 Localization of the sampling sites of mushrooms from the Inner Mongolia and Yunnan provinces in China

samples representing each species, sampling location and time of collection. Mushroom parts were dried at 65°C to constant mass (Ultra FD1000 dehydrator, Ezidri, Australia), finely powdered in a porcelain mortar, passed through an 80-mesh sieve, and stored in screw sealed plastic (low density polyethylene) bags under dry conditions.

Two pooled samples of topsoil (0–10 cm layer; 150 g whole weight each) were cleaned from any visible pebbles, leaves and twigs, soil samples, air dried under clean condition, ground (porcelain mortar), sieved (2 mm mesh plastic sieve), and stored in sealed polyethylene bags.

Directly before analysis, the mushroom and soil materials were prophylactically deep frozen and lyophilized (Labconco Freeze Dry System, Kansas City, MO, USA) for three days to ensure full dehydration.

Instrumental analysis. The analytical methodology applied has been presented in detail before [43, 67, 68] but a summarized description is given below. In brief, activity concentrations of ^{137}Cs, ^{134}Cs and ^{40}K were measured using a γ-spectrometer with a coaxial HPGe detector with a relative efficiency of 18% and a resolution of 1.9 keV at 1.332 MeV of ^{60}Co (with associated electronics) (Detector GC 1819 7500 SL, Canberra Packard, Poland, Warsaw). The measurements of the fungal materials in this study were preceded by

Table 1 ^{137}Cs and ^{40}K activity concentration (± an instrumental counting error) and estimated K in mushrooms collected from the provinces of China

Province and species	Location	Year	n#	^{137}Cs, Bq kg^{-1} dw		^{40}K, Bq kg^{-1} dw		K, g kg^{-1} dw	
				Caps	Stipes	Caps	Stipes	Caps	Stipes
Inner Mongolia province	Xilin Gol League								
Agaricus arvensis	West Ujimqin [1]*	2015	80	< 8.7	19 ± 4	2100 ± 240	1700 ± 250	72 ± 8	58 ± 8
A. arvensis	Bayanhushu [2]	2015	60	7.2 ± 1.7	7.6 ± 1.7	875 ± 140	1100 ± 140	30 ± 1	37 ± 5
A. arvensis	Bayanhushu [2]	2014	60	< 4.1	< 7.1	1500 ± 190	1100 ± 180	51 ± 6	37 ± 6
Calocybe gambosa	Jinhe [3]	2015	14	9.7 ± 1.7	12 ± 2	1250 ± 100	1200 ± 120	43 ± 3	41 ± 4
Calvatia gigantea	Bayanhushu [2]	2015	4	(10 ± 2)		(1400 ± 170)		(41 ± 5)	
Macrolepiota excoriata	Bayanhushu [2]	2015	2	15 ± 4	< 23	1600 ± 320	1400 ± 620	55 ± 11	48 ± 21
Lepista personata	Baiyinhua [4]	2015	10	< 8.7	19 ± 4	1400 ± 93	1300 ± 110	48 ± 3	44 ± 4
L. personata	Jinshan [5]	2015	10	6.4 ± 1.4	< 4.1	1200 ± 100	1200 ± 110	41 ± 3	41 ± 4
Yunnan province									
Auricularia delicate	Meng'a, Xishuangbanna [6]	2016	7	(< 1.4)		(540 ± 61)		(16 ± 2)	
Baorangia bicolor	Mojiang, Pu'er [7]	2015	5	4.9 ± 4.7	ND	1000 ± 200	ND	29 ± 6	ND
B. bicolor	Yuxi [8]	2015	11	(< 3.6)		(900 ± 100)		(26 ± 3)	
Boletus bainiugan	Ning'er, Pu'er [9]	2016	5	< 2.8	2.7 ± 0.9	870 ± 88	520 ± 68	25 ± 3	15 ± 2
B. bainiugan	Jiuxi, Yuxi [10]	2015	17	6.1 ± 1.2	6.6 ± 1.1	ND	ND	ND	ND
B. bainiugan	Dayingjie, Yuxi [11]	2015	12	< 2.9	ND	810 ± 76	ND	24 ± 2	ND
B. bainiugan	Ning'er, Pu'er [9]	2016	30	5.3 ± 1.1	< 2.5	780 ± 89	690 ± 66	27 ± 3	24 ± 2
Boletus ferrugineus	Midu, Dali [13]	2016	10	17 ± 1	13 ± 1	730 ± 85	600 ± 74	21 ± 2	18 ± 2
Boletus impolitus	Jiuxi, Yuxi [10]	2016	2	41 ± 3	9.5 ± 1.8	1000 ± 130	910 ± 120	29 ± 4	27 ± 3
Boletus reticulatus	Jiuxi, Yuxi [10]	2015	1	21 ± 6	< 3.1	1500 ± 440	2000 ± 850	44 ± 13	59 ± 25
Boletus speciosus	Yuxi [8]	2015	7	(5.0 ± 1.1)		(720 ± 74)		(21 ± 2)	
Boletus tomentipes	Yuxi [8]	2015	12	(69 ± 4)		(1300 ± 210)		(38 ± 6)	
B. tomentipes	Hongta, Yuxi [12]	2015	7	35 ± 9	< 19	4000 ± 680	1800 ± 520	120 ± 20	53 ± 15
Caloboletus calopus	Jiuxi,Yuxi [10]	2015	12	< 4.2	ND	960 ± 110	ND	33 ± 4	ND
C. calopus	Hongta, Yuxi [12]	2015	10	9.8 ± 1.8	ND	1000 ± 110	ND	29 ± 3	ND
C. calopus	Midu, Dali [13]	2015	11	7.2 ± 1.3	3.2 ± 1.2	640 ± 95	380 ± 78	19 ± 3	11 ± 2
Neoboletus brunneissimus	Yuxi [8]	2015	11	(< 3.6)		(1000 ± 95)		(29 ± 3)	
N. brunneissimus	Midu, Dali [13]	2015	9	5.7 ± 1.3	9.6 ± 1.5	940 ± 87	960 ± 91	28 ± 3	28 ± 3
Retiboletus griseus	Yuxi [8]	2015	10	(4.3 ± 1.4)		(1400 ± 94)		41 ± 3	
R. griseus	Luohe, Yuxi [14]	2016	7	9.7 ± 2.7	< 5.4	1300 ± 250	950 ± 140	38 ± 7	28 ± 4
R. griseus	Midu, Dali [13]	2015	14	9.4 ± 1.3	< 2.6	1100 ± 81	940 ± 73	32 ± 2	28 ± 2
Rubroboletus sinicus	Jiuxi, Yuxi [13]	2015	9	< 6.2	13 ± 2	1100 ± 160	750 ± 140	37 ± 5	26 ± 5
R. sinicus	Yuxi [8]	2015	11	< 4.9	ND	1100 ± 140	ND	33 ± 3	ND
R. sinicus	Jiuxi, Yuxi [10]	2015	9	2.4 ± 0.3	ND	1000 ± 90	ND	32 ± 4	ND
Sutorius magnificus	Dayingjie, Yuxi [11]	2016	7	18 ± 2	45 ± 3	1300 ± 120	1000 ± 120	38 ± 3	29 ± 3
Sutorius obscureumbrinus	Yuxi [8]	2015	12	(3.9 ± 3.7)		(1200 ± 130)		(35 ± 4)	
S. obscureumbrinus	Gasa, Xishuangbanna [15]	2016	16	< 2.7	3.0 ± 0.7	1300 ± 100	975 ± 74	45 ± 4	33 ± 3
Laccaria vinaceoavellanea	Baoshan city, Baoshan [16]	2016	7	(< 3.2)		(1200 ± 93)		(35 ± 3)	
Lactarius deliciosus	Zhengyuan, Pu'er [17]	2014	5	< 5.3	< 10	800 ± 150	1100 ± 290	23 ± 4	32 ± 8
L. deliciosus	Lianhuachi, Yuxi [18]	2016	20	8.1 ± 1.6	17 ± 2	580 ± 110	720 ± 140	17 ± 3	21 ± 4
Lactarius hatsudake	Lianhuachi, Yuxi [18]	2016	10	6.2 ± 1.3	15 ± 3	830 ± 80	710 ± 210	24 ± 2	21 ± 6
L. hatsudake	Lianhuachi, Yuxi [18]	2016	4	12 ± 3	20 ± 5	1000 ± 160	1100 ± 350	29 ± 5	32 ± 10
Lactarius hygrophroides	Lianhuachi, Yuxi [18]	2016	2	< 19	ND	1500 ± 64	ND	44 ± 2	ND
L. hygrophroides	Lianhuachi, Yuxi [18]	2016	6	< 5.0	29 ± 7	1200 ± 140	1400 ± 500	35 ± 4	41 ± 15
L. hygrophroides	Jiulongchi, Yuxi [19]	2016	9	130 ± 5	60 ± 5	920 ± 150	1300 ± 260	27 ± 4	38 ± 8
Lactarius volemus	Jiulongchi, Yuxi [19]	2016	17	210 ± 13	67 ± 7	1000 ± 99	760 ± 97	30 ± 23	22 ± 3
L. volemus	Yongping, Dali [20]	2016	8	< 3.5	6.1 ± 1.6	920 ± 100	830 ± 102	31 ± 3	28 ± 3
Lentinus edodes	Wuding, Chuxiong [21]	2015	70	5.2 ± 1.4	12 ± 3	810 ± 110	910 ± 270	24 ± 3	27 ± 8
L. edodes	Longyang, Baoshan [22]	2015	100	12 ± 2	22 ± 4	1200 ± 140	1100 ± 240	35 ± 4	32 ± 7
Lentinula edodes	Northeast of China	2016	30+	5.3 ± 1.4	4.5 ± 1.2	880 ± 110	640 ± 88	26 ± 3	19 ± 3
L. edodes	Baise, Guangxi province [23]	2016	30+	6.9 ± 1.7	< 3.6	790 ± 110	690 ± 82	23 ± 3	20 ± 2
Leccinum rugosiceps	Ning'er, Pu'er [9]	2016	30	6.3 ± 1.1	4.0 ± 0.8	815 ± 84	781 ± 90	27 ± 3	27 ± 3
Morchella esculenta	Midu, Dali [13]	2016	30	(< 3.4)		(1200 ± 140)		(35 ± 4)	
Russula compacta	Midu, Dali [13]	2016	5	(4.6 ± 1.0)		(940 ± 80)		(28 ± 2)	
Boletus sp.	Baoshan city, Baoshan [16]	2016	5	8.3 ± 1.4	9.3 ± 1.5	ND	ND	ND	ND
Boletus sp.	Midu, Dali [13]	2016	7	5.2 ± 1.2		1000 ± 83		29 ± 2	
Boletus sp.	Midu, Dali [13]	2016	6	5.9 ± 1.2		1200 ± 92		35 ± 3	
Boletus sp.	Baoshan city, Baoshan [16]	2016	9	9.0 ± 1.2		690 ± 78		20 ± 2	

Continuation of Table 1

Province and species	Location	Year	n#	^{137}Cs, Bq kg^{-1} dw		^{40}K, Bq kg^{-1} dw		K, g kg^{-1} dw	
				Caps	Stipes	Caps	Stipes	Caps	Stipes
Boletus sp.	Baoshan city, Baoshan [16]	2016	5	< 2.7	3.0 ± 0.7	1300 ± 100	975 ± 74	39 ± 3	29 ± 2
Boletus sp.	Midu, Dali [13]	2016	6	7.7 ± 1.7		1100 ± 130		32 ± 4	
Boletus sp.	Changning, Baoshan [24]	2016	5	5.7 ± 1.4		860 ± 98		25 ± 3	
Boletus sp.	Baoshan city, Baoshan [16]	2016	7	9.6 ± 1.4		760 ± 96		22 ± 3	
Boletus sp.	Baoshan city, Baoshan [16]	2016	6	< 4.1		780 ± 110		23 ± 3	
Boletus sp.	Changning, Baoshan [24]	2016	7	9.6 ± 2.2		1100 ± 150		32 ± 4	
Boletus sp.	Baoshan city, Baoshan [16]	2016	5	7.9 ± 1.4		790 ± 100		23 ± 3	
Boletus sp.	Midu, Dali [13]	2016	6	6.0 ± 1.5		1100 ± 120		32 ± 3	
Boletus sp.	Changning, Baoshan [24]	2016	6	4.4 ± 0.9		960 ± 73		28 ± 2	
Boletus sp.	Changning, Baoshan [24]	2016	5	7.4 ±1.4		810 ± 93		24 ± 3	
Boletus sp.	Changning, Baoshan [24]	2016	7	18 ± 2		990 ± 97		29 ± 3	
Tricholoma sejunctum	Liqi, Yuxi [25]	2016	14	7.7 ± 2.0	6.3 ± 2.0	1400 ± 140	1700 ± 170	41 ± 4	50 ± 5
T. sejunctum	Yiwanshui, Yuxi [26]	2016	20	9.0 ± 1.4	23 ± 1	1400 ± 92	1200 ± 79	41 ± 3	35 ± 2
T. sejunctum	Lianhuachi, Yuxi [18]	2016	5	20 ± 3	15 ± 4	2000 ± 270	1900 ± 340	59 ± 8	56 ± 10

*ID of the sampling site (see also in Fig. 1); #Quantity of specimens (fruit bodies) in a pool; ND – no data

background measurement (time 80 000 s) and counting time was similar (> 22 h).

The instrument was calibrated using a multi-isotope standard by validated methodology. The reference solution (Standard solution of gamma emitting isotopes, code BW/Z-63/48/16), obtained from the IBJ-Świerk near Otwock in Poland, was used to prepare reference samples for equipment calibration. The radionuclides used in the reference solution during equipment calibration were ^{241}Am (1.2%), ^{109}Cd (2.1%), ^{57}Co (0.80%), ^{51}Cr (1.55%), ^{113}Sn (2.0%), ^{85}Sr (1.2%), ^{137}Cs (1.5%), ^{54}Mn (1.55%), ^{65}Zn (1.2%) and ^{60}Co (0.8%). The same geometry of cylindrical dishes with a 40-mm diameter was used for the analysis of the fungal material extracts as well as for the reference samples during equipment calibration organized by IAEA-RML-2018-01. Detailed results of the intercalibration are available in the publication [50].

Minimum detectable activity was determined by the Currie method. This method is based on two basic parameters: (a) critical level, which is defined as a level below which the detection signal cannot be reliably recognized and (b) detection limit specifying the smallest signal that can be quantitatively reliable. The measurement results obtained were recalculated for dehydrated materials and decay corrected back to the time of collection. Total potassium content was calculated from the original ^{40}K activity concentration data (using mean value of 29.32 Bq g^{-1}) in natural K, which is in the range from 27.33 to 31.31 Bq g^{-1} of K (percentage abundance of ^{40}K atoms in natural K is 0.0117%) [51].

RESULTS AND DISCUSSION

^{137}Cs and ^{134}Cs in mushrooms and soil. All species collected from Inner Mongolia in this study were saprobic. ^{134}Cs activity was not detected in any of the study samples. It was possibly due to the negligible impact from the Fukushima's fallout in 2011 as wells as a relatively short half-life of this isotope ($t_{0.5}$ = 2.1 years) and small impacts from the Chernobyl's fallout in 1986 and preceding, the nuclear weapons detonations in the atmosphere.

The values of the activity concentration of ^{137}Cs in caps and stipes of the fruiting bodies of *Agaricus arvensis*, *Calocybe gambosa*, *Lepista personata* and *Macrolepiota excoriata* and in the whole fruiting bodies of *C. gigantea* were in the range from < 4.1 to 19 ± 4 Bq kg^{-1} dw (Table 1). There is no prior data for these species from regions of Asia other than Inner Mongolia [44, 53, 54]. The low levels of ^{137}Cs contamination in the studied mushrooms from the Inner Mongolian region reflects low activities of this nuclide in local soils as well as a lower potential of these species to bio-accumulate this nuclide.

In this study, a composite sample of the upper (0–10 cm) layer of soil collected in parallel with *A. arvensis* from the Bayanhushu site (altitude 920 m a.s.l.) showed ^{137}Cs activity concentration of 6.8 ± 0.7 Bq kg^{-1} dw. This result obtained for the sample from 2015 is around 2 to 4-fold lower than earlier results cited for topsoils collected in Inner Mongolia in 1982–1987, and is close to the activity values reported in 1–5 cm layer of forest topsoils sampled from the Changning and Mengman sites in Yunnan in 2016 (4.9 ± 0.6 and 7.5 ± 0.7 Bq kg^{-1} dw) [53].

Because of colder weather in the mountains, soil and the mushrooms can be specifically affected with radiocaesium, which is scavenged from the contaminated plumes by wet precipitation [53–55]. Forest topsoil collected at 3000 m above sea level from the Minya Konka (Gongga Shan) mountain in Sichuan province of China in 2012 showed ^{137}Cs at level from 41 ± 1 to 79 ± 2 Bq kg^{-1} dw. This result is well in excess

of what has been noted in topsoil from Inner Mongolia in this study or other studies of soils from China [32, 33, 53].

As given in Table 1, the determined activity concentrations of ^{137}Cs in fruiting bodies of the saprobic and perhaps a little parasitic species of *Auricularia delicate*, the caps and stipes of fruiting bodies of the saprobic decomposer *Lentinula edodes*, the saprobic *Morchella esculenta* as well as over 20 species of mycorrhizal mushrooms collected in Yunnan were low and roughly in the range of values noted in mushrooms from Inner Mongolia.

The only exception was individuals of *Lactarius hygrophoroides* collected from the region of Jiulongchi in Yuxi prefecture in central Yunnan in the summer of 2016. They showed activity concentrations of ^{137}Cs from 130 ± 5 to 210 ± 13 Bq kg dw^{-1} in caps and from 60 ± 5 to 67 ± 7 Bq kg dw^{-1} in stipes (Table 1). These relatively high levels of ^{137}Cs activity in *L. hygrophoroides* from the Jiulongchi site were in the range of activities determined previously in several species of ectomycorrhizal mushrooms collected at 2900–3600 m above sea level from the Minya Konka summit in 2012 [53].

Many other species of mushrooms collected from the prefecture of Yuxi and across other regions from Yunnan and elsewhere in China (Zhangzhou in the Fujian province) in 2010–2018 were substantially less contaminated than *L. hygrophoroides* from the Jiulongchi site or even mushrooms from the subalpine regions on the eastern slope of the Minya Konka summit [12, 16, 42, 44, 47, 52, 53, 56]. The exception was *Turbinellus floccosus* (Schwein.) Earle ex Giachini & Castellano [previous name *Gomphus floccosus* (Schw.) Singer] collected from the region of Mangshi (98°24' E, 24°22' N) in the western part of Yunnan during August 2012 to July 2013, which showed a ^{137}Cs activity concentration of 212 (148–339) Bq kg^{-1} dw in the whole fruiting bodies [44, 57].

Elevated activity concentrations of ^{137}Cs in *L. hygrophoroides* from the Jiulongchi site in this study can possibly be explained by weather conditions (episodic rain) scavenging nuclides from the radioactive plume after the Fukushima (Japan) nuclear power plant accident in early 2011.

The radioactive incident took place in Tongchuan, Shaanxi Province, south of the central region of Inner Mongolia (approximate distance from the sampling sites mushrooms there is 1200 km). Some ^{137}Cs from a measuring instrument (lead ball – a major component of a nuclear scale) when dismantling a cement factory has gone missing. In a later investigation, radioactivity from ^{137}Cs was found at a steel refinery in Shaanxi's Fuping county. Possibly, a lead ball with scrap metal was melted down into the steel [58]. Information on possible, if any, ground pollution in the region from this accident is not available.

A recent (2021) study showed that the activity concentration of ^{137}Cs in 66 out of 68 of wild mushrooms (17 species) collected from the northeast regions of China in 2017–2020 ranged from < 0.6 to 26 Bq kg^{-1} dw (data rounded), and only in single *Lactarius deliciosus* and *Lepista nuda* (Bull.) Cooke specimens collected in 2020, was 46 ± 3 Bq kg^{-1} dw and 130 ± 9 Bq kg^{-1} dw, respectively [59].

The maximum activity concentration of ^{137}Cs noted in *L. nuda* in the above mentioned study was close to values determined in *Lactarius hygrophroides* and *Lactarius volemus* from Jiulongchi, Yuxi (Yunnan) (Table 1), while the results are not very comparable due to only two single specimens examined by Wang *et al*. [59].

The radiocaesium contamination of land, the oceans and biota, including edible wild growing mushrooms has thus far, occurred in three main waves. The first one arose from the nuclear weapons detonations in the atmosphere in the period from 1945 to 1980 and resulted in wide-spread aerial diffusion of radiocaesium and other nuclides including ^{14}C, ^{137}Cs, ^{90}Sr, $^{239–240}$Pu, ^{241}Am and ^{3}H [60]. With time, the resulting depositions of longer lasting ^{137}Cs affected every region of the world [1, 60].

Data on radiocaesium in mushrooms for the period before 1986 is scarce [10–13, 42, 61]. Fifteen years before the Chernobyl accident, a solely fruiting body of *Tricholoma terreum* collected from the Czech Republic in 1971 showed ^{137}Cs at a level of 40 Bq kg^{-1} dw [61]. Additional historical data on ^{137}Cs in mushrooms was recorded in 1984, in Poland for the Poison Pax (*Paxillus involutus*), which showed ^{137}Cs at a level of 2700 Bq kg^{-1} dw, with lower levels noted for the King Bolete (*Boletus edulis*) (95 and 104 Bq kg^{-1} dw) and Slippery Jack, *Suillus luteus* (125 and 150 Bq kg^{-1} dw) collected in 1984 and 1985, respectively [10].

Data on the radiocaesium concentration activities accumulated in wild mushrooms growing in Asia from the period before the Chernobyl accident are absent in the available literature. Effectively, there is also nothing published on radiocaesium in wild mushrooms from mainland Asia in the period between the Chernobyl and Fukushima incidents.

The Chernobyl emission of radioactivity caused an extreme and long-lasting radiocaesium pollution of wild growing mushrooms in the regions of Europe, and particularly in the neighbor areas collapsed nuclear power plant [12, 16, 17, 62–65]. Japanese researchers have published a large volume of data on artificial radioactivity accumulated in wild mushrooms growing in the country, both from the post-Chernobyl and post-Fukushima emissions, which have recently been evaluated by Komatsu *et al*. and Prand-Stritzko and Steinhauser [25, 66]. The activity in these wild mushrooms collected in the period up to March 2011 was largely from accumulated radiocaesium (^{137}Cs) due to the global fallout from nuclear weapons detonations, with a small proportion being attributed to the Chernobyl emissions [54]. The more recent emissions

from the Fukushima incident changed the pattern of radionuclide contamination of wild mushrooms in Japan. However, as shown in this study (Table 1) and in a few other reports, the emissions could have only a small impact on mainland Asia or elsewhere [44, 53, 68–69].

^{40}K and K in mushrooms and soil. The topsoil from the Bayanhushu site showed ^{40}K activity concentration of 595 ± 41 Bq kg^{-1} dw and total K content of 17 000 ± 1000 mg kg^{-1} dw, which were higher than previously determined in topsoils sampled from several forested areas in Yunnan (150 ± 14 to 340 ± 19 Bq kg^{-1} dw) [53].

In the study by Zhang *et al*., the means of ^{40}K activity concentrations in topsoils (0–10 cm) in Inner Mongolia and Yunnan in 1982–1987 were 755 (866–1066 Bq kg^{-1} dw) and 487 Bq kg^{-1} dw (149–1010 Bq kg^{-1} dw), respectively [70]. In another national survey performed during 1983–1990, the area-weighted mean and the point-weighted mean of ^{40}K were 655.6 and 624.6 Bq kg^{-1} dw, respectively, for soils in Inner Mongolia, while the two values for soils from Yunnan were 532.0 and 518.6 Bq kg^{-1} dw, respectively [71].

The activity concentrations of ^{40}K in mushrooms from Inner Mongolia were in the range of 875 ± 140 to 1600 ± 320 Bq kg^{-1} dw in caps and from 1100 ± 180 to 1400 ± 620 Bq kg^{-1} dw in stipes (Table 1). In the case of mushrooms from Yunnan, *A. delicate* (ear-like jelly fungus), which grows on wood, they had a lower activity concentration of ^{40}K (540 ± 61 Bq kg^{-1} dw) than *L. edodes* (Table 1), which also grows on wood. The *L. edodes* showed activities in the range of 790 ± 110 to 1200 ± 140 Bq kg^{-1} dw in the caps, which are culinary valued, and from 640 ± 88 to 1100 ± 240 Bq kg^{-1} dw in the stipes, which are largely discarded. This species collected from Yunnan and examined by other authors, demonstrated the mean value of ^{40}K activity concentration to be 629 Bq kg^{-1} dw (from 396 to 1010 Bq kg^{-1} dw; n = 11) [44]. ^{40}K values in the caps of terrestrial mushrooms from Yunnan were from 580 ± 110 Bq kg^{-1} dw in *L. deliciosus* to 4000 ± 680 Bq kg^{-1} dw in *Boletus tomentipes*, while stipes showed activities from 380 ± 78 Bq kg^{-1} dw in *L. deliciosus* to 1900 ± 340 Bq kg^{-1} dw in *Tricholoma sejunctum*.

Potassium (total K) is the major metallic element in mushrooms and occurs in dried fungal materials in quantities of up to several percent, while the natural nuclide ^{40}K forms only a small proportion (makes up 0.012%) of the total. Hence, mushrooms collected from areas that are only mildly affected by ^{137}Cs depositions or mushrooms without a high species-specific ability to bioconcentrate this nuclide, e.g. like some species from the genus *Cortinarius*, contained natural ^{40}K in high excess relative to ^{137}Cs (Table 1) [12].

The amounts of K in the caps, stipes, or whole fruiting bodies of the species in this study were in the range 16 000 to 120 000 mg kg^{-1} dw (1.6 to 12 g kg^{-1} dw). Potassium is indispensable for mushrooms, for the uptake and osmotic regulation of water in the cytoplasm of cells and is a co-factor in certain enzymes [72]. However, the same species, i.e. *A. arvensis*, *Boletus bainiugan*, *Retiboletus griseus*, *Rubroboletus sinicus*, *Caloboletus calopus*, *L. hygrophoroides*, *L. edodes* and *T. sejunctum* collected from different sites could differ around twofold in the content of K (Table 1).

The daily adequate intake of K for adults is 2300 mg for females and 3400 mg for males [73]. Thus, the mushroom species examined in this study and assuming absorption rate at around 90% could be considered as potentially good sources of dietary potassium, especially when stir-fried with oil, which is a common culinary technique in SW China [67].

Potential risk from ionizing radiation doses. In this study, a total of 70 lots of several species of edible mushrooms collected from 26 locations in Yunnan were examined and in 63 lots, the contamination with ^{137}Cs of the caps or the whole mushrooms was well below 20 Bq kg^{-1} dw (Table 1). There were three of 70 lots that were more contaminated with ^{137}Cs than the others. Those lots were the gilled mushroom *B. tomentipes* (of 69 ± 4 Bq kg^{-1} dw), caps of the lamellar mushroom *L. hygrophoroides* (130 ± 5 Bq kg^{-1} dw), and caps of lamellar *L. volemus* (210 ± 13 Bq kg^{-1} dw) (Table 1). Assuming that the moisture content in fruiting bodies is 90%, the estimated ^{137}Cs activities in these three species were 6.9, 13, and 21 Bq kg^{-1} on a wet weight basis. Therefore, these amounts were much lower than the maximum permitted levels for import of mushrooms from third countries [specific 13 countries affected by the Chernobyl's radioactive fallout for which the regulation applies] to the European Union (600 Bq kg^{-1}) [74].

In Yunnan, the main way to cook mushrooms is stir-frying in vegetable oil in a wok pan [75]. It is interesting that stir-fried mushroom meals showed about 2 to 5-fold higher activity concentrations of ^{137}Cs than the raw mushrooms on a whole weight (wet) basis [67, 68].

Therefore, a 100-g portion of stir-fried *L. volemus* caps from the most contaminated lot in this study could include from 4.2 to 10.5 Bq of ^{137}Cs (equivalent to ionizing radiation dose from 56×10^{-3} to 140×10^{-3} μSv *per capita* or 0.49×10^{-3} to 2.35×10^{-3} μSv per kg body mass; 60 kg body mass). These estimates are low, taking into account the risk associated with the doses of ionizing radiation received by consumers in Yunnan, even if stir-fried mushrooms are consumed daily for longer periods during the mushrooming season.

In comparison, the natural ^{40}K nuclide contained in mushrooms (Table 1) introduces much higher doses of ionizing radiation than ^{137}Cs for locals in Inner Mongolia and Yunnan provinces but is not considered as a hazardous nuclide for consumers due to homeostasis of K in human body.

CONCLUSION

The activity concentrations of ^{137}Cs in lamellar mushrooms from the Inner Mongolia province of China

and the local soil were low. ^{137}Cs contamination of the lamellar and gilled mushrooms from Yunnan province in China was also low, i.e. well below one tenth of statutory limits, and mushroom meals there can be considered as a negligible source of ^{137}Cs for their consumers.

In view of the results from this study, the accident in the Fukushima nuclear power plant had little or negligible effect on radioactive contamination of edible and medicinal fungi in the regions of China. Natural nuclide ^{40}K contained in mushrooms is not considered as hazardous for mushroom meal consumers. Wild mushrooms can be considered as a good source of dietary potassium for consumers.

CONTRIBUTION

Michał Saniewski: resources, methodology, investigation, validation, data curation and analysis, writing – review & editing. Jerzy Falandysz: conceptualization, resources, investigation, formal analysis, data curation, graphics, supervision, writing – original draft, writing – review & editing. Tamara Zalewska: resources, methodology, investigation, validation, data curation and analysis.

CONFLICT OF INTEREST

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

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Improving the nutritional properties of *Brassica* L. vegetables by spontaneous fermentation

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Abstract:
Introduction. *Brassica* L. vegetables are rich in fiber, minerals, and bioactive compounds. Lactic fermentation can improve their nutritional value. The goal of this study was to evaluate phytase, calcium, phytic acid, total phenolic content, and antioxidants during spontaneous fermentation of white cabbage, red cabbage, and Chinese cabbage.
Study objects and methods. The research featured samples of water extract, methanol extract, and brine. The procedure involved monitoring lactic bacteria and pH during cabbage fermentation. Diphenyl-1-picrylhydrazyl radical (DPPH) scavenging assay and cupric reducing antioxidant capacity (CUPRAC) assay were used to measure the antioxidant activity and Folin-Ciocalteau method to determine total phenolic content in the water and methanol extracts. In the brine samples, we studied calcium, phytic acid, and phytase activity.
Results and discussion. The samples of white and red cabbage displayed the highest phytase activity on days 5–10 and had a maximal decrease of phytic acid and increase of calcium concentration, while in Chinese cabbage these processes occurred gradually throughout the fermentation. The total phenolic content in the brine and extracts was very similar for all the cultivars throughout the fermentation process. A continuous release from the solid phase to brine could be observed during the first ten days of fermentation. DPPH and CUPRAC assays revealed a similar phenomenon for the total phenolic content. The antioxidant capacity decreased in the water and methanol extracts and increased in the brine. At the end of fermentation, the red cabbage samples demonstrated a significant increase in the total phenolic content and total antioxidant activity, which was less prominent in the Chinese cabbage. The samples of white cabbage, on the contrary, showed a decrease in these parameters.
Conclusion. Fermentation made it possible to increase the concentration of free calcium in white, red, and Chinese cabbages, as well as improve the antioxidant capacity of red and Chinese cabbages.

Keywords: Phytase activity, total phenolics, antioxidant activity, lactic acid bacteria, fermentation, cabbage

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INTRODUCTION

Brassicaceae family comprises a large number of plant species distributed all over the world except Antarctica. This family includes approximately 338 genera and 3700 species [1]. Fresh or preserved, cabbage has been part of human diet since ancient times [2].

Brassica oleracea L. and *Brassica rapa* L. are the most popular representatives of *Brassica* vegetables. They are almost completely edible, e.g. leaves, inflorescence, root, stem, and seed. Their excellent adaptability makes it possible to cultivate them in different seasons and environments. In the Occident,

consumers prefer *B. oleracea* var. *capitata*, especially white cabbage and red cabbage. Oriental cuisine features mostly *B. rapa* var. *glabra* Regel (Chinese cabbage) or *B. rapa* ssp. *chinensis* (L.) Hanelt (pak choi) [3].

Brassica vegetables have low concentrations of protein and fat, which makes them popular low calorie foods. In addition, they are rich in fiber, such minerals as calcium and iron, and such bioactive compounds as polyphenols and glucosinolates [4]. Calcium is essential for human diet. Dairy products are great sources of calcium, both qualitatively and quantitatively. However, people with lactose intolerance and vegans refrain from dairy products, which makes *Brassica* vegetables an excellent source of the recommended daily calcium intake. Indeed, cabbage has high concentrations of calcium, iron, selenium, copper, manganese, and zinc. Unfortunately, it also contains phytates that may form complexes with calcium, thus reducing its bioavailability and nutritional value [5].

The past decade has seen an increase in scientific interest to the antioxidant properties of dietary plant polyphenols. These secondary metabolites can act as reducing agents (free radical terminators), metal chelators, singlet oxygen quenchers, and hydrogen donors [6]. Furthermore, epidemiological studies strongly suggest that long term consumption of plant polyphenols prevents degenerative diseases associated with oxidative stress [7]. Some recent studies also showed that cruciferous vegetables decrease the risk of several types of cancer, which makes cabbage a functional food [2].

Cabbage can be consumed raw as part of salads, condiments, or juice. It can be subjected to thermal processing, e.g. steaming, boiling, roasting, microwaving, etc., or fermentation (sauerkraut, kimchi, etc.) [2]. Recent studies demonstrated that *Brassica* vegetables lose their nutrient and health-promoting properties if overheated during domestic cooking [4, 8]. However, fermentation is known to enhance their nutritional properties [9]. Fermentation is one of the oldest ways of food processing and preservation. It is a spontaneous process carried out by lactic bacteria present in vegetables tissues. Fermentation increases the safety, sensory properties, and shelf-life of foods. It also promotes the release of bioactive compounds and reduces anti-nutritional factors [10].

Food safety and shelf-life are associated with microbial competition and the synthesis of inhibitory metabolites, such as lactic acid, acetic acid, hydrogen peroxide, diacetyl, ethanol, bacteriocins, and biosurfactants [10]. Lactic fermentation improves the nutritional value of cabbage, as well as its antioxidant activities. Lactic fermentation reduces phytates, thus improving the bioavailability of essential dietary nutrients, such as minerals, e.g. Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, and $Fe^{2+/3+}$, proteins, and amino acids [11].

Different databases feature the same nutritional data on *Brassica* vegetables (energy, fat, protein, mineral content, and carbohydrates) [12]. However, the situation is very different when it comes to the content of bioactive compounds [2]. The profile and concentration of phytochemicals depend on the cultivar, fertilization, agricultural conditions, environment, sowing season, and processing [13]. Furthermore, different studies report different effects of fermentation on the total phenolic compound and antioxidant activity [4, 9, 10, 14]. So far, no studies have featured the changes in the total phenolics and antioxidant activity that occur between cabbage tissue and brine.

The research objective was to evaluate the effect of spontaneous fermentation on: 1) phytase activity, calcium, and phytic acid concentrations; 2) total phenolic content and antioxidants activity of methanol extracts, water extracts, and brine throughout the fermentation of three Brassicaceae cabbages harvested in Patagonia (Argentina).

STUDY OBJECTS AND METHODS

Preparing the ferments. Chinese cabbage (*Brassica rapa* var. *glabra* Regel), white cabbage (*Brassica oleracea* var. *capitate* f. *alba*), and red cabbage (*B. oleracea* var. *capitata* f. *rubra*) were obtained from a local farm of Valle Inferior del Río Chubut located in Patagonia, Argentina. The cabbages were planted in March 2020 and harvested in June 2020. Before the fermentation, each cabbage head was stripped of dry outer leaves. The cleaned cabbage heads were chopped in a shredder into 2 mm thick strips and mixed with 3.0 % (w/w) of salt. Sterile water homogenized the medium (5 mL/100 g of cabbage). Each cabbage was spontaneously fermented at 18°C for 30 days. The fermentation was performed in duplicate.

Fermentation parameters. The total content of lactic bacteria and pH were monitored during the fermentation process on days 0, 1, 2, 3, 4, 5, 10, 15, 20, 25, and 30. At the beginning of the process, these parameters were examined after 6 and 12 h. The pH of the ferments was measured using a pH meter (model Orion 410A). The lactic bacteria count was monitored by incubating on MRS agar at 30°C for 48 h [15]. The results were expressed as colony forming units per milliliter of experimental sample (CFU/mL).

Preparing the solvent extracts and brine. During fermentations, the solid and liquid samples were withdrawn on days 0, 1, 3, 5, 10, 15, 20, 25, and 30. To prepare the solvent extracts, solid samples were dried at 37°C until constant weight to avoid degradation of thermal-sensitive compounds. After that, they were ground. Methanol and distilled water (1:10 m/V dilution) were used to prepare the extracts. For the methanol extract, the mixes were incubated for 3 h at 37°C under stirring. For the water extract, they were autoclaved for 15 min at 120°C. Both extracts were centrifuged at 13 000×g for 15 min at 25°C. The supernatants were stored at –20°C, while the brine samples (liquid material) were stored at –20°C.

Measuring calcium. The o-cresolftaleín complexone colorimetric method was used to determine the

amount of calcium in the cabbage brines. Briefly, 50 µL of sample were mixed with 950 µL of reaction mix composed by 3.7 mM of cresolphtalein complexone and 0.2 mM of amino methyl propanol solution (pH 11). The calcium content in the brines was determined against the calcium standard curve (0–55 µg Ca/mL). The absorbance was measured at 570 nm using a Jenway spectrophotometer (UK). The results were expressed as mg calcium per 100 mL brine (mg Ca/100 mL).

Phytic acid determination. The content of phytic acid was evaluated using an enzymatic method kit (Megazyme International, Ireland), based on its hydrolysis and further determination of free phosphorus. The procedure followed the manufacturer's instructions. The phosphate released from phytic acid was measured using a modified colorimetric molybdenum blue assay described by McKie *et al.* [11]. The color reagent was prepared with a solution of 0.6 M sulfuric acid (32 mL/L), ammonium molybdate (5 g/L), and ascorbic acid (20 g/L). After enzymatic treatment, 1.0 mL of color reagent was added to 50 µL of supernatant. The system was incubated for 30 min at 50°C, and the absorbance was measured at 820 nm. A standard curve was constructed with dipotassium phosphate (K_2HPO_4) (0–0.4 µg/mL). The results were expressed as mg K_2HPO_4/100 mL brine. The concentration of phytic acid was calculated on the basis of free phosphorus using the formula suggested by McKie *et al.* [11].

Phytase activity of the brine. Phytase activity was determined by measuring the amount of inorganic phosphate released from sodium phytate as proposed by De Angelisa *et al.* [16]. Briefly, 180 µL of reactive contained 5 mM of sodium phytate and 200 mM of sodium acetate buffer (pH 5.0). This amount was added to 20 µL of brine. After 15 min of incubation at 37°C, the reaction was stopped by adding an equal volume of 15% trichloroacetic acid. Afterward, the phosphate released was determined by the previously described ammonium molybdate method. One unit of phytase activity was defined as 1 µmol of phosphate produced per min per mL of brine under the assay conditions. The results were expressed as milli-units (mU).

Measuring the total phenolics. The total phenolic content was determined using the Folin-Ciocalteau reagent according to previously published procedures, with minor modifications [17]. An aliquot of 50 µL of extract was mixed with 100 µL of Folin-Ciocalteu´s phenol reagent and kept for 10 min. Then, Na_2CO_3 (1.0% m/V; 1.0 mL) was added and kept for 90 min at 25°C. The absorbance was measured at 750 nm. A calibration curve was based on gallic acid as standard. The results were expressed as milligram gallic acid equivalents per 100 g of dry weight (mg GAE/100 g DW).

Determination the antioxidant activity. ***Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay.*** The free radical scavenging activity of the samples was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) method as described by Chen *et al.*, with some modifications [18]. Briefly, 900 µL of an ethanolic DPPH solution (100 µM) was added to 100 µL of sample at various concentrations. After 30 min of incubation in the dark at 25°C, the absorbance was measured at 517 nm using a spectrophotometer. A standard curve was constructed with Trolox as a reducing agent (15–250 µg/mL). The results were expressed as milligram Trolox equivalents per 100 g of dry weight (mg TE/100 g DW).

Cupric reducing antioxidant capacity (CUPRAC) assay. Cupric reducing antioxidant power (CUPRAC) was used to determine the antioxidant capacity of the sample as described by Gouda *et al.*, with minor modifications [19]. An aliquot of 100 µL of sample was mixed with 900 µL of reaction mix. The reaction mix consisted of 2 mL of Neocuproine solution (5 mM), 1 mL of Cl_2Cu (0.01 M), and 3 mL of acetate buffer (50 mM, pH 5.0). After shaking and incubating for 1 h in the dark, the mix was tested for absorbance at 450 nm. A calibration curve was prepared using Trolox as standard (15–250 µg/mL). The results were expressed as mg of Trolox equivalent per 100 g of dry weight (mg TE/100 g DW).

Total antioxidant capacity. The total antioxidant capacity of the ferments was calculated by adding partial antioxidant activity of extracts and liquid phase (brine) contained in 100 g of edible material to simulate the antioxidant activity per sample. The same procedure was repeated for each vegetable and antioxidant parameter, i.e. DPPH, CUPRAC, and total phenolics. The results were expressed as milligram Trolox equivalents per 100 g of fresh weight ferment (mg TE/100 g FW).

Statistical analysis. All assays were carried out in duplicate, unless mentioned otherwise. The data were analyzed by ANOVA, and the means were compared by the minimum significant difference test at $P < 0.05$, using the Statgraphics Centurion XVI software.

RESULTS AND DISCUSSION

Fermentation parameters. Lactic bacteria and pH helped monitor the evolution of the fermentation process. Spontaneous fermentation of cabbage relies on autochthonous lactic bacteria present on the raw substrate. Organic acids decrease pH and increase the titratable acidity of the raw material.

The pH of raw white cabbage, red cabbage, and Chinese cabbage were 6.0, 5.9, and 6.1, respectively (Fig. 1). The samples of red and Chinese cabbage demonstrated a similar decrease in pH. In both cultivars, the lowest values were observed on day 4 and remained stable over 30 days (Figs. 1b and 1c). The white cabbage showed no sharp decrease of pH during fermentation. The lowest value was achieved on day 10 and remained stable (Figs. 1a vs 1b and 1c).

The initial population of lactic bacteria was 2.1, 2.1, and 2.5 log CFU/mL in the white, red, and Chinese cabbages, respectively (Fig. 1). This trend confirms previous reports by R. Di Cagno *et al.* and J. Beganović *et al.* [10, 20]. While the

highest count was observed on day 5 (9.9 log CFU/mL), the red cabbage sample approached its maximal counts on day 3 (9.4 log CFU/mL) (Fig. 1b). A similar curve was observed for the Chinese cabbage fermentation; however, the maximal counts were detected after day 4 (9.7 log CFU/mL) (Fig. 1c). Regarding the white cabbage, lactic bacteria population increased slower than in other samples and reached its maximum (9.2 log CFU/mL) on day 5 (Fig. 1a). In all the cases, once the peak was reached, the bacteria populations began to decrease. On day 30, the lactic bacteria cell counts were 5.0, 5.9, and 5.7 log CFU/mL for red cabbage, white cabbage, and Chinese cabbage, respectively (Fig. 1).

Calcium, phytic acid, and phytase activity. Figure 2 shows the changes in the phytase activity and calcium and phytic acid concentrations that occurred in the brine during fermentation. The raw samples of red and Chinese cabbage (Figs. 2b and 2c, respectively) contained comparable amounts of water-soluble calcium, whereas the white cabbage appeared to have a much lower concentration (Fig. 2a).

The initial level of phytic acid was almost the same for all three cultivars. The raw sample of Chinese cabbage showed the highest phytase activity (Fig. 2c). The initial specific activities of white and red cabbages were 39.54 ± 18.67 (Fig. 2a) and 56.71 ± 8.20 mU (Fig. 2b), respectively. The enzymatic activity was supplied exclusively by vegetal tissue during early

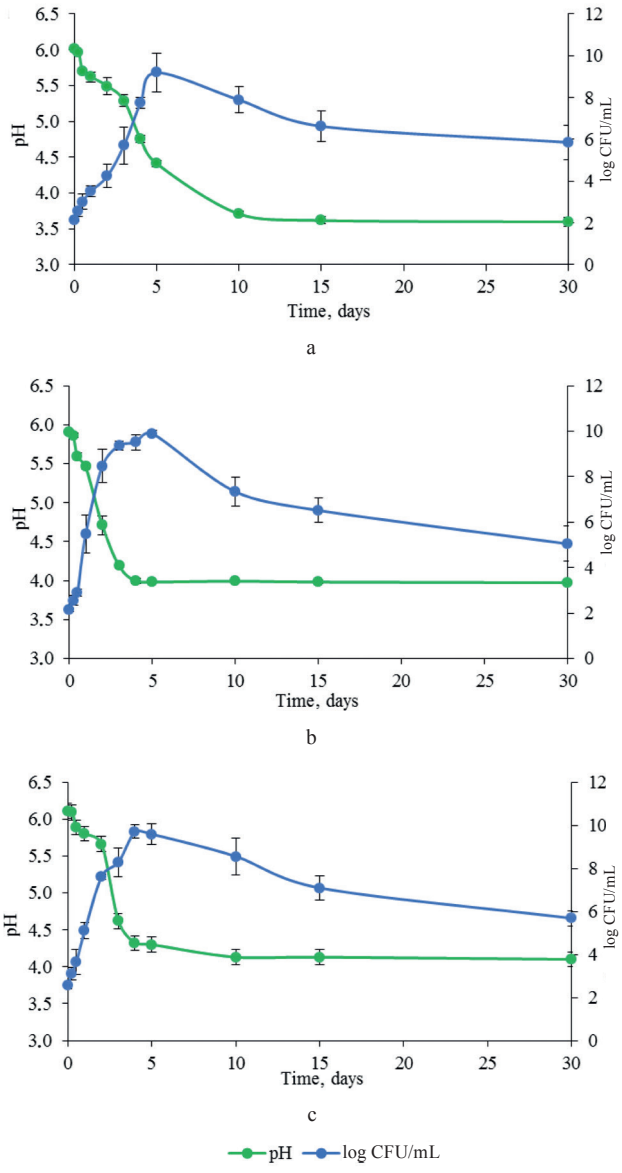

Figure 1 pH and total lactic acid bacteria counts grown on MRS agar in sauerkraut brine during spontaneous fermentation for white cabbage (a), red cabbage (b), and Chinese cabbage (c). Each value is mean ± SD of two measurements

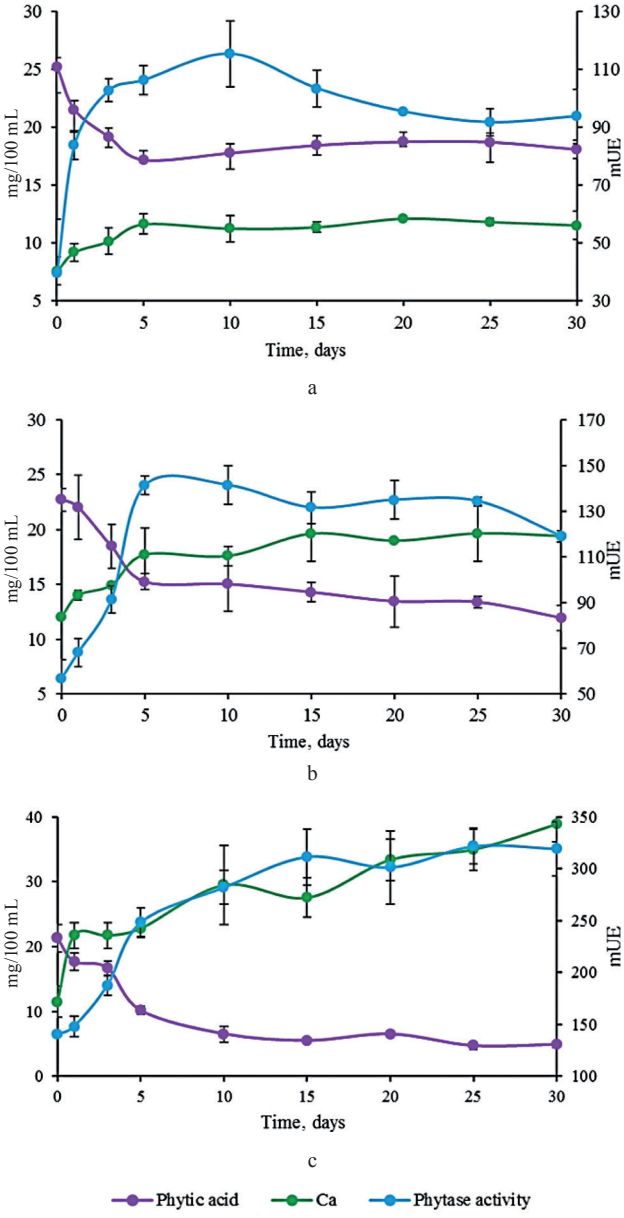

Figure 2 Calcium, phytic acid, and phytase activity during fermentation for white cabbage (a), red cabbage (b), and Chinese cabbage (c). Each value is mean ± SD of two measurements

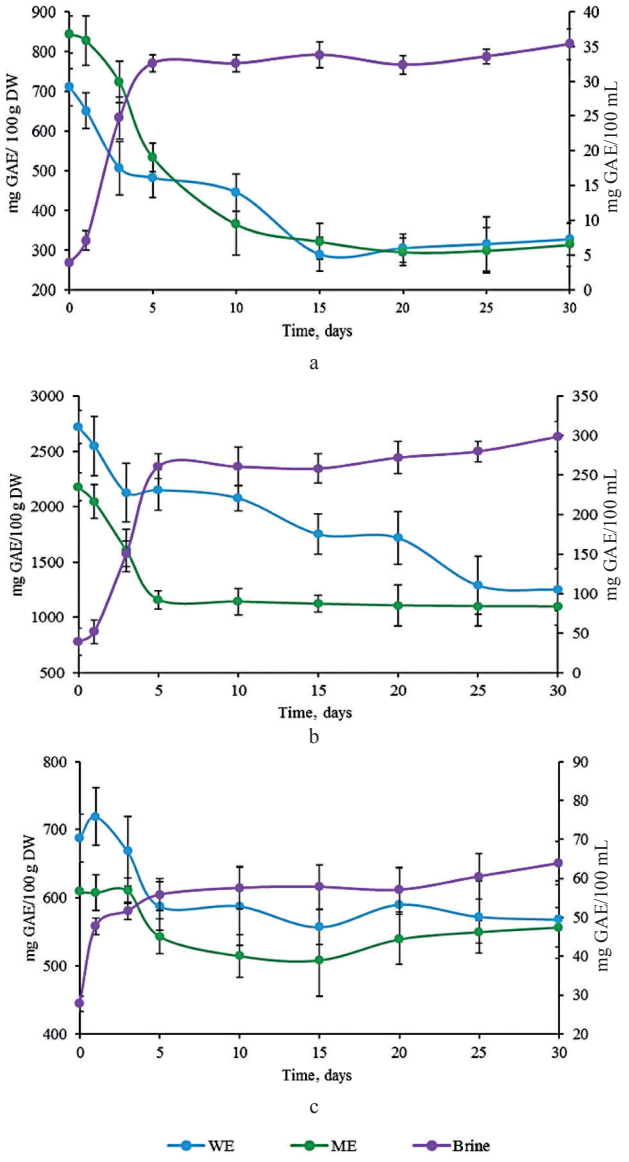

Figure 3 Total phenolic content in methanol extract (ME), water extract (WE), and brine during fermentation for white cabbage (a), red cabbage (b), and Chinese cabbage (c). Each value is mean ± SD of two measurements

fermentation, and then bacterial phytase brought about phytate hydrolysis [21].

The highest phytase activity was detected between days 5 and 10 in the samples of white and red cabbage, when the population of lactic bacteria reached its maximum (Figs. 2a and 2b). After that, the values remained constant. In the sample of Chinese cabbage, the maximal activity was detected on day 10 (Fig. 2c), which coincided with the maximal viable cell count of lactic bacteria.

As the fermentation process advanced, the phytate concentration decreased and the amount of soluble calcium increased in all the samples. This phenomenon was more pronounced in Chinese cabbage when the phytase activity had its highest value. The lowest phytate concentration and the highest calcium concentration were achieved on day 30. The assays for all the samples proved that the highest phytase activity occurred under acidic conditions.

Phytic acid (myo-inositol-6-phosphate) is the major storage form of phosphorous and represents 50–85% of total phosphorous in plants [21]. This compound and its derivatives are the main inhibitors of divalent mineral absorption in the gastrointestinal tract due to the formation of insoluble and indigestible complexes [22]. Hence, it may decrease the calcium bioavailability in cabbage [21]. However, this point of view is now controversial since several studies demonstrated that the myo-inositol-6-phosphate consumption may be associated with some health benefits. The antinutrient effect of phytic acid has not been fully demonstrated *in vivo*. On the other hand, phytic acid exerts anti-inflammatory and anticancer activities and diminishes the risk of osteoporosis [23].

Phenolic compounds. Phenolic composition and antioxidant activity depend mainly on the type of extraction solvent. The choice of solvent depends mainly on the chemical nature and polarity of the compounds to be extracted. Methanol and water are widely used as solvents in vegetable and plant tissues [14]. In this study, methanol and water helped measure phenolic compounds and antioxidant activity in the cabbage samples during fermentation.

Figure 3 shows the total phenolic content in the extracts (methanol and water) and brines of white, red, and Chinese cabbages. Regarding the white cabbage sample, the water and methanol extracts exhibited a similar total phenolic content. However, the total phenolic content in the water extracts of red and Chinese cabbages was much higher than in the methanolic extract (Figs. 3b and 3c). Probably, the solubility of phenolic compounds depended on extraction conditions, e.g. the chemical structure of solvents, dielectric constant, time, temperature, phytochemical properties, etc. However, thermal treatment is known to damage some phenolics [24].

The total phenolic content in the extracts and brine of red cabbage was higher than in the samples of white and Chinese cabbage. This trend was in agreement with previous studies. For instance, Tabart *et al.* [25] reported 1851 mg GAE/100 g DW in red cabbage; Vicas *et al.* [26] – 980–1220 mg GAE/100 g DW in white cabbage; Seong *et al.* [27] – 347.46 ± 32.17 mg GAE/100 g DW in Chinese cabbage. In vegetables, phenolics exist mostly in conjugated forms through hydroxyl groups with sugar as glycosides. Lactic bacteria possess an enzymatic battery that can convert phenolics to aglycone forms, which are simpler and biologically more active [28]. Furthermore, during fermentation, pectic enzymes may soften cabbage texture, thus releasing phenolics compounds from the solid to the liquid phase [27].

Lactic fermentation promoted a significant decreased in the total phenolic content in the red and white cabbage extracts (methanol and water) after 3–5 days of incubation (Figs. 3a and 3b).

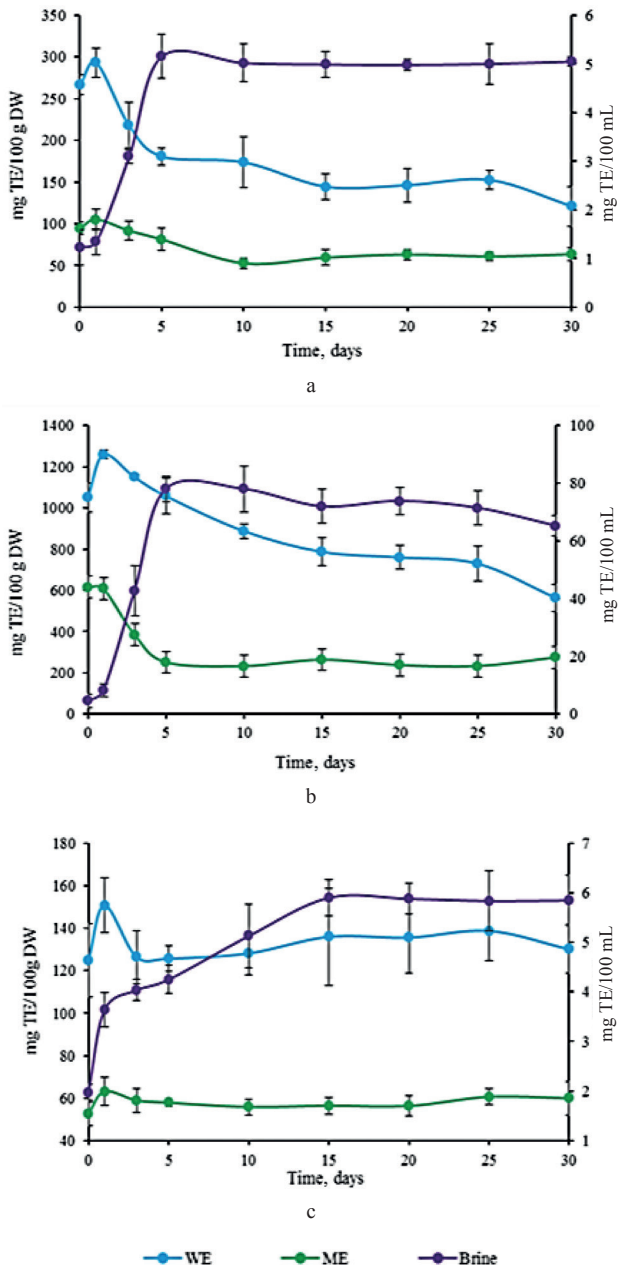

Figure 4 Antioxidant activity (DPPH assay) in methanol extract (ME), water extract (WE), and brine during fermentation for white cabbage (a), red cabbage (b), and Chinese cabbage (c). Each value is mean ± SD of two measurements

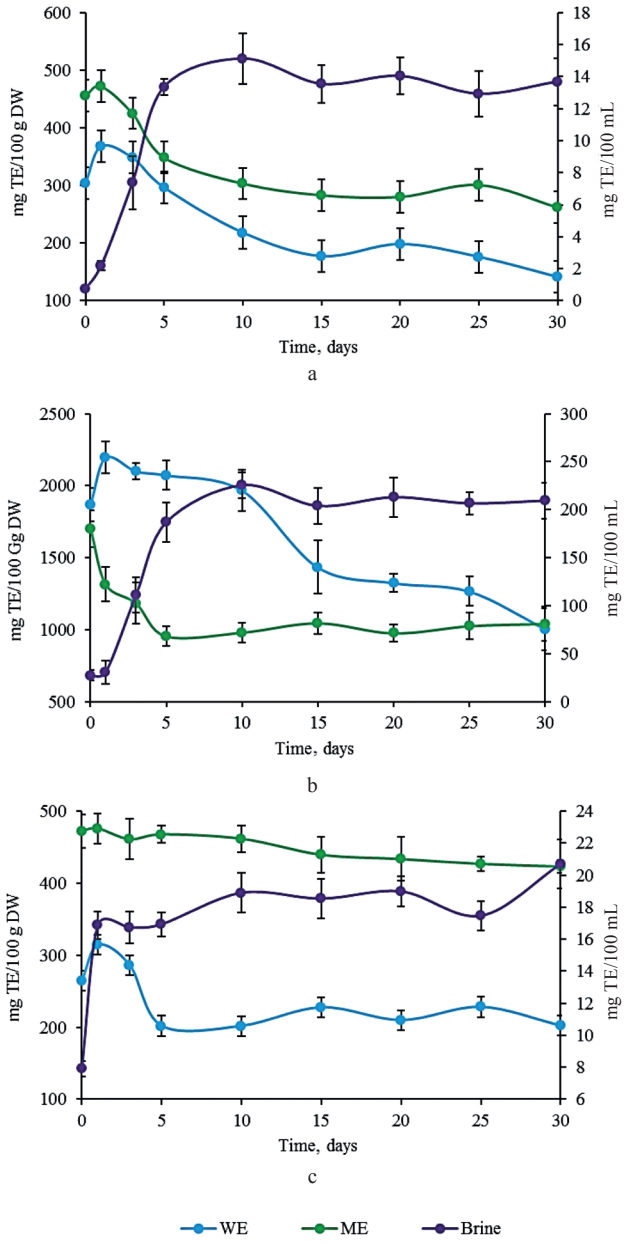

Figure 5 Antioxidant activity (CUPRAC assay) in methanol extract (ME), water extract (WE), and brine during fermentation for white cabbage (a), red cabbage (b), and Chinese cabbage (c). Each value is mean ± SD of two measurements

Afterward, the total phenolic content dropped slowly and remained almost constant until the end of lactic fermentation. The total phenolic content in the methanol and water extracts decreased almost by half. On the contrary, the brine samples demonstrated a significant increase between days 3 and 5, and then the concentration remained almost stable until the end of storage. The Chinese cabbage sample showed a slight decrease in the total phenolic content in methanol and water extracts throughout the fermentation (Fig. 3c).

Antioxidant activity. The antioxidant activity was evaluated by DPPH radical scavenging assay and CUPRAC reduction assay. Both are electron transfer-based methods, frequently used to determine the antioxidant activities of phenolic compounds [6]. Figure 4 illustrates the antiradical activity of methanol and water extracts against DPPH radical. The methanol extract contained significantly less reduction power than the water extract in all the cabbage samples. Probably, this solvent failed to provide efficient extraction of compounds with antioxidant activity. The raw

Table 1 Contribution of water extract and brine to the total phenolic content of each individually fermented cabbage

Total phenolic content, mgGAE/100 mL			
Time, days	White cabbage	Red cabbage	Chinese cabbage
0	36.27 ± 2.26	24.35 ± 10.82	51.88 ± 2.01
1	35.57 ± 2.13	32.17 ± 9.00	68.31 ± 4.09
3	40.41 ± 3.18	92.95 ± 10.05	69.78 ± 4.77
5	42.24 ± 2.34	161.45 ± 9.83	70.30 ± 5.46
10	40.48 ± 2.21	161.23 ± 15.23	71.81 ± 7.81
15	33.67 ± 1.99	159.73 ± 11.67	70.85 ± 6.24
20	33.74 ± 1.72	168.33 ± 12.43	71.48 ± 1.96
25	35.09 ± 3.25	173.17 ± 7.98	73.77 ± 2.15
30	36.21 ± 1.85	184.75 ± 11.90	76.51 ± 1.75

*Each value is mean ± standard deviation of three measurements

The values were expressed in mg of Gallic Acid Equivalents (GAE)/100 g of fresh weight

sample of red cabbage showed the highest antioxidant capacity with 1050.44 ± 71.33 TE/100 g DW and 616.63 ± 49.80 mg TE/100 g DW in water and methanol extracts, respectively (Fig. 4b). Afterwards, these values declined until the end of fermentation.

On the contrary, in the brine, the values kept rising until day 5 and then remained stable. The extracts of white and Chinese cabbages displayed a significantly lower DPPH radical scavenging activity than the extracts of red cabbage (Figs. 4a and 4c). These trends confirmed previous reports [29]. The methanol and water extracts of white cabbage exhibited a slight decrease in the antiradical activity, while its brine demonstrated an increase during the first 5 days of fermentation (Fig. 4a). However, no significant differences in the antioxidant activities were observed in the Chinese cabbage extracts. A significant increase was detected in the brine during the first 10 days of fermentation, but it remained constant until the end of fermentation (Fig. 4c).

In all the cases, the values of antioxidant capacity obtained with CUPRAC assay (Fig. 5) were higher than those obtained with DPPH method. This trend could be explained by the ability of CUPRAC method to measure hydrophilic and lipophilic antioxidants simultaneously, while DPPH detects only those molecules that are soluble in organic solvents, particularly in alcohols [30].

The antioxidant capacity of the red and white cabbages decreased significantly in the methanol and water extracts during day 1 and increased significantly in the brine (Figs. 5a and 5b). In the white cabbage, these changes occurred between days 5 and 10. For the red cabbage, the decrease was observed on day 5 in the methanol extract and on day 15 in the water extracts. The maximal value in brine was achieved after 5 days.

Regarding the Chinese cabbage samples, a comparable trend could be observed between the values obtained with DPPH radical scavenging assay and CUPRAC method. The concentration of reducing agents in dry matter decreased slowly in the water extract, while the methanol extract showed no significant differences. A slight but significant increase in the concentration was detected in the brine (Fig. 5c).

The antioxidant capacity presented a sharp increase on day 1 (Figs. 4 and 5). This trend was due to the high driving force produced by concentration gradients of the substance that tends to equilibrate the medium. In this process, water flows from the solid phase to the liquid phase and brings some solutes from the vegetables. This phenomenon is due to transfer rates that increase or decrease until equilibrium is reached [31].

Overall evaluation of total phenolics and antioxidant activity. The total phenolic content and antioxidant activity in the white and red cabbage samples decreased in the dry matter and increased in the liquid phase. This phenomenon was less pronounced in the Chinese cabbage sample. However, these data alone cannot estimate the total variation of the antioxidant capacity throughout the process: both phases contributed to the phenolic content and scavenging activity since the cabbages were not to be consumed dry.

Table 2 Contribution of water extract and brine to the total antioxidant capacity (DPPH and CUPRAC) of each individually fermented cabbage

	DPPH assay, mgTE/100 mL			CUPRAC assay, mgTE/100 mL		
Time, days	White cabbage	Red cabbage	Chinese cabbage	White cabbage	Red cabbage	Chinese cabbage
0	13.04 ± 0.79	52.15 ± 4.85	6.44 ± 0.52	15.01 ± 1.04	104.94 ± 2.13	16.84 ± 0.36
1	14.10 ± 1.01	64.27 ± 2.32	8.86 ± 0.19	18.66 ± 0.80	122.38 ± 12.63	26.46 ± 0.62
3	11.94 ± 1.56	80.45 ± 6.05	8.28 ± 0.65	20.94 ± 0.12	166.01 ± 6.19	25.24 ± 1.03
5	11.37 ± 0.68	97.98 ± 6.88	8.44 ± 0.46	22.20 ± 0.93	213.06 ± 7.82	22.23 ± 2.01
10	11.04 ± 1.17	89.93 ± 6.61	9.28 ± 0.16	19.60 ± 1.80	231.88 ± 15.18	23.92 ± 1.21
15	9.64 ± 0.55	81.44 ± 0.41	10.24 ± 1.17	16.70 ± 1.70	193.52 ± 20.26	24.59 ± 2.92
20	9.79 ± 1.11	81.32 ± 5.57	10.21 ± 0.91	17.99 ± 0.28	194.01 ± 15.47	24.30 ± 0.01
25	10.07 ± 0.43	78.42 ± 5.80	10.29 ± 0.01	16.26 ± 0.05	187.33 ± 13.73	23.69 ± 2.62
30	8.68 ± 0.91	66.81 ± 0.85	9.98 ± 0.00	15.08 ± 1.87	176.77 ± 4.79	24.48 ± 3.11

* Each value is mean ± SD of three measurements

The values were expressed in mg of Trolox Equivalents (TE)/100 g of fresh weight

Tables 1 and 2 show the results obtained by adding the values of dry matter and brine. These results can be considered the total polyphenol content and the total antioxidant activity of the fermented cabbages. Regarding the samples of red and Chinese cabbages, the total phenolic content and the total scavenging activity in the water extracts and brine gradually increased and reached plateau after about 5–10 days, which coincided with the highest population of lactic bacteria. In the white cabbage samples, the total phenolic content and the total antioxidant capacity in the water extract and liquid phase exhibited slight changes. By the end of fermentation, the total phenolic content and the antioxidant activity were similar or smaller, in the case of radical scavenging activity measured by DPPH.

To sum up, the fermentation increased the total phenolic content and the antioxidant activity in the liquid phases of red and Chinese cabbages. The red cabbage sample had the highest total phenolic content.

CONCLUSION

Fermentation was able to significantly improve the quality and functionality of *Brassica* L. cabbages. The test samples showed a significant increase in phytase activity, which promoted the decrease of phytic acid and the increase of free calcium. Fermentation raised the total phenolic content and the antioxidant activity because of the individual contribution of the solid and liquid phases to total scavenging capacity.

CONTRIBUTION

Romina Parada is responsible for conceptualization, methodology, software, validation, formal analysis, investigation, reviewing, proofreading, and visualization. Emilio Marguet is responsible for conceptualization, methodology, formal analysis, investigation, and drafting. Carmen Campos is responsible for conceptualization, software, formal analysis, writing-reviewing, and editing. Marisol Vallejo participated in conceptualization, methodology, writing, reviewing, editing, and visualization.

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 adulterated palm cooking oil on the quality of fried chicken nuggets

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Abstract:

Introduction. There is a rising concern over food safety caused by an increasing trend towards adulterating fresh cooking oil with used cooking oil in Malaysia. Recent decades have seen more cases of high-quality edible cooking oil adulteration with reused oil, driven by high market demand and profit margins. In this study, we aimed to analyze the properties of vegetable oils and their effect on the quality of fried chicken nuggets.

Study objects and methods. We determined free fatty acid contents and characterized the properties of fresh palm olein, used cooking oil, and adulterated oil. We also compared the sensory quality attributes of chicken nuggets fried in fresh and adulterated oils.

Results and discussion. The content of free fatty acids consistently increased with rising adulteration levels. The FTIR spectral analyses revealed significant differences between fresh, used, and adulterated oils at 3006, 2922, 2853, 2680, 1744, 1654, 987, 968, and 722 cm^{-1}. The oil samples with high adulterant concentrations demonstrated a linear increasing trend in K_{232} and K_{270} values, where higher absorbance values indicated severe deterioration in the oil quality. The sensory evaluation showed no significant effect ($P > 0.05$) of adulteration with used cooking oil on the quality of fried chicken nuggets.

Conclusion. Our findings filled in a gap in the previous studies which only focused on the effects of adulteration on the oil properties. The study also provides valuable information to regulatory authorities on the reliability of quality parameters and modern instruments in edible oil adulteration detection.

Keywords: Adulteration, fresh palm olein, used cooking oil, food safety, sensory evaluation, frying, chicken

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INTRODUCTION

Vegetable oils are a group of fats that are extracted from different parts of a plant, such as seeds, nuts, cereal grains, and fruits [1]. They play a significant role in our diet as the main source of dietary fat and nutrients, as well as a flavor enhancer. In Malaysia, palm oil has been widely employed in the frying process, particularly in deep frying, owing to its high stability [2, 3].

Deep-fried foods have become popular due to the ease and speed of thermal treatment, as well as unique flavor, taste, and texture induced during the frying process [4]. The quality of oil has become a major concern to the deep frying industry since it affects the sensory quality of fried food, such as fried chicken nuggets [5].

However, the authenticity of cooking oil has been a serious issue since old times [6, 7]. According to statistics, 26.5% of all food fraud incidents (n = 1648) in 1980–2012 were associated with cooking oils [8]. Vegetable oil adulteration can be defined as an addition of cheaper, inferior, harmful, or unnecessary substances to oil that could affect its nature and quality [9]. High profit often drives this kind of fraudulent practice. Lim *et al.* and Alagesh reported a rising concern over food

safety in light of the increasing trend of fresh cooking oil adulteration in Malaysia [10, 11].

Used cooking oil (UCO), also known as waste cooking oil or yellow grease, is the oil that has already been used in food preparation processes [10]. In order to reduce expenses, most food business operators and caterers tend to reuse the oils repeatedly, toping them up with fresh oil to mask the effects of degraded oil. Moreover, by using a series of simple and low-cost processes, including preliminary filtration, boiling, and refining, they are able to recover the quality of waste cooking oil to a certain extent to make it resemble that of fresh oil [12]. Since the past decade, cooking oil adulteration with refined waste oil has been rampant in Asian countries, particularly in mainland China, followed by other countries [13–16]. The situation is worsened by the low purchase cost of UCO, its wide availability, and high profit gain over the price difference.

Various analytical techniques and parameters have been developed to determine cooking oil adulteration. The most common of them is a free fatty acid (FFA) test [3, 17]. However, this test only measures the overall levels of titratable acids, without identifying the profiles of FFAs. In recent years, many sophisticated analytical methods have been studied intensively, including the FTIR fingerprint spectroscopic method. They have proven fit to unravel the menace of adulteration in high quality fresh oil [10, 18–20]. According to Amereih *et al.* and Hashem *et al.*, the UV-Vis spectrophotometric method is also effective enough in adulteration detection and quantification [20, 21].

Therefore, we aimed to study the properties and quality of palm cooking oil adulterated with used cooking oil. Palm oil was chosen as the most common frying medium in Malaysia. In addition, we determined the effects of adulteration on the sensory quality of fried chicken nuggets, adding to former studies that mainly report its effects on the oil properties.

STUDY OBJECTS AND METHODS

Oil sample collection. Fresh palm olein (FPO) and frozen chicken nuggets were purchased from the local market in Pagoh Jaya, Johor, Malaysia. Pre-filtered used cooking oil (UCO) was collected from a local feedstock trading company.

Formulation and preparation of adulterated oil (AO). Sets of pure FPO and UCO samples were prepared without any adulteration. A set of AO samples was prepared by mixing FPO with 20, 40, 60, and 80% (v/v) of UCO. The mixtures were vortexed to ensure complete homogenization.

Determination of free fatty acid (FFA) content. The FFA content was determined using a conventional acid-base titration method developed by the Malaysian Palm Oil Research Institute, as previously reported by Abdul Wahab *et al.*, with slight modification [22, 23]. A 500 mL volumetric flask was filled with 50 mL of 1.0 M sodium hydroxide solution that was diluted with distilled water to the graduation mark. The solution was standardized by titrating with a standard KHP solution. Then, 2-propanol solution was heated to approximately 80°C and mixed with 1 mL of phenolphthalein indicator. The heated alcohol solution was then neutralized by adding the 0.1 M sodium hydroxide solution drop by drop until the first permanent light pink color was obtained. Subsequently, an oil sample was mixed with the neutralized alcohol solution and shaken vigorously to ensure an even mixture. Finally, the still hot mixture was titrated against the 0.1 M sodium hydroxide solution until another permanent light pink color was obtained. The amount of sodium hydroxide consumed during titration was recorded and used to determine the FFA content (Eq.(1)). The results were expressed in mean ± standard deviation in triplicate.

$$\text{FFA}\,\% = \frac{28.2\text{VM}}{\text{W}} \quad (1)$$

where V is the volume of NaOH, mL; M is the molarity of NaOH, M; W is the weight of the oil sample, g.

Measurement of ATR-FTIR spectra. The procedure followed the method described by Poiana *et al.* [19]. The ATR-FTIR spectra of each oil sample were scanned and recorded using a Spectrum Two FT-IR spectrometer (PerkinElmer, United States) equipped with an ATR accessory. A drop of each oil sample was placed on the crystal at room temperature (25°C). All the spectra were measured at the mid-infrared region ranging from 4000 to 650 cm^{-1} with a scanning time of 60 s and 4 cm^{-1} resolution. The ATR-FTIR spectra were obtained against the air background spectrum. After every scan, a new reference air background spectrum was performed. The ATR plate surface was gently wiped with a soft tissue soaked in acetone to remove any residues of the previous oil sample before placing a new one. The FTIR spectra of all the oil samples were recorded as an absorbance value in triplicate.

Measurement of UV-Vis absorption at 232 and 270 nm (K_{232} and K_{270}). The procedure was based on the method reported by Amereih *et al.* and Chong, with slight modification [21, 24]. The absorption spectra of all the oil samples were obtained at 200 to 800 nm using a U-3900H UV-Vis spectrophotometer (Hitachi High-Tech Corp., Japan). A quartz cuvette (1 cm path) was filled with 1% of an oil sample in isooctane solution. The absorption was measured against a blank of isooctane. The maximum absorption values obtained at 232 nm and 270 nm were subsequently used to determine the specific extinction coefficients, K_{232} and K_{270} respectively, as outlined in Eq. (2).

$$K_{\lambda} = \frac{A\lambda}{\text{cL}} \quad (2)$$

where K_{λ} is the specific extinction coefficient at wavelength λ; A_{λ} is the absorption measured at wavelength λ; c is the concentration of the oil sample in solvent, g/100 mL; L is the path length of the cuvette, cm.

Deep frying and sensory evaluation. The frozen chicken nuggets were weighed and made into batches of 300 g. FPO was added into an EF-102T electric dual tank deep fryer (Wibur, China) to reach its minimum capacity of about 4 kg of oil. The FPO was heated for about 20 min up to 175 ± 5°C. After pre-heating, the first batch of frozen chicken nuggets was deep-fried for 3 min until the nuggets turned golden brown. The subsequent frying cycles started at an interval of 20 min. At the end of a frying cycle, the nuggets fried in FPO were taken for sensory evaluation. Due to health concerns regarding reused oil, the use of AO in preparing fried chicken nuggets for sensory evaluation was limited to 60%. It simulated the AO that could be commonly found in the local night market. The new frying cycles began by replacing the FPO with AO containing 60% UCO (w/w). Similarly, the fried chicken nuggets prepared in 60% AO were then evaluated for sensory acceptance.

The fried chicken nuggets prepared in FPO and 60% AO were evaluated for sensory attributes such as color, flavor, juiciness inside, crispiness outside, taste, and overall acceptability. The 9-point hedonic scale was employed differently for each attribute, namely for flavor, taste, and overall quality: 1 = extremely dislike, 5 = neither like nor dislike, and 9 = extremely like; for crispiness outside: 1 = soft and 9 = crispy; for juiciness inside: 1 = dry and 9 = juicy; and for color: 1 = dark brown and 9 = golden yellow.

Statistical analysis. All the tests were conducted in triplicate. The volume of titrant and % FFA were recorded and expressed in mean ± standard deviation in triplicate. The functional groups and their vibration modes, as shown in the IR spectra, were matched to the respective characteristic bands in FPO, AO, and UCO. The absorbance intensities of the bands were evaluated by comparing the peak heights. Each spectrum and maximum absorbance at 232 nm and 270 nm were reported in mean ± standard deviation. An independent t-test was performed using Microsoft Excel to examine the differences between the oil samples ($P < 0.05$). ANOVA was used to analyze the sensory evaluation data to determine significant effects between the fried chicken nuggets prepared in different oils.

RESULTS AND DISCUSSION

Effect of adulteration with reused oil on FFA content. In all the oil samples under analysis, FFA contents increased with higher adulterant concentrations (Table 1). The FFA level was the lowest in FPO (0.90 ± 0.16) and the highest in UCO (3.25 ± 0.06). Banani *et al.* and Alias *et al.* reported that used or waste cooking oil had high FFA values, which subsequently led to high acidity and viscosity values [25, 26]. This finding was similar to those by Abdul Wahab *et al.* and Panadare and Rathod, who found higher FFA contents (2.33–6.42%) in waste cooking oil compared to fresh cooking oil [23, 27].

Table 1 FFA content in oil samples with different adulteration levels

Oil sample	Adulterant concentration, %	Free fatty acid, %
Fresh palm olein	0	0.90 ± 0.16
20% adulterated oil	20	1.34 ± 0.14
40% adulterated oil	40	1.69 ± 0.00
60% adulterated oil	60	2.12 ± 0.05
80% adulterated oil	80	2.58 ± 0.04
Used cooking oil	100	3.25 ± 0.06

The relatively higher FFA content in UCO, which was attributed to darker oil color, might be due to the exposure to prolonged heating and moisture from food, which induced the hydrolysis reaction of triglycerides [23]. Since the FFA content in UCO was less than 15%, it was classified as yellow grease. This finding was similar to the results reported by Abdul Wahab *et al.*, Panadare and Rathod, and Rosnelly *et al.* [23, 27, 28]. All the findings indicated the deteriorating quality of the UCO subjected to repeated heating cycles. As a result, it was no longer suitable for frying or human consumption due to increased oil acidity, which is potentially harmful to human health. This observation was in agreement with the results reported by Ahmad Tarmizi *et al.*, Maskan and Bagci, and Chong [3, 29, 30].

Used or waste frying oil is an end product of frying. It is subjected to harsh frying conditions and prolonged exposure to excessive heat and atmospheric air due to repetitive use. The chemical changes induced by frying, such as hydrolysis and oxidation, generate reactions in its by-products, such increasing FFA values, which gives rise to off flavors and odors. This justifies the high FFA content in the UCO in our study. We also found that increasing adulterant concentrations corresponded to high FFA values, which makes the adulterated oils unsafe for frying or human consumption.

Characterization of oil properties using FTIR spectra. We found no significant differences between the spectral features, despite slight changes in the absorbance of some bands and a few shifts in their exact position. Figure 1 shows the FTIR spectra of the FPO and UCO samples at ambient temperature. Both the FPO and UCO displayed some typical spectral features associated with oils. Both spectra were similar in terms of shape, position of the characteristic bands, and the presence of peaks. These similarities can be explained by the same origin of the oils and the presence of identical principle components in their composition, which are triglycerides [10, 31].

However, variation in the oil composition is an important factor that influences the exact position of the bands, as well as shifts in the spectra [19, 31–33]. The variation in both spectra could be due to the quality degradation caused by adulteration. Table 2 summarizes the significant aberrations observed in the FTIR

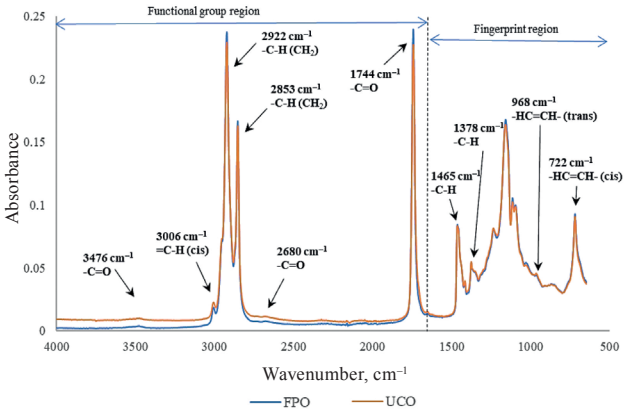


Figure 1 FTIR spectra for fresh palm olein (FPO) and used cooking oil (UCO) at 4000–650 cm^{-1}

spectra of all the oil samples in response to adulteration with UCO.

The FTIR spectrum is divided into two distinctive regions. They are functional group and fingerprint regions corresponding to 4000–1650 and < 1650 cm^{-1}, respectively. The entire spectra that we obtained for all the oil samples were seemingly identical because of their similar fatty acid compositions.

Nevertheless, we found that the UCO and AO samples with increasing adulteration levels demonstrated a slight aberration at 3006, 2922, 2853, 2680, 1744, 1654, 987, 968, and 722 cm^{-1} in terms of absorption bands and absorbance intensity. The adulteration of FPO with UCO resulted in a shift of the 3006 cm^{-1} band (Fig. 2a). This finding was in agreement with [19, 32, 34, 35] showing that the exact band position was determined by oil composition and unsaturation level. FPO recorded its highest absorbance at 3007 cm^{-1}, while AO reached its maximum at 3006 cm^{-1} due to reduced unsaturation. This suggested that exposure of frying oil to high heat had an effect on its unsaturation degree. Compared to FPO, the AO and UCO samples, which had been heated repeatedly, showed higher absorbance at 3006 cm^{-1}. This observation was consistent with a previous study by Alshuiael and Al-Ghouti, which proved that high heat application caused oil to become more unsaturated by losing hydrogen atoms [35].

We also observed strong and sharp absorption bands at 2922 and 2853 cm^{-1} due to the symmetric stretching vibration of aliphatic groups (-CH). The bands are attributed to the presence of aliphatic fatty acid chains. The high absorption peak of FPO at around 2922 cm^{-1} was determined by its unique fatty acid composition.

Apart from that, we identified a weak absorption band at around 2680 cm^{-1}, which could be attributed to carbonyl ester (-C=O) caused by Fermi resonance (Fig. 2b). The absorption band at 2680 cm^{-1} indicated the presence of aldehyde containing the O=C-H group. The increment of carbonyl aldehyde correlated with the adulteration incidence. As we can see in Fig. 2b, the concentration of aldehydes in the UCO samples and in most AO samples was far higher than that in FPO, except for 80% AO.

The concentration of volatile aldehydes is associated with a degree of oxidative degradation of oil, as aldehydes are major volatile compounds emitted upon heating as thermal degradation products [36, 37]. Volatile compounds such as aldehydes, ketones, alcohols, and acids are generated during oil degradation. They create unfavorable aroma and flavor, shorten the oil's shelf life, and may induce health problems [38]. We found that the UCO exposed to repeated frying gave high absorbance intensity at 2680 cm^{-1}, which might be attributed to an increment of volatile aldehydes due to lipid oxidation that consequently degrades the oil quality.

In relation to that, we observed a strong and sharp absorption band at 1744 cm^{-1} (Fig. 2c) due to the presence of the C=O group of triglycerides caused by stretching vibration. This was due to the decomposition of unstable primary hydroperoxides, which formed upon oxidation, into stable secondary oxidation products such as aldehydes and ketones, which cause an absorbance

Table 2 Significant aberrations in the FTIR spectra for oil samples in response to adulteration

Description of spectra feature	Significance
Slight shift of band near 3006 cm^{-1}	Reduced degree of unsaturation caused by diminution of *cis*- olefinic double bonds (=CH)
Strong band at 2922 and 2853 cm^{-1}	Presence of aliphatic methylene (-CH_2) group indicative of saturation level
Weak band at 2680 cm^{-1}	Presence of saturated aldehydes as a marker of advanced oxidation
Strong band at 1744 cm^{-1}	Appearance of carbonyl compounds and other secondary oxidation products
Increased absorbance of band at 1680–1630 cm^{-1} (or decreased absorbance of band at 1654 cm^{-1})	Presence of *trans*- and *cis*- isomers due to *cis-trans*- isomerization upon thermal stress
Maximum absorption at 987 and 968 cm^{-1}	Formation of *trans*- isomers (conjugated *trans* and non-conjugated *trans*- respectively) induced by conjugation and *cis-trans*- isomerization due to heat
Appearance of band at 968 cm^{-1}	Possible presence of secondary oxidation products (aldehydes, ketones) with isolated *trans*- double bond indicative of advanced oxidation
Progressive decrease in absorbance of band at 700–725 cm^{-1} (or at 722 cm^{-1})	Disappearance of *cis*- double bonds indicative of reduced unsaturation

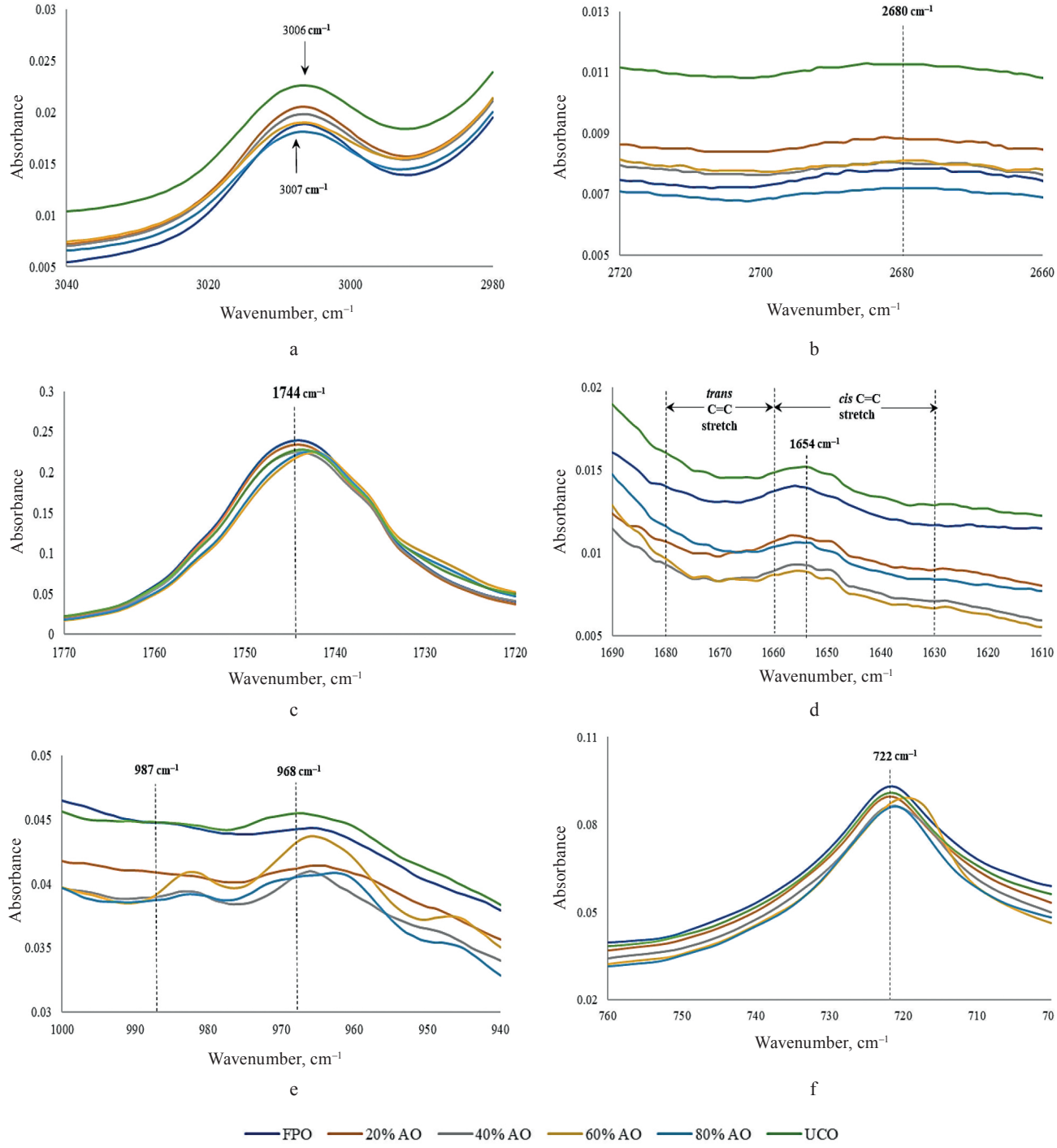


Figure 2 Significant aberrations in fresh palm olein (FPO), used cooking oil (UCO), and adulterated oil (AO) spectra at: (a) 3006, (b) 2680, (c) 1744, (d) 1680–1620, (e) 987–968, and (f) 722 cm^{-1}

near 1744 and 1728 cm^{-1}. High absorbance of FPO at this band could be explained by prolonged storage, which intensified the oxidative reaction [39].

The absorption band near 1680–1620 cm^{-1} could be assigned to the C=C stretch (Fig. 2d). The peak at 1654 cm^{-1} showed a general declining trend in absorbance with increasing adulteration levels, implying the disappearance of the *cis*- carbon-carbon double bond within the molecular structure. This could be due to the thermal and/or oxidative degradation of the oil samples.

The accumulation of *trans*- fatty acids in all the samples was further evident through an increasing trend in absorbance from the *cis*- C=C stretch band region (1660–1630 cm^{-1}) to the *trans*- C=C stretch band region (1680–1660 cm^{-1}). This might be explained by the occurrence of *cis-trans*- isomerization induced

by thermal stress, changing the initial *cis*- geometric configuration into *trans*- and resulting in *trans*- fatty acids accumulation [19, 40, 41]. This finding was further reinforced by the absorption bands at 968 and 722 cm^{-1}, corresponding to bending vibration of -HC=CH- in *trans*- and *cis*- configuration, respectively (Fig. 2e and 2f).

The UCO generally demonstrated a higher absorbance intensity than the FPO at 968 cm^{-1}. This observation related to the increment of *trans*- composition caused by *cis*-*trans*- isomerization, resulting in deteriorated oil quality. Meanwhile, the band near 722 cm^{-1} was responsible for the *cis*- double bonds of disubstituted olefins. The UCO showed lower absorbance values compared to the FPO, which was probably due to the reduction of *cis*- C=C double bonds of unsaturated fatty acids. This observation was consistent with a previous study [19] that showed a progressive decline in absorbance at 722 cm^{-1} indicative of *cis*-*trans*- isomerization in unsaturated fatty acids, which subsequently resulted in double bonds vanishing from the *cis*- conformation.

In addition, we found a very weak absorption band at 987 cm^{-1}, which indicated the presence of *trans*-, *trans*- and/or *cis*-, *trans*-conjugated diene groups of hydroperoxides. Oil oxidation causes *cis*- double bonds to disappear, also leading to the isomerization of *cis*- fatty acids to *trans*- isomers and hydroperoxide (primary oxidation products) generation [42]. Unstable hydroperoxides decompose into aldehydes, ketones, and other secondary oxidation products, which are more stable. These volatile compounds are responsible for the off-odor of the oxidized oils.

According to Guillen and Cabo, the absorption band at 967 cm^{-1} indicates the possible presence of secondary oxidation products such as aldehydes and ketones, which contain isolated *trans*- double bonds [43]. As can be seen in Fig. 2e, the UCO showed a relatively higher absorbance at 987 and 967 cm^{-1} compared to the FPO. This was due to the generation of *trans*- isomers that contributed to conjugated *trans*- isomers caused by the exposure of UCO to harsh frying that advanced oxidation.

This observation was in agreement with the studies by Lim *et al.* and Poiana *et al.* [10, 19]. Nevertheless, we also used the UV-Vis spectroscopy as an exceptional alternative to the FTIR spectroscopy in detecting the presence of primary (232 nm) and secondary (270 nm) oxidation products, which will be discussed later.

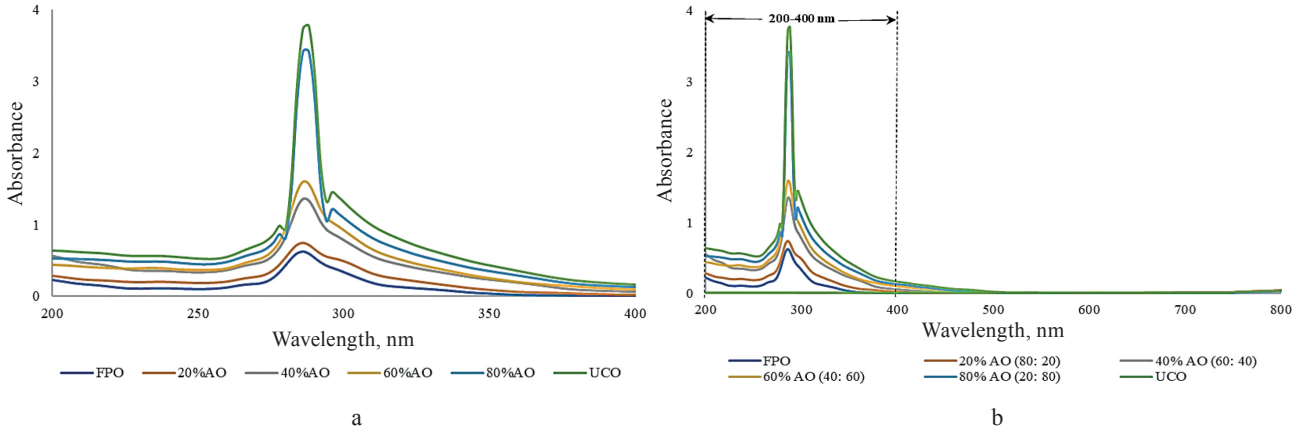
Detection and quantification of FPO adulteration using UV-Vis spectrophotometry. The UV-Vis spectrophotometry is a simple analytical method to detect and quantify oil adulteration incidence. This method evaluates the authenticity of oils by measuring absorption bands between 200 and 400 nm [21]. The UV-Vis spectra from 200 to 400 nm are considered to be directly related to oil quality [20, 21].

Figure 3a illustrates a significant peak that we observed within this range, from 200 to 400 nm. This finding was consistent with the previous studies [20, 21], which detected oil adulteration incidence by observing the molecular absorption of UV-Vis spectra within the designated range.

Figure 3b shows an enlarged view of the UV-Vis spectra. We found that the maximum absorption at 232 and 270 nm was related to the presence of conjugated dienes and trienes, which served as the best indicator of oil quality. This is because conjugated dienes and trienes are substances that form at an advanced oxidation stage, indicative of degraded oil quality.

Oxidation products absorption at 232 and 270 nm (K_{232} and K_{270}). As mentioned above, the maximum absorption at 232 and 270 nm correlate with the presence of oxidation products that are exceptionally powerful in determining the adulteration incidence in oil. These absorptions are typically expressed as specific extinctions at 232 and 270 nm denoted by K_{232} and K_{270}, respectively [20].

Traditionally, the peroxide value and the anisidine value are often used together to measure the oxidative status of edible oils. They reflect the concentration of primary (hydroperoxides) and secondary (aldehyde



a b

Figure 3 (a) UV-Vis spectra of fresh palm olein (FPO), adulterated oil (AO), and used cooking oil (UCO) at 200–800 nm; (b) enlarged view of UV-Vis spectra of oil samples at 200–400 nm

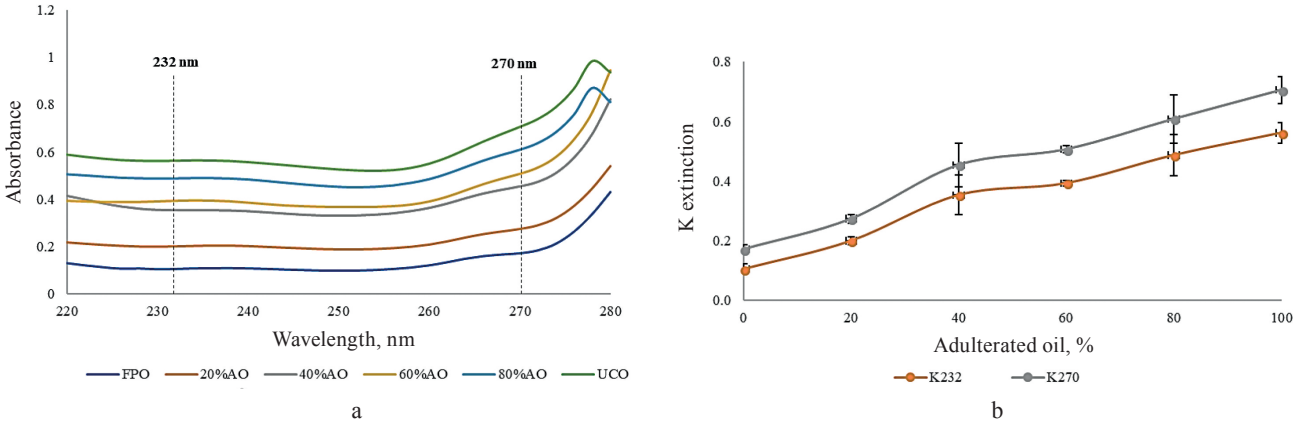


Figure 4 (a) Maximum absorption of fresh palm olein (FPO), used cooking oil (UCO), and adulterated oil (AO) with increasing adulterant concentrations at 232 and 270 nm, (b) Direct relationship between adulterant concentrations and UV absorbances at 232 and 270 nm

and ketones) oxidation products, respectively [44, 45]. Repeated frying accelerates the accumulation of oxidative products, thus contributing to higher peroxide and anisidine values indicative of deteriorated oil quality [46].

Xu *et al.* reported that palm olein exhibited a significant increment in the peroxide and anisidine values with increasing frying cycles [47]. Therefore, in counterfeit oil, their significant increase could be considered a result of quality degradation. Nevertheless, these chemical analyses are lengthy, expensive, and involve hazardous chemicals [48]. Thus, we preferred to use spectrophotometry to determine oxidative products in the oil samples.

Figure 4a shows maximum absorption of all the oil samples at 232 and 270 nm, while Fig. 4b shows an increasing trend of K_{232} and K_{270} values with adulterant concentrations. We found the absorbances at 270 nm to be significantly higher than those at 232 nm.

We found a linear relationship between adulterant concentrations and absorbances at 232 and 270 nm. The AO samples showed higher absorbances with increasing adulterant concentrations, while the FPO and the UCO samples had the lowest and highest values, respectively.

We also observed a tendency for all the samples to show much higher absorption at 270 nm, compared to 232 nm. This was due to the formation of relatively unstable hydroperoxides (primary oxidation products), which directly correlated with absorbance at 232 nm and could decrease in number over time [10]. They tended to decompose into more stable, complex forms of secondary oxidation products including aldehydes, ketones, and alcohols, which corresponded to the absorption at 270 nm.

This observation was in agreement with that reported by Lim *et al.*, Maskan and Bagci, and Jolayemi *et al.* [10, 29, 49]. This experimental finding also supported the application of specific absorbances in the ultraviolet region at 232 and 270 nm to detect adulteration. They can serve as an oil quality indicator through the measurement of primary and secondary oxidation indicative of oxidative deterioration [50]. This finding was consistent with that reported by Amereih *et al.*, where high absorbance at these particular wavelengths indicated oil adulteration [21]. Thus, high quality oil shows low absorbances at 232 and 270 nm and vice versa.

Effect of adulterated oil on the quality of fried chicken nuggets. In this study, chicken nuggets were fried in two sets of oil samples, FPO and 60% AO. Motivated by health concern, we only used 60% AO to simulate the adulterated palm cooking oil that was commonly found in the night market. Figure 5 compares the average scores of sensory attributes for the chicken nuggets fried in FPO (code 831) and 60% AO (code 524).

We observed no significant difference ($P > 0.05$) between the chicken nuggets fried in FPO and those fried in 60% AO in terms of sensory attributes including flavor, color, juiciness, taste, and overall acceptability. However, there was a significant difference ($P < 0.05$) in crispness. These observations concluded that adulterated oil with 60% UCO did not have a significant effect ($P > 0.05$) on the sensory perception of chicken nuggets

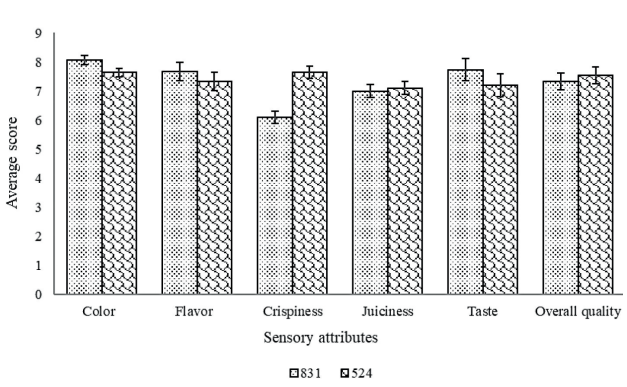


Figure 5 Comparison of sensory attributes for chicken nuggets fried in different sets of oil samples

fried in it, compared to FPO. Our findings were similar to those by Enriquez-Fernandez *et al*., who reported an insignificant difference ($P > 0.05$) between the foods fried in used oil and fresh oil in terms of sensory evaluation [51].

Color differences were insignificant ($P > 0.05$) between the nuggets fried in FPO and those fried in 60% AO, both having a golden brown color. However, we observed that the 60% AO-fried nuggets were cooked faster and therefore turned golden brown in a shorter time than those fried in FPO. 60% AO was much darker and intense in color compared to FPO. Thus, our study showed a negligible effect of frying oil on the color of chicken nuggets. This finding was in line with the results by Ahmad, but opposite to those reported by Li, who emphasized that the color of frying oil influenced the color of fried foods [52, 53].

Although taste differences were insignificant ($P > 0.05$) between the chicken nuggets fried in FPO and those fried in 60% AO, we observed an appreciable gap in the scores. Some panelists mentioned an unpleasant rancid taste of the samples coded 524, which were fried in 60% AO. This rancid taste became more obvious and intense over time. This observation was further enhanced by Okparanta *et al*., who reported that rancid oil led to abnormal rancid taste in fried foods [54].

However, there was a significant difference ($P < 0.05$) in crispiness, a desirable textural quality of fried foods. The chicken nuggets fried in 60% AO tended to be perceived with increased crispiness, compared to those fried in FPO. This observation might be due to a considerable time gap between the frying process of the samples and their sensory evaluation. The prolonged exposure to atmospheric air could have a noticeable influence on the sensory crispiness of both the FPO- and 60% AO-fried nuggets.

This finding was consistent with those by Antonova and Sung [55, 56]. In particular, Antonova reported a correlation between increased holding time under ambient conditions and decreased crispiness perceived by the panelists [55]. Holding time, which is defined as the minimum and maximum time after frying that a product can be used for sensory evaluation, should be determined for fried chicken nuggets to minimize variation in the test results. The previous studies suggested that breaded fried chicken nuggets should be served for sensory evaluation within 10 min after frying, under ambient conditions, to avoid variation in the test results [55]. Any longer than the suggested holding time can have an impact on the panelists' sensory perception.

However, it is worth noting that the chicken nuggets fried in adulterated oil with 60% used oil (7.53 ± 1.28) were found to be preferred in terms of overall quality, compared to those fried in fresh palm olein (7.33 ± 1.15). This finding can be supported by Bluementhal and Bordin *et al*., suggesting that the optimum quality of fried food can be achieved with moderately altered and reused frying oil, instead of fresh oil [57, 58]. This is because of the role of surfactant compounds in the frying process. These compounds accumulate in increasingly abused oils and facilitate the contact between foods and oil, thus contributing to better characteristics of fried food products.

CONCLUSION

In conclusion, our study showed the effects of adulteration with used cooking oil on both the oil properties and the quality of fried chicken nuggets. We observed higher FFA contents in the oils as adulterant concentrations increased. Pure UCO recorded the highest FFA value and reached the discard point set by legislation.

The chemical characterization of oil properties by using the FTIR spectral analyses determined some differences between FPO, UCO, and AO in terms of the exact position of band appearance and absorbance intensities. Significant aberrations in the FTIR spectra were observed at 3006, 2922, 2853, 2680, 1744, 1654, 987, 968, and 722 cm^{-1}.

The UV-Vis spectral analysis used absorbances at 232 and 270 nm (K_{232} and K_{270}, respectively) as an indicator of oil adulteration. We found a linear increasing relationship between the adulterant concentrations and the K extinction values, which enabled the detection and quantification of adulteration with UCO.

The sensory evaluation of the chicken nuggets fried in FPO and AO showed no significant effects of adulteration with UCO on their quality.

CONTRIBUTION

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

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Properties of serum albumin in electrolyzed water

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Abstract:
Introduction. Electrochemical activation of water controls the physicochemical parameters of aquatic food environment without any reagents. Electrolyzed water affects the properties of macronutrient solutions. The present research studied the effect of anodic and cathodic fractions of electrochemically activated water on protein molecules and their interaction patterns.
Study objects and methods. The study featured bovine serum albumin and its properties in electrochemically activated water with non-standard redox and acidity values. The aqueous solution of bovine serum albumin was studied by viscometry, UV spectrometry, time-of-flight secondary ion mass spectrometry, and electrophoresis.
Results and discussion. By knowing the interaction patterns of electrochemically activated water and protein molecules, food producers can control the properties of biological raw materials. Bovine serum albumin was studied in metastable fractions of electrochemically activated water obtained in the anode or cathode chamber of an electrochemical reactor. Both fractions of electrochemically activated water appeared to modify the properties of bovine serum albumin. The oxidized fraction of electrochemically activated water (anolyte) converted the protein solution into a more homogeneous molecular composition. The solution of bovine serum albumin in the reduced fraction of electrochemically activated water (catholyte) had an abnormally negative redox potential (–800 mV). The aqueous solution of bovine serum albumin in catholyte retained its initial viscosity for a long time, and its level was lower than in the control sample. This effect was consistent with other physicochemical characteristics of the solution.
Conclusion. The research revealed some patterns that make it possible to apply reagent-free viscosity regulation to protein media in the food industry.

Keywords: Electrochemical activation, water, bovine serum albumin, protein-containing food medium, viscosity, molecular mass spectrometry (ToF-SIMS)

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INTRODUCTION

Economical and environmentally friendly methods of food processing require novel technological solutions to maintain the high quality of finished products [1–3]. Food science helps understand the impact of innovative approaches on the properties of substances in the production chain, from raw materials and by-products to finished products and waste disposal issues [4–6].

The structure and properties of food ingredients depend on such physical conditions as temperature, pressure, stirring speed, etc., as well as on chemical interactions with other nutrients, e.g. water [7–9]. A targeted effect on the water base can develop the desired characteristics of the semi-finished or finished product [10, 11]. The food industry uses electrochemical activation as a relevant method of reagent-free control of physicochemical and rheological properties.

Electrochemical activation, or electrolysis, is a unipolar electrochemical processing of water or aqueous electrolyte solutions. It occurs in the anode or cathode chamber of a diaphragm or membrane electrochemical reactor [12, 13]. Electrolysis happens as a result of electrochemical and electrical processes in water in a double electric layer of electrodes with a non-equilibrium electric charge transfer. Water is treated with a constant electric current,

and electric potentials exceed its decomposition voltage (+1.25 V). As a result, water passes into a metastable state with non-standard electron activity, redox potential, and other physicochemical parameters. Electrochemically activated water is able to retain this metastable state for a long time and resists the thermodynamic equilibrium with the environment [12].

Metastable compounds with a high oxidizing (anolyte) or reducing (catholyte) ability undergo a series of spontaneous structural, energetic, and chemical transformations and gradually stabilize during storage. They are highly reactive to chemicals and biological objects. Metastable compounds enhance acidic and oxidizing properties of anolyte, as well as the alkaline and reducing properties of catholyte [14–17]. Electrochemical nonequilibrium leads to multiple changes in the reactivity of ions but does not affect their concentration. In electrochemically activated water, the pH values of catholyte and anolyte correspond to the equilibrium concentrations of alkali and acid that exceed the content of salts in this water. The redox values also go beyond the chemical control capabilities for a given electrical conductivity [12].

Electrochemically activated water and its solutions owe their chemical activity to electrically active microbubbles of electrolysis gases. These microbubbles are 0.2–5.0 μm in size, and their concentration can reach 10^6–10^7 mL^{-1}. They are stabilized by uncompensated electric charges at the interface of gas and liquid phases [12, 14, 18].

Electrochemically activated water and its solutions have non-standard physicochemical parameters of pH and redox potential, which makes them biologically active [13, 18, 19]. Electrochemically activated water solutions of both low and high molecular weight compounds differ from similar solutions of non-electrolyzed water [12, 16].

Electrochemically activated water and its solutions behave differently in technological processes. For instance, electrochemically activated water and ultrapure water are known to affect apricot protein extraction [20]. At the same pH = 9.5, electrochemically activated water had a better extraction efficiency than ultrapure water. Foaming ability and stability of the electrochemically activated water emulsions were 11.17% and 36.33 min, whereas in the ultrapure water samples they were 4.75% and 23.88 min, respectively. Electrochemically activated water had a more ordered secondary structure than ultrapure water. The ordered structures of α-helix and β-sheet were 7.5 and 60.2%, while the disordered structures and random turns were 8.4 and 23.8%, respectively. The extraction method increased the yield of the product, minimized the structural degradation, and improved the functional properties of apricot protein [20].

Electrochemical activation proved an effective means of extracting protein from canola meal [21]. Under the electric field, the cathode chamber produced an alkaline solution from a sodium chloride (NaCl) solution. The alkaline solution had better extractive properties compared to the samples subjected to chemical alkalization. The extracted proteins had a better extractability, composition, and secondary structure. The concentration of NaCl was 0.01–1 M, electroactivation time – 10–60 min, current – 0.2 and 0.3A. The experiment was conducted in a three-chamber cell separated by ion-exchange membranes.

The resulting solutions underwent an extraction procedure. The maximal protein extract of 34.32 ± 1.21% occurred when the electrolyzed solution was generated at 0.3A, regardless of the activation time. The standard extraction (pH 7–10) yielded 31.18 ± 1.89% proteins under the same conditions. The Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) showed that the electrophoretic profiles of electrolyzed protein concentrates and isolates differed from those obtained with the conventional method. The Fourier Transform Infrared Spectroscopy (FTIR) showed significant differences in the secondary structures of proteins depending on the pH and salt concentration. The electrochemically activated samples had a lower denaturation [21].

Electric field can change the properties of aqueous protein solutions due to their electrical conductivity and the chemical structure of polyampholytic polyelectrolytes. Their amino acid units have ionogenic side groups, and their acidic groups alternate with basic ones, which provides macromolecules with specific electric, configuration, and hydrodynamic properties [22]. Molecular conformation, volume, and rheology depend on the concentration of the polyelectrolyte in the solution, e.g. temperature, pressure, low molecular weight substances, pH value, etc. [23–27].

Animal blood proteins can serve as an example of such relationships. Serum proteins have a lot of beneficial nutritional properties, which makes them part of many food formulations. A globular molecule of bovine serum albumin consists of several hundred amino acid residues. Its three-dimensional structure is labile, mobile, and sensitive to exogenous factors [23, 26, 28]. A bovine serum albumin solution contains protein fragments of different dimensions. Its monomers and aggregates are in a state of dynamic equilibrium, and the weight of polypeptides increases as the albumin concentration in the solution rises [26].

The dissolution of crystalline albumin depends on the contact time of the phases: it can change its conformation, develop intermolecular bonds, or destroy them. The structure of albumin solutions and their surface properties depend on the pH of the solution and the pH value of the isoelectric point. The closer to the isoelectric point, the more turbid the solutions are and the lower their viscosity gets. This phenomenon can be explained by the minimal energy of electrostatic repulsion between the side chains of albumin molecules

and the molecules themselves. The resulting aggregates are denser, more compact, and larger in size. They have less effect on the flow and increase light scattering. During structuring, the turbidity and viscosity of the solutions change nonlinearly, depending on the protein concentration [24, 27].

The surface activity of albumin increases together with proton concentration. In an acidic environment, more non-polar groups emerge on the surface of the molecule than in a neutral or slightly alkaline environment. Obviously, the surface activity of albumin molecules is minimal at physiological pH values [27].

Denaturation and aggregation of serum protein isolates depend on the pH of the medium. This effect is widely used in food technology. When acidity pH drops to 1, it leads to the denaturation of bovine serum albumin with a conformational transition. This process is caused by the loss of the tertiary structure, which occurs as the polypeptide chain of the bovine serum albumin molecule unfolds and the aggregates increase in size [26].

A strong alkaline environment has a more pronounced texturing effect, e.g. 2N NaOH solution with a pH of 12.4 ± 0.4 or alkaline electrolyzed water with a pH of 11.5 ± 0.4. In an acidic environment, the effect is less pronounced, e.g. 2N HCl with a pH of 2.0 ± 0.2 or acidic electrolysis water with pH 2.5 ± 0.2 [29]. Albumin is a polyelectrolyte with a high conformational mobility. In an electrochemically activated solution, it should be sensitive both to the acidity of the solution and its redox potential. The present research objective was to study the effect of electrochemically activated water on the properties of serum albumin in protein solutions.

STUDY OBJECTS AND METHODS

Sample preparation. The research featured bovine serum albumin BSA 100 (Merck, Sigma-Aldrich). Preparations with casein proteins and instant food gelatin were used as control (Dr. Oetker, OOO Oetker, Russia, TU 20.59.60-011-42450906-2018).

The research involved UV spectrometry, time-of-flight secondary ion mass spectrometry (ToF-SIMS), and electrophoresis of an 1% protein aqueous solution, which was then diluted with water or electrochemically activated water at a ratio of 1:4. Fractions of electrolyzed water, catholyte (pH 8.2, redox –800 mV), and anolyte (pH 2.2, redox +800 mV) were obtained in a fresh drinking water purification unit by means of direct electrochemical action in diaphragm modular electrochemical cells (LLC Delfin Aqua, Russia). Artesian water (pH 7.2, redox +360 mV) from the city water supply served as control. In the viscosity test, electrolyzed water with a negative redox value was obtained using an Izumrud-K1 installation (NPO Ekran OJSC, Russia). Tap water passed through a number of stages:

1. Anode chamber of a flow-type electrochemical module. Here the water was disinfected due to peroxide and chlorine-oxygen compounds, then saturated with oxygen and ozone to kill microorganisms and oxidize organic impurities.

2. Reaction-flotation reactor. It removed coagulated products of anodic treatment from electrolytically obtained microbubbles of oxygen and ozone.

3. Heterophase catalytic reactor. The procedure removed active chlorine compounds and produced active oxygen compounds.

4. Cathode chamber. Here the residual ions of iron, copper, magnesium, etc. were converted into insoluble hydroxides, which were then removed in the flotation and electrokinetic reactors. During the cathodic treatment, molecular hydrogen and free hydroxyl groups entered the water and gave it a negative redox value and antioxidant properties.

The electrochemically activated water had pH 7.3 and redox –223 mV, while for the initial water these values were 7.3 and +190 mV, respectively. The acidity (pH) and redox potential of the solution were measured using a SevenExellence S470 multivariable device (METTLER TOLEDO, Switzerland) with a pH electrode (Inlab Routine Pro, Mettler Toledo, Switzerland) and a redox electrode (Inlab Redox Pro, Mettler Toledo, Switzerland). Depending on the concentration of bovine serum albumin, its pH value ranged 7.2–7.6 for electrolyzed water and 7.9–8.6 for tap water.

UV spectrometry. Spectrometry was used to define the effect of electrolyzed water on aqueous protein solutions. Its optical density was recorded in the absorption spectrum of the sample in the ultraviolet region at a wavelength of 235 and 280 nm using a Shimadzu UV-2401PC spectrophotometer (Japan).

Time-of-flight secondary ion mass spectrometry (ToF-SIMS). To obtain samples for mass spectrometry, 2 μL of bovine serum albumin solution in water or water fractions were applied to a clean glass substrate. Its surface was covered with a conductive ITO-indium tin oxide film (Sigma-Aldrich). After drying in an stream, the sample was transferred to the chamber of a ToF-SIMS 5 secondary ion mass spectrometer (ION-ToF GmbH, Germany). The preparation was ionized with a 200 nm beam of primary Bi^{3+} ions at 30 keV. After 70 ns of exposure, secondary ions were registered (~ 80 μs) and the beam moved to the next point. The primary ion irradiation did not exceed 5×10^{12} ions/cm^2. The principal component analysis helped to assess the differences between the obtained mass spectra [30, 31].

Measuring the kinematic viscosity of protein solutions. This parameter was measured using an Ostwald VPZh-4m capillary viscometer (VPZh-4m viscometer, LABTECH LLC, Russia) at 20°C. The viscosity value was calculated as follows:

$$V = \frac{(g*K*t)}{9.8} \quad (1)$$

where V is the kinematic viscosity of the liquid, mm^2/s; K is the constant of the viscometer, mm^2/s^2; t is the flow time, sec; g is the gravity acceleration, 9.8 m/s^2.

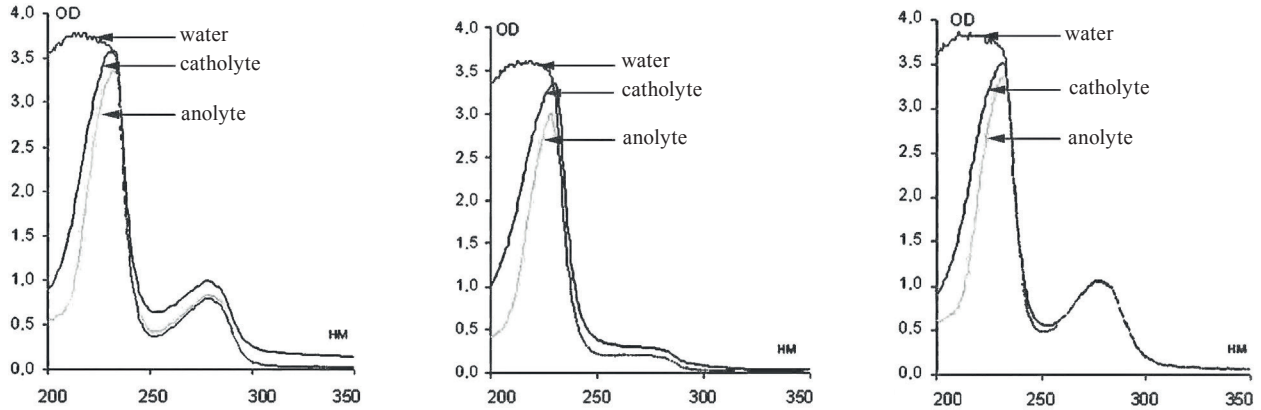


Figure 1 Nonspecific UV absorption spectra of 0.20% solutions of bovine serum albumin (a), food gelatin (b), and casein (c) in water or electrochemically activated water fractions (catholyte, anolyte)

The protein content in the diluted solution was 0.01, 0.05, 0.1, and 0.2%; in the concentrated solution – 1, 3, 5, and 10%.

Protein electrophoresis. This parameter was measured using standard methods and the following ingredients. Acrylamide 8% separating gel contained 0.375 M Tris HCl (pH 8.8), 0.1% PSA (ammonium persulfate 0, 0.1% DS-Na (Dodecylsulfat Na-salz), and 0.01% TEMED (tetramethylethylenediamine). Acrylamide 5% focusing gel included 0.125 M Tris HCl (pH 6.8), 0.1% PSA, 0.1% DS-Na, and 0.01% TEMED. The electrode buffer included 0.025 M Tris HCl (pH 8.3) and 0.19 M glycine.

The protein was diluted in buffer: 2% DS-Na, 10% glycerin, 5% 2-mercaptoethanol, 0.004% bromophenol blue, 0.063 M Tris HCl, pH 6.8. After that, it was boiled in a 100% water bath for 5 min. The solution was applied to the gel, where electrophoresis was carried out at 20 mA for 2 h. The resulting preparation was stained with Cumassi R 250.

RESULTS AND DISCUSSION

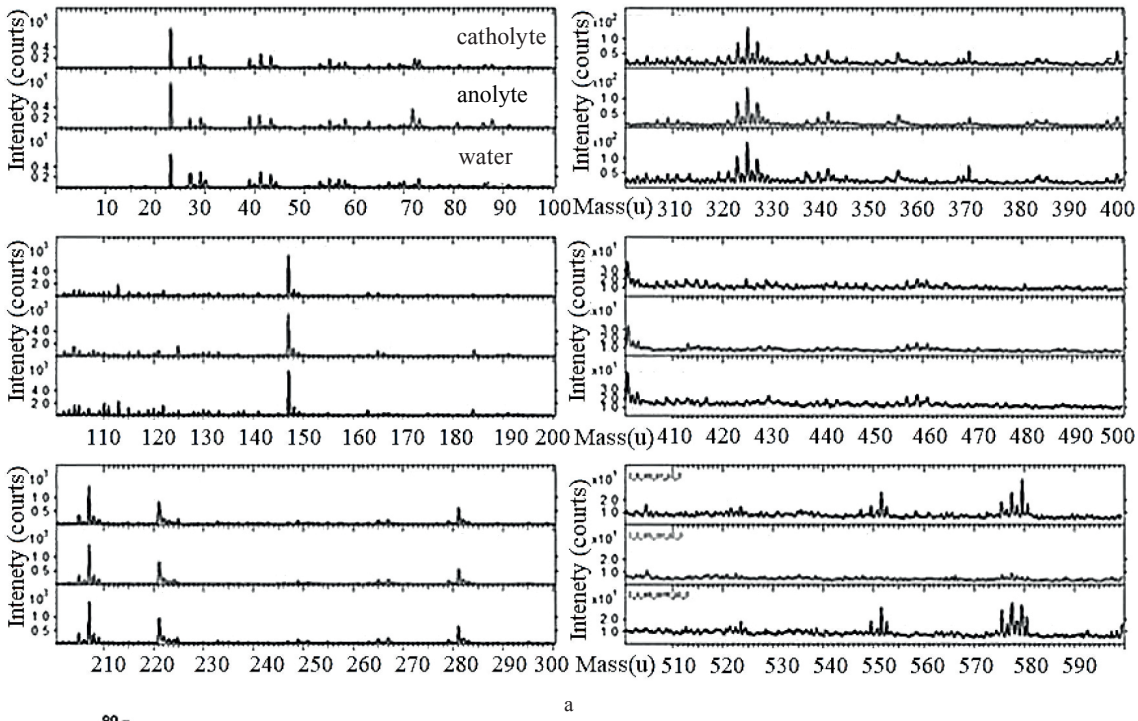
UV spectrometry. The method of UV spectrometry of aqueous solutions was used to study the effect of electrochemically activated water on protein. The UV spectrum was not specific for biomolecule solutions, but it made it possible to perform a comparative analysis of integral changes in the sample. The UV spectrometry test featured bovine serum albumin, food gelatin, and casein (Fig. 1).

Figure 1 demonstrates that the obtained absorption spectra were identical for all the proteins in the experiment. Unlike the conventional water solutions, the solutions of electrochemically activated water fractions had a lower optical density, and their absorption peak was in 235–280 nm. All the samples of electrochemically activated water had a slightly higher absorption level of the protein solution in catholyte. These changes were more obvious in the solution of biochemically pure albumin (Fig. 1a) than in the samples of food gelatin (Fig. 1b) and casein (Fig. 1c). The UV spectrometry demonstrated the modification of the protein in the solution of electrolyzed water fractions. The time-of-flight molecular mass spectrometry (ToF-SIMS) provided additional data on the state of albumin in the solutions.

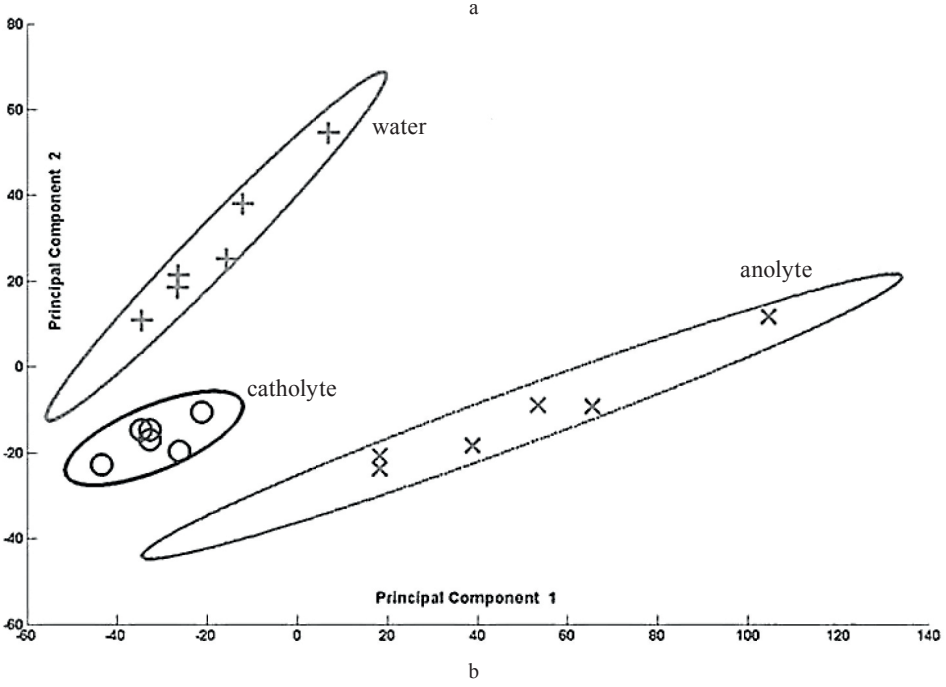
Time-of-flight secondary ion mass spectrometr (ToF-SIMS). This method was used to perform the molecular analysis of protein samples. A droplet of each solution was dried on a cover glass in a stream of clean air. ToF-SIMS provided information about chemical composition, molecular orientation, surface order, chemical bonding, and purity. Mass spectra of each preparation were compared using various data classification techniques. The principal component analysis is one of the most popular techniques used in mass spectrometry. It features the most intense peaks in mass spectra and provides a 95% confidence interval [30, 31].

Briefly, the program received 20 principal components: the higher the component number, the more variation in the data it reflected. Such a number of coordinates was unnecessary, so the space of the first two components was used to analyze the similarity of the samples. All the samples of biological macromolecules in this research underwent the same ToF-SIMS preparation procedure and the same principal component analysis. Figure 2 shows the results of the molecular mass spectrometry.

Figure 2a illustrates a typical mass spectrum of bovine serum albumin dissolved in water or electrolyzed water fractions. Figure 2b clearly demonstrates a significant difference between all three samples. The catholyte-treated protein showed significant heterogeneity compared to the control and especially the anolyte-treated sample. Anolyte treatment had a focusing effect on the protein samples, if compared to the heterogeneous group of samples obtained from the albumin solution in catholyte or water. The change in the mass spectrum may be due to the development of new



a



b

Figure 2 ToF-SIMS analysis of bovine serum albumin samples in conventional water (control) and electrochemically activated fractions (anolyte, catholyte): (a) ranges of molecular weights: bottom mass spectrum – control (water), medium – after catholyte treatment, upper – after anolyte treatment; (b) ellipses – 95% confidence areas for n = 6 measurements for each group

peaks and/or a change in the intensity of similar peaks. The results of molecular analysis statistically confirmed the UV spectrometry data (Fig. 1a): electrochemically activated water fractions really modified the albumin. The total change in the physicochemical properties of protein could change in the rheological properties (viscosity) of the solution.

Kinematic viscosity of bovine serum albumin. The viscosity of aqueous solutions of hydrocolloids is an important characteristic of food systems. For instance, the viscosity of protein solutions is one of the most serious problems when highly concentrated protein formulations or milk powder. The viscosity of food systems is controlled both by physical methods and by additives. A small amount of such low molecular weight additives as salt reduces the viscosity that results from electrostatic repulsion and attraction. Arginine hydrochloride (ArgHCl) is known to act as a chaotropic agent. It destroys the network of hydrogen bonds between water molecules, thus suppressing hydrophobic

attraction and clustering, which can reduce the viscosity of the solution [32].

Hydrodynamic cavitation served as a technological tool to reduce the viscosity of serum protein concentrate before spray drying. Whey protein concentrate (31% dry matter) underwent various hydrodynamic cavitation treatments. The samples were tested for viscosity during 14 days of storage. The enthalpy of denaturation was estimated using differential scanning calorimetry, while the particle size was measured using dynamic light scattering. The hydrodynamic cavitation treatment appeared to reduce the viscosity by 7–8%, and this effect remained constant for 14 days of storage. According to the particle size distribution, the destruction of aggregates decreased the number of large particles and thus caused the drop in viscosity [33].

The viscosity of protein solution depends not only on the size, but also on the shape, morphology, and structure of the particles. For instance, flow behavior of partially denatured serum protein aggregates showed a complex dependence on the microstructural morphology of particles, their concentration, and shear rate [24]. Even though the protein content in the solution was the same, particles with an open fibrillar/tubular structure had a higher viscosity than compact aggregates. Rough and uneven particles appeared to form solutions of higher viscosity than smooth particles of the same size. Serum proteins of various sizes and denaturation degrees produced solutions of different viscosity, probably, as a result of interactions between protein aggregates. Partial denaturation technology could control the structure of serum protein aggregates to achieve specific viscosity characteristics [24].

Protein is a biopolymer. Hence, the viscosity of its solution depends not only on its properties and concentration, but also on the solvent. Temperature, pH, impurities, and dissolved gases affect the viscosity of solutions. For example, negative redox potential can affect the quality and interaction efficiency of dissolved macromolecules [34, 35].

This part of the experiment featured electrolyzed water with a standard acidity value (pH ~ 7.3) but extremely low redox potential (–223 mV) at the starting point. The redox potential value of tap water in the control sample remained constant (+190 mV) at 20°C during 24 h. However, the redox potential value of electrolyzed water (–223 mV) gradually increased as the metastable state relaxed. The highest relaxation rate occurred in the first 6 h after treatment, and then the process slowed down. After 24 h, the redox potential reached +69 mV, which was much lower than in the control sample. As the concentration of albumin kept growing from 0.01 to 0.2%, the relaxation rate increased gradually. After bovine serum albumin dissolved, the redox potential index increased from –194 to –162 mV. When the albumin concentration reached 1–3%, the effect intensified abruptly and reached plateau at –90 mV. Adding bovine serum albumin speeded up the recovery of the redox potential of the electrolyzed water. The kinetics of the process depended on the concentration. After 24 h, the maximal value was +125 mV at 0.2% of bovine serum albumin, which was much lower than the redox potential of the control sample (+190 mV).

The results clearly showed the dependence of the redox potential of electrochemically activated water solutions on albumin concentration. Such interactions may affect the kinematic viscosity of the solution: the molecular conformation change and/or intermolecular bonds are distorted. The structure of water in the electrochemical reactor changes, thus resulting in a negative redox potential of the water. These changes can also affect the behavior of macromolecules, i.e. solubility, interaction, conformation, repulsion or attraction, as hydrogen, hydrophobic, or electrostatic non-covalent bonds get stronger or weaker [36, 37].

Figure 3 shows the changes in the kinematic viscosity of the solutions of tap water and electrolyzed water at different concentrations of bovine serum albumin from 1 to 10%.

Electrochemical treatment of tap water decreased the viscosity of the solution at all the concentrations of bovine serum albumin (Fig. 3). The tap water solution became more viscous over time, while the electrolyzed water with the same concentration of albumin remained almost the same. The viscosity of the solution usually

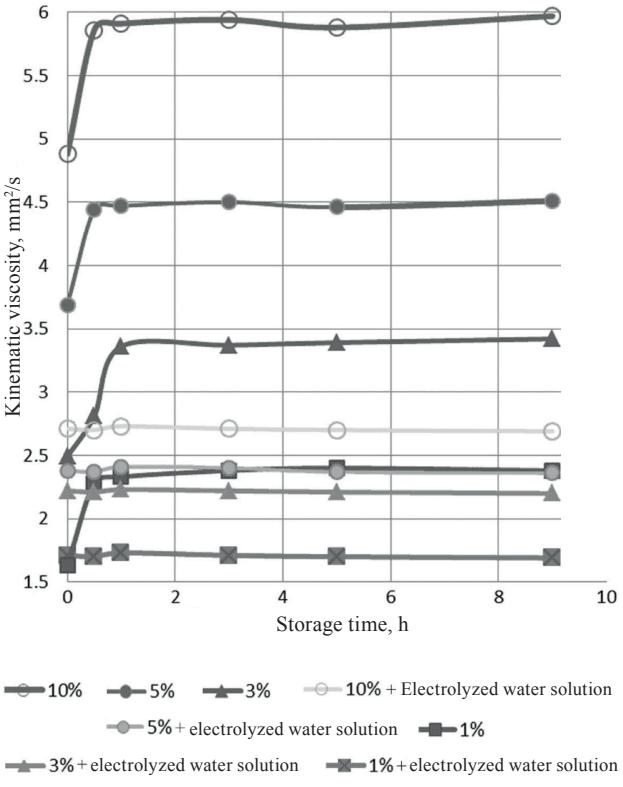


Figure 3 Kinematic viscosity of water solution vs. electrochemically activated water solution at different concentrations of bovine serum albumin, %

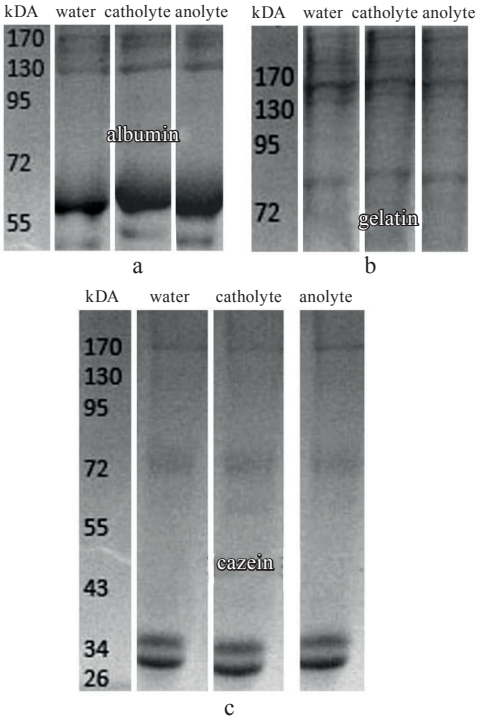


Figure 4 Gel electrophoresis of protein solutions in water or in the fraction of electrochemically activated fractions (catholyte, anolyte): (a) albumin, (b) gelatin, (c) casein

increases together with the increase in the protein content, but electrolyzed water reduced the initial value of this parameter, especially in the 10% solutions of bovine serum albumin. This effect might have resulted from the increased electrostatic repulsion between albumin molecules. At an isoelectric point of pl 4.9 and pH 7.3–8.6, the total charge of the protein became negative due to additional dissociation of carboxyl groups caused by albumin molecular conformation.

Both the protein monoproduct sample (albumin) and the food protein composition samples (gelatin and casein) changed when dissolved in the electrolyzed water fractions. The observed effects might be a consequence of changes in the structure of the protein and/or its fragmentation. Figure 4 shows the results of gel electrophoresis.

Protein electrophoreses differed (Fig. 4), but the solutions of the same protein in water or electrolyzed water fractions showed no significant differences. A high performance liquid chromatography (HPLC) confirmed this observation. The data of gel electrophoresis differed from the results of molecular analysis (ToF-SIMS) because these two methods are based on different physical principles. Electrophoresis features proteins and their large fragments in an electric field while mass spectrometry registers amino acid ions and small peptides. The decrease in molecular weight under the action of electrolyzed water was insignificant, but it could still affect the peptide structure both in the oxidized (pH 2.2, redox +800 mV) and reduced (pH 8.2, redox –800 mV) fractions of electrolyzed water. When proteins dissolved in the anodic and cathodic fractions of purified drinking water, they got neither fragmented nor structurally changed.

The effect of the anodic and cathodic fractions of electrolyzed water on the properties of serum albumin confirmed the prospects of the targeted use of electrochemical activation in the food industry as a means of condition monitoring.

The research results correlated with other studies that state the importance of water for reagent-free control of protein quality in the food industry. According to [38], polyphosphate (50%) can be partially replaced with alkaline electrolyzed water (1.25 g/L sodium tripolyphosphate, 0.3 g/L sodium metapolyphosphate, 0.4 g/L sodium polyphosphate, pH = 11.4). The replacement improved the quality of catfish fillet: its weight and water retention capacity increased. A higher phosphate content had a similar result (2.5 g/L sodium tripolyphosphate, 0.6 g/L sodium metapolyphosphate, 0.8 g/L sodium polyphosphate, pH = 9.0). However, the experiment established no change in hardness and elasticity. The test samples improved in color and oxidation resistance, though [38].

Electrochemical activation proved to be an effective sustainable technology to produce acidic and alkaline (anolyte and catholyte) extraction solutions that could replace hydrochloric acid and sodium hydroxide. For example, a combination of electrolyzed water and ultrasonic treatment improved the efficiency of extracting proteins from sea krill [39]. Unlike a similar combined method with deionized water, the electrolyzed water method reduced NaOH consumption by 30.9% w/w. Electrochemically activated water with a negative redox potential –(800–900 mV) showed good antioxidant properties, which protected the active groups of proteins (carbonyl, sulfhydryl) from oxidation. Ultrasonic treatment provided an additional increase in the extraction yield, raised the solubility, reduced the particle size, changed the structure, and improved the functional properties of krill proteins, e.g. emulsifying and foaming capacity, foam stability, etc. [39].

A combination of electrochemically activated water, isoelectric precipitation, as well as isoelectronic precipitation and electrochemically activated water treatment (IP-EWT) provided a high recovery rate (≥ 50%) of protein concentrate from heat stabilized defatted rice bran [40]. The protein fraction contained 65.1 wt% protein and had a high amino acid value (76.6%). A Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis and an immunoblotting analysis showed no signs of allergenic rice protein or heavy metals in the protein fractions. The combined IP-EWT process was environmentally friendly. It yielded highly concentrated and safe protein from plant materials without enzymes or chemicals, e.g. organic solvents, buffering agents, surfactants, etc.

Electrochemically activated water proved effective in the extraction of dry material from soybean meal [41]. Solutions of anolyte and catholyte had a

high ability to extract proteins, carbohydrates, and especially minerals. The extracted proteins had a well-balanced amino acid composition, which meant they could serve as ingredients in various functional foods. Electrochemically activated water gives the food industry an important alternative to chemical reagents. In future, it can become an effective tool for functional modification of proteins [41, 42].

CONCLUSION

The present research involved viscometry, UV spectrometry, time-of-flight mass spectrometry of secondary ions, and electrophoresis of bovine serum albumin. All the methods confirmed a multifaceted effect of the anodic and cathodic fractions of electrochemically activated water on the structure and properties of protein in aqueous solutions. The protein monoproduct (serum albumin) was subject to modification when interacting with fractions of electrolyzed water. The oxidized fraction of electrochemically activated water (anolyte) made the protein solution more homogeneous in terms of molecular composition. The research registered a significant unified effect of anolyte with a high concentration of hydrogen peroxide on the disulfide bond of amino acid residues, e.g. cysteine.

However, the mechanism of action of the reduced fraction of electrochemically activated water (catholyte) still remains unclear. The catholyte has a pronounced antioxidant activity, but the activity of antioxidants in biological systems can be studied only by the compensation of oxidative stress to the normal level of the redox potential of the medium (~ 360 mV). The catholyte-based solutions of bovine serum albumin had an abnormally negative potential (–800 mV), which was not induced under physiological conditions or pathological changes. Unlike the control samples, the experimental samples with electrochemically activated water retained the initial viscosity for a long time. Their viscosity was lower than that of the protein solution in non-electrochemically activated water. This effect was consistent with other physicochemical changes.

The obtained patterns revealed good prospects for reagent-free control of protein food media in technological processes. Less food additives and technological aids during processing means the possibility to modify the functional properties of protein food ingredients, e.g. texturing isolates. Electrochemically activated water can serve as a water base for liquid protein-fortified products. The method helps maintain the desirable viscosity, consistency, and sensory properties of functional foods.

CONTRIBUTION

A.G. Pogorelov supervised the project and proofread the final manuscript. L.G. Ipatova wrote and improved the manuscript. M.A. Pogorelova obtained and analyzed the data. A.L. Kuznetsov interpreted the data. O.A. Suvorov designed the research concept.

CONFLICT OF INTEREST

The authors claim there is no conflict or interests regarding the publication of this article.

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Enzyme complexes for activating yeast generation and ethanol fermentation

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Abstract:
Introduction. Recent studies have shown the benefits of phytolytic enzymes to prepare grain wort in ethanol production. However, there is a lack of data on the effect of phytases and their amount on the conversion of grain polymers, the ionic composition of wort and mash, and the efficiency of yeast generation and ethanol fermentation.
Study objects and methods. Wheat and corn wort samples were treated with a complex of hydrolases, including phytases. Capillary electrophoresis determined the ionic composition of wort and mash. Gas chromatography measured the content of volatile metabolites.
Results and discussion. The key enzymes were phytases and proteases. They improved the conversion of grain polymers and stimulated the growth and metabolism of yeast cells. Their synergism enriched the wort with assimilable nitrogen, phosphorus, and other valuable minerals. In addition, it intensified the growth of the *Saccharomyces cerevisiae* yeast, increased the rate of carbohydrate consumption, and reduced the formation of side metabolites 1.7–1.9 times, mainly due to higher and aromatic alcohols. The concentration of phosphates remained practically unchanged during the fermentation of grain wort treated with phytases. However, by the end of fermentation, it was 2.4–5.1 times higher than in the mash samples without phytolytic treatment. Finally, we identified a complex of enzymes and optimal amounts of phytases that have a stimulating effect on ethanol fermentation.
Conclusion. Phytases, whether used individually or together with proteases, enriched grain wort with soluble macro- and microelements, improved yeast metabolism, directed ethanol synthesis, and decreased the formation of fermentation by-products.

Keywords: Wort, phytase, protease, mash, yeast, metabolism, ethanol fermentation

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INTRODUCTION

Recent studies have proven the effectiveness of mechanical and enzymatic treatment of grains (at temperatures under 100°C) with complex enzyme preparations in ethanol production [1–4]. This "soft" technology for grain wort preparation can significantly reduce heat and power consumption and increase profitability. It is based on controlled biocatalytic conversion of grain polymers (starch, xylans, β-glucans, and proteins) with the formation of easily digestible carbohydrates and nitrogenous substances that yeast cells need for normal metabolism [2, 4–7].

However, the studies hardly took into account the presence of phytic acid and its salts (phytates) in grains that contain up to 80% of phosphorus in a bound state [8–10]. The bioavailability of phosphorus can be increased by using phytolytic enzymes. Grains contain enzymes that catalyze the destruction of intracellular polymers. Normally in a latent state, these enzymes are activated during germination. Studies show that the amount of phytolytic enzymes in grain is insufficient for the complete release of phosphorus [11, 12]. Therefore, researchers have recently focused on obtaining enzyme preparations – sources of phytases – based on microorganisms. Using genetic engineering, they have developed highly productive recombinant strains of fungi, yeasts, and bacteria that synthesize phytolytic enzymes [13–15].

Phytolytic enzymes of microbial origin have been widely studied, namely their use in fodder production for the release of phytate phosphorus. The biocatalytic conversion of plant phytates has been shown to improve the digestibility of nutrients in fodder and stimulate the growth of farm animals and poultry [16–18]. Much attention has been paid to the use of phytases in the food industry to increase the digestibility and bioavailability of food components [19–21]. However, there is a lack of data concerning the catalytic action of microbial phytases on grain polymers in fermentation.

Previous studies have shown a positive effect of phytases on the growth and development of yeast cells cultivated on grain media, including beer production from sorghum [22–24]. Some authors who studied the effect of phytolytic enzymes reported better rheological properties of rye and triticale wort, as well as improved fermentation [6, 25, 26].

However, there is a lack of research into their effect on the biochemical parameters of grain wort and mash, especially those from wheat and corn [2, 23]. What needs studying is changes in the ionic composition of grain wort depending on the substrate specificity of enzyme systems used for its preparation, as well as the assimilation of the released phytate phosphorus by the *Saccharomyces cerevisiae* yeast during ethanol fermentation [1–3, 22–25]. Hardly studied is the effect of phytases on the hydrolytic capacity of enzymes with substrate specificity in relation to starch and proteins, on the conversion of these polymers in grain wort preparation, and on the efficiency of yeast generation and ethanol fermentation.

Thus, literature analysis revealed a lack of studies into the potential of phytic substances with bound phosphorus for the conversion of grain to ethanol. A number of papers reported that phytates – strong chelating agents – bind not only phosphorus, but also metal cations (calcium, magnesium, manganese, zinc, iron, etc.) [19, 21, 27–29]. Low bioavailability of macro- and microelements in grains can have a negative effect on the supply of yeast with minerals and on the catalytic activity of some metal-dependent enzymes.

The *S. cerevisiae* yeast is a key factor in the efficiency of ethanol fermentation. The yield and quality of ethanol, as well as the process duration, largely depend on the fermentation activity and yeast productivity. The metabolism of *S. cerevisiae* is significantly affected not only by the strains' genetic characteristics, but also by the conditions of their cultivation (nutrient medium with easily assimilated nutrition) [2, 30–32].

Metabolism involves all enzymatic reactions that occur in the cell to regulate the composition and synthesis of target and secondary metabolites. Therefore, there is a need for research to select effective enzyme systems that contribute to deep destruction of grain polymers. It is especially true of phytolytic enzymes. Recent studies have revealed that phytic acid forms stable complexes with carbohydrates and proteins [33–35]. Apparently, this can reduce the hydrolytic action of carbohydrases and proteases on carbohydrate and peptide polymers. Phosphate groups of phytic acid bind to basic amino acids (arginine, histidine, and lysine) and form strong protein-phytate complexes.

However, the studies of phytate-carbohydrate complexes have ambiguous results. They show that the interaction occurs either through direct binding to starch or indirectly, through starch-associated proteins [34–37]. Therefore, to ensure a steadily high yield of ethanol, it is important to select optimal parameters for preparing grain media to produce high-quality wort [1–3].

In connection with the above, we can assume that the biocatalytic destruction of phytic substances will contribute to the release of phosphorus and other valuable microelements. It will also stimulate the conversion of carbohydrate and protein polymers of grain through the use of hydrolases with substrate specificity in relation to the main polymers of grain.

We aimed to study the effect of hydrolytic enzymes with proteases and phytases on the efficiency of yeast generation and metabolism during the fermentation of wheat and corn wort.

STUDY OBJECTS AND METHODS

Our study objects included wheat and corn wort prepared with enzyme preparations that served as sources of hydrolases with different substrate specificity and action. They were used for:

– dextrinization and saccharification of starch α-amylase (EC 3.2.1.1.) and glucoamylase (EC 3.2.1.3.);
– destruction of xylanase non-starch polysaccharides (EC 3.2.1.8, 3.2.1.32, 3.2.1.37, 3.2.1.72);
– hydrolysis of protein substances of the protease complex (EC 3.4.11-3.4.15, EC 3.4.21-3.4.24); and
– hydrolysis of phytase substances (EC 3.1.3.8).

Yeast, organic acids, and dietary supplements were obtained from the Biotechnology Department. Ultraconcentrates of culture liquids were used as enzyme preparations to produce thermostable α-amylase (*Bacillus licheniformis* sp., Amilolikheterm), glucoamylase and xylanase (VKM F-4278D, a recombinant strain of *Aspergillus awamori*, Glucavamorin-Xyl), a protease complex (VKPM F-931, a mutant strain of *Aspergillus oryzae*, Protoorizin), and phytase (Phytaflow, Novozymes, Denmark) [38–40].

Enzyme activity was determined by the existing methods. A unit of amylolytic activity (AA) was defined as an amount of enzyme that catalyzes the hydrolysis of 1 g of soluble starch to dextrins of various molecular weights under standard conditions (30°C, pH 6.0, 10 min). A unit of glucoamylase activity (GA) was an amount of enzyme that is capable of catalyzing starch hydrolysis at 30°C (pH 4.7) and releasing 1 μmol of glucose per minute. A unit of xylanase activity (XA) was an amount of enzyme that acts on xylan from birch wood and releases 1 μmol of reducing sugars (in glucose equivalent) per minute under standard conditions

Table 1 Biochemical composition of wheat and corn

Components	Content, %	
	Wheat	Corn
Proteins	13.9 ± 0.5	10.2 ± 0.4
Mono- and disaccharides	3.0 ± 0.1	4.1 ± 0.2
Starch	57.4 ± 2.6	65.8 ± 3.2
Hemicellulose	4.4 ± 0.2	3.0 ± 0.1
Cellulose	2.9 ± 0.1	3.3 ± 0.1
Phytates	1.30 ± 0.05	2.30 ± 0.09

Values are expressed as means ± SD

(50°C, pH 5.0). A unit of total proteolytic activity (PA) was an amount of enzyme that brings hemoglobin into a TCA non-precipitated state equivalent to 1 μmol of tyrosine per minute (30°C, pH 5.3). A unit of phytase activity (PhA) was an amount of enzyme that catalyzes the hydrolysis of sodium phytate to produce 1 μmol of inorganic phosphate per minute under standard conditions (37°C, pH 5.5, 15 min).

Grain wort was prepared by the enzymatic-hydrolytic processing of grain (40–90°C, hydromodule 1:3). For this, 50 g of grain flour and 150 cm^3 of water were mixed in 750-cm^3 Erlenmeyer flasks and regularly stirred in a PE-4300 water bath (Ekros, Russia).

The water-grain mixture was prepared for 2 h (40–90°C) using a thermostable *α*-amylase (0.6 AA units/g starch) to liquefy starch. When the mixture was cooled to 60°C (56–60°C, 1 h), EPs with glucoamylase (9.0 GA units/g starch) and xylanase (0.4 XA units/g grain) activity were used to saccharificate starch and hydrolyze non-starch polysaccharides. They served as a control. The experimental samples contained EPs with phytase (1.0–3.0 PhA units/g grain) and proteolytic activity (0.1 PA units/ g grain), in addition to those with amylolytic, glucoamylase, and xylanase activity.

Wort was fermented using a selected race of the *Saccharomyces cerevisiae* 985-T yeast with thermo-tolerant and osmophilic properties (34–35°C, 68 h) [41].

Methods. The Instructions for the Technochemical Control of Alcohol Production were followed to determine the contents of starch, protein, and non-starch polymers of grain, the concentrations of yeast cells and reducing carbohydrates in the grain mash, as well as ethanol concentration and yield [42]. The concentration of amine nitrogen in the grain wort was measured by iodometric titration (Pharmaceutical Regulations 1.2.3.0022.15). The ionic composition of wheat and corn wort, as well as mash, was determined using a PrinCE-560 capillary electrophoresis system (Netherlands) equipped with a conductometric detector. The composition and content of volatile metabolites synthesized by yeast were measured using an HP Agilent 6850 gas chromatograph (USA).

Data obtained in triplicate were statistically processed in Microsoft Excel using the Student's coefficient with a 0.95 confidence interval.

RESULTS AND DISCUSSION

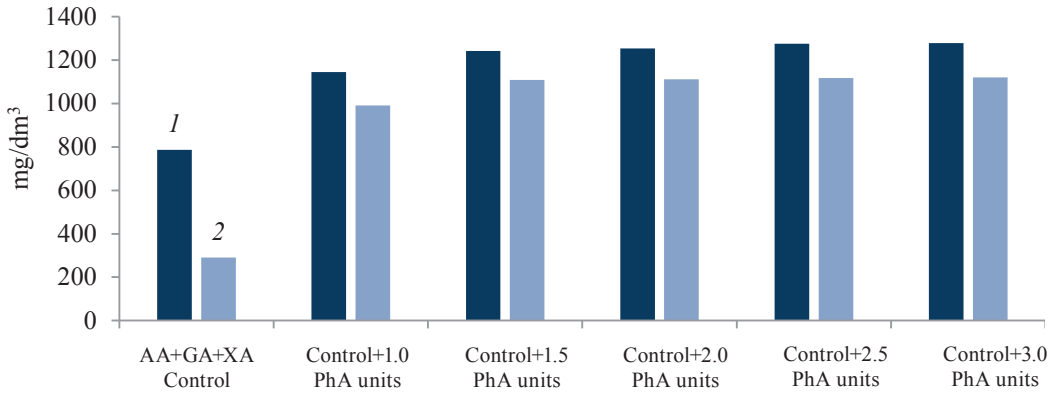
Ground wheat and corn were used as a substrate to prepare wort. Table 1 compares the compositions of the main polymers contained in the grains. According to their caryopsis composition, wheat and corn, like all grains, are classified as multicomponent starchy plant raw materials, in which starch is the main polymer that determines ethanol yield [10, 25, 42].

Corn had a higher content of starch (65.8%), while wheat was richer in protein (13.9%). In addition, the grains under study contained non-starch polysaccharides (hemicelluloses, cellulose), protein substances, and phytates (Table 1). This multicomponent composition of grain polymers determined the selection of enzyme preparations with a given substrate specificity to prepare grain wort (Table 2). Amilolicheterm (thermostable *α*-amylase, 330 AA units/g) was used to liquefy and dextrinize starch. Glucavamorin-Xyl (glucoamylase, 7700 GA units/g; xylanase, 350 XA units/g) was used to saccharify starch and hydrolyze non-starch polysaccharides. Protoorizin (protease, 580 PA units/g) was used for protein proteolysis and Phytaflow (30 000 PhA units/g) was selected to convert phytic substances.

Protoorizin contained at least five proteolytic enzymes that differ in action [39]. In previous studies, the authors used neutral protease or Glucavamorin-Xyl, which contained proteinases to catalyze the hydrolysis of proteins to peptides with different molecular weights [24, 25]. In contrast to them, Protoorizin contains a complex of proteinases and peptidases that hydrolyze proteins to low-molecular-weight peptides and free amino acids [40, 43].

Table 2 Enzyme preparations by activity of major and minor enzymes

Enzyme preparation	Enzyme activity, units/g				
	Amylolytic activity	Glucoamylase activity	Xylanase activity	Proteolytic activity	Phytase activity
Amilolicheterm	330 ± 8	0	25 ± 5	7.0 ± 0.1	0
Glucavamorin-Xyl	110 ± 4	7700 ± 240	350 ± 13	6.0 ± 0.1	0
Protoorizin	0	0	0	580 ± 26	0
Phytaflow	0	0	0	0	30000 ± 1200



AA – amylolytic activity, GA – glucoamylase activity, XA – xylanase activity, PhA – phytase activity

Figure 1 Effects of phytolytic enzymes on phosphate release in wheat (*1*) and corn (*2*) wort

Enzyme preparations were used at the stage of grain wort preparation in the amounts specified above (see "Study objects and methods"). The control wort contained Amilolicheterm and Glucavamorin-Xyl preparations, the sources of carbohydrases that catalyze the hydrolysis of polysaccharides. Their proteolytic enzymes had practically no effect on the degree of protein conversion due to the low level of their activity. The concentration of proteases in the control was under 0.02 PA units/g grain, which was 5 times lower than in the experimental samples. Therefore, Protoorizin (0.1 PA unit/g grain) and Phytaflow (1.0–3.0 PhA units/g grain) were additionally added to the experimental samples to ensure an efficient conversion of grain polymers and activate yeast generation and ethanol fermentation.

We found that the ionic composition of grain wort changed depending on the type of grain and the substrate specificity of the enzymes used to hydrolyze grain polymers. In the wheat wort treated with a complex of amylolytic and xylanase enzymes (AA + GA + XA), the phosphate content was 2.7 times higher than in the corn wort (Fig. 1). The concentrations of other cations, such as potassium, calcium, sodium, and magnesium, were also different. The wheat wort was richer in potassium, while the corn wort had a higher content of calcium and magnesium (Table 3).

Phytolytic enzymes had a more significant effect on the release of phytate phosphorus. Increasing their amount to 1.5–2.0 PhA units/g grain led to higher concentrations of released phosphates due to the catalytic destruction of phytic substances. We found that the content of phosphorus ions increased 1.6 times in the wheat wort and 3.8 times in the corn wort. A further increase in the phytase concentration to 3.0 PhA units/g grain had practically no effect on the content of soluble phosphates in the wort (Fig. 1).

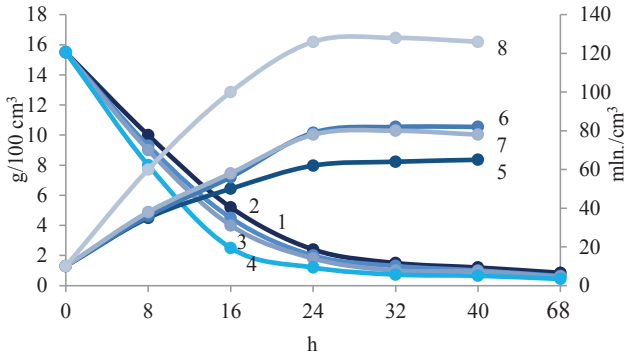
The catalytic synergism of phytases and proteases stimulated the release of phosphorus ions. Their content increased 1.8 times in the wheat wort and 4.9 times in the corn wort (Table 3).

In addition to the release of phosphorus ions, phytases increased the concentration of cations in the wheat and corn wort samples: potassium by 12 and 13%,

Table 3 Effects of hydrolytic enzymes on amine nitrogen and basic ions in wheat and corn wort

Enzyme composition	Type of wort	Amine nitrogen, mg%	Ion content, mg/dm³				
			Phosphorus	Potassium	Calcium	Sodium	Magnesium
Control (AA+GA+XA)	Wheat	53.0 ± 2.5	787.1 ± 28.2	811.4 ± 33.4	12.1 ± 0.2	16.5 ± 0.4	131.1 ± 3.7
	Corn	40.3 ± 1.2	290.0 ± 13.2	780.3 ± 24.7	16.1 ± 0.4	16.1 ± 0.3	179.6 ± 5.4
Control+0.1 PA units	Wheat	85.7 ± 3.2	960.5 ± 29.4	825.1 ± 34.5	12.9 ± 0.3	16.9 ± 0.5	141.9 ± 4.2
	Corn	70.4 ± 2.6	330.4 ± 13.3	834.6 ± 28.8	18.0 ± 0.4	19.0 ± 0.7	199.3 ± 8.2
Control+1.5 PhA units	Wheat	53.5 ± 1.8	1242.1 ± 44.2	907.0 ± 34.9	13.9 ± 0.5	23.0 ± 0.8	151.8 ± 5.8
	Corn	41.3 ± 1.6	1109.0 ± 38.4	878.0 ± 26.3	21.8 ± 0.6	20.9 ± 0.7	218.5 ± 8.9
Control+2.0 PhA units	Wheat	53.9 ± 2.1	1232.3 ± 51.2	909.3 ± 31.4	14.1 ± 0.3	23.9 ± 0.8	152.1 ± 6.7
	Corn	41.1 ± 1.6	1112.3 ± 36.7	881.0 ± 28.4	22.9 ± 0.7	21.7 ± 0.7	222.3 ± 6.4
Control +1.5 PhA units+0.1 PA units	Wheat	93.9 ± 3.8	1379.6 ± 51.4	918.7 ± 34.6	14.0 ± 0.4	24.2 ± 0.8	152.9 ± 4.8
	Corn	76.5 ± 2.6	1423.6 ± 56.7	888.9 ± 42.5	23.4 ± 0.8	23.9 ± 0.7	225.6 ± 7.4

AA – amylolytic activity, GA – glucoamylase activity, XA – xylanase activity, PA – proteolytic activity, PhA – phytase activity



1 and 5 – treated with amylases and xylanase (control);
2 and 6 – treated with phytases (control + 1.5 PhA units/g grain);
3 and 7 – treated with phytases (control + 2.0 PhA units/g grain);
4 and 8 – treated with phytases and proteases
(control + 1.5 PhA units + 0.1 PA units)

PA – proteolytic activity; PhA – phytase activity

Figure 2 Changes in carbohydrate consumption (1–8) and *Saccharomyces cerevisiae* 985-T yeast growth (5–8) during wheat wort fermentation for 68 h

calcium by 15 and 35%, sodium by 39 and 30%, and magnesium by 16 and 22%, respectively (Table 3).

We found that the content of cations depended on the substrate specificity of the enzymes involved in the bioconversion of grain polymers. The combined action of proteolytic and phytolytic enzymes contributed not only to the accumulation of phosphates and minerals, but also to a significant increase in amine nitrogen. Its concentration was 1.8 times as high in the wheat wort and 1.9 times as high in the corn wort, compared to the control.

Grain wort enriched with phosphates and minerals was used as a nutrient medium to cultivate the ethanol yeast *Saccharomyces cerevisiae* (race 985-T). We studied the process of yeast generation on grain wort prepared with hydrolytic enzymes differing in substrate specificity and found that the presence of phytase in the enzyme complex had a positive effect on the growth and reproduction of yeast cells (Fig. 2).

As we know, mineral and nitrogenous substances are essential for the biochemical reactions of yeast cells [24, 25, 43]. Magnesium and calcium ions activate the catalytic ability of almost all intracellular metalloenzymes, including phosphofructokinase, which is involved in glucose metabolism. Potassium, sodium, and calcium ions have a regulatory effect on the metabolism of yeast cells. Potassium also plays an essential role in oxidative phosphorylation and glycolysis processes [43]. It activates yeast aldolase and, together with magnesium ions, catalyzes pyruvate carboxylase. Like nitrogen, potassium can also affect yeast lipid metabolism.

We analyzed the processes of yeast generation and carbohydrate consumption and found that the presence of phosphorus and other minerals in the medium intensified the growth of *S. cerevisiae* yeast. In the lag phase (first 18–24 h of growth), the concentration of yeast cells increased 1.2–1.3 times, alongside a rising rate of carbohydrate consumption. A 1.3-fold increase in phytolytic enzymes (up to 2.0 PhA units/g grain) during grain wort preparation had no significant effect on the yeast growth and the assimilation of reducing carbohydrates (Fig. 2).

The catalytic synergism of phytase and protease significantly enriched the wheat wort with mineral and nitrogenous nutrition and resulted in the most active growth of yeast cells (2-fold) and a more intensive consumption of carbohydrates (Fig. 2, curves 4 and 8). A similar pattern was observed with the corn wort.

Thus, our results confirmed that the nutrient medium has a significant effect on yeast generation and physiological activity, particularly the presence of soluble macro- and microelements in addition to easily digestible carbohydrates and nitrogenous substances.

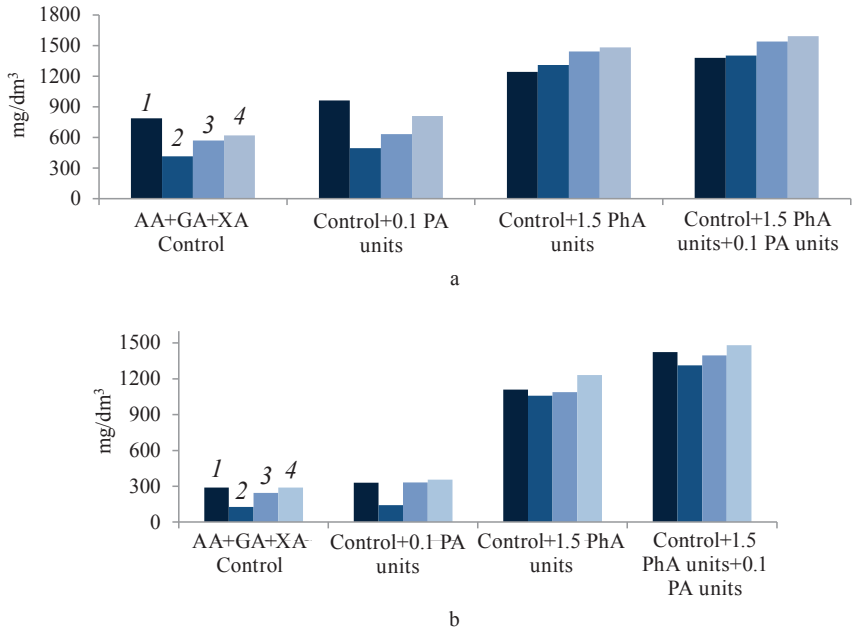
At the next stage, we analyzed changes in the concentration of phosphates in the mash against the amount of phytolytic enzymes used in the preparation of wheat and corn wort (Figs. 3a and 3b). We found that the content of phosphorus ions significantly decreased during the fermentation of the control wort, which was not treated with phytases. In the logarithmic phase of yeast cell growth (on day 1), it declined 1.9 times in the wheat wort and 2.3 times in the corn wort. The wort samples treated with protease in addition to carbohydrases showed the same trend – a sharp decrease in phosphates after 24 h of fermentation, followed by a slight rise.

In the experimental samples treated with phytolytic enzymes, the concentration of phosphates hardly changed during the fermentation of grain wort. This might be due to the ongoing biocatalytic hydrolysis of phytic substances and an extra release of phosphorus. By the end of fermentation, the content of phosphates slightly increased, which might be associated with autolytic processes in the cell (Fig. 3).

We found an increase of 2.4–2.6 times and 4.3–5.1 times in the residual content of phosphates in the wheat mash and the corn mash, respectively. This indicated that phytolytic enzymes not only enriched the grain wort with assimilable phosphorus and other valuable minerals, but also improved the value of grain stillage, a waste product of ethanol production that is used in the diet of farm animals.

Apart from the main fermentation products (ethanol and carbon dioxide), yeast cells synthesize accompanying metabolites: secondary (organic acids, aldehydes, and esters) and by-products (higher alcohols) [43].

We studied the metabolism of *S. cerevisiae* 985-T yeast during its cultivation on wheat and corn wort treated with phytolytic enzymes and found a decrease of 18–21 and 20–23%, respectively, in total metabolite formation that accompanies ethanol synthesis (Fig. 4).



AA – amylolytic activity, GA – glucoamylase activity, XA – xylanase activity, PA – proteolytic activity, PhA – phytase activity

Figure 3 Changes in the concentration of phosphates (mg/dm^3) after 0 (*1*), 24 (*2*), 48 (*3*), and 68 h (*4*) during the fermentation of wheat (a) and corn (b) wort depending on the amount of enzymes

These results indirectly confirmed the improvement of phosphorus metabolism in the cell.

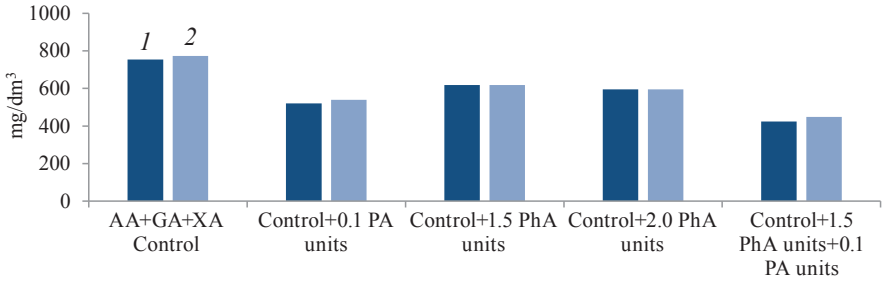
The use of proteases reduced the synthesis of side and secondary metabolites by more than 30%. The greatest effect was caused by the combined catalytic action of phytases and proteases during yeast generation on the media enriched with assimilable phosphates and amine nitrogen. In particular, the total content of accompanying volatiles in the wheat and corn mash decreased by 44 and 42%, respectively, compared to the control.

Higher alcohols (mostly isoamylol, isobutanol, and 1-propanol) dominated among side and secondary metabolites synthesized by *S. cerevisiae* 985-T during the wort fermentation. In addition, the mash contained aromatic alcohols (β-phenylethyl, p-hydroxyphenylethyl) and a small amount of secondary by-products, primarily aldehydes and esters (Fig. 5).

Our study showed that treating the nutrient media with phytolytic and proteolytic enzymes decreased the synthesis of higher and aromatic alcohols (1.9 and 1.4 times, respectively) (Fig. 5). Exposure to phytases reduced the amount of higher alcohols and proteases (1.3–1.4 and 1.5–1.6 times, respectively). In addition, we observed a slight decrease in aldehydes and esters.

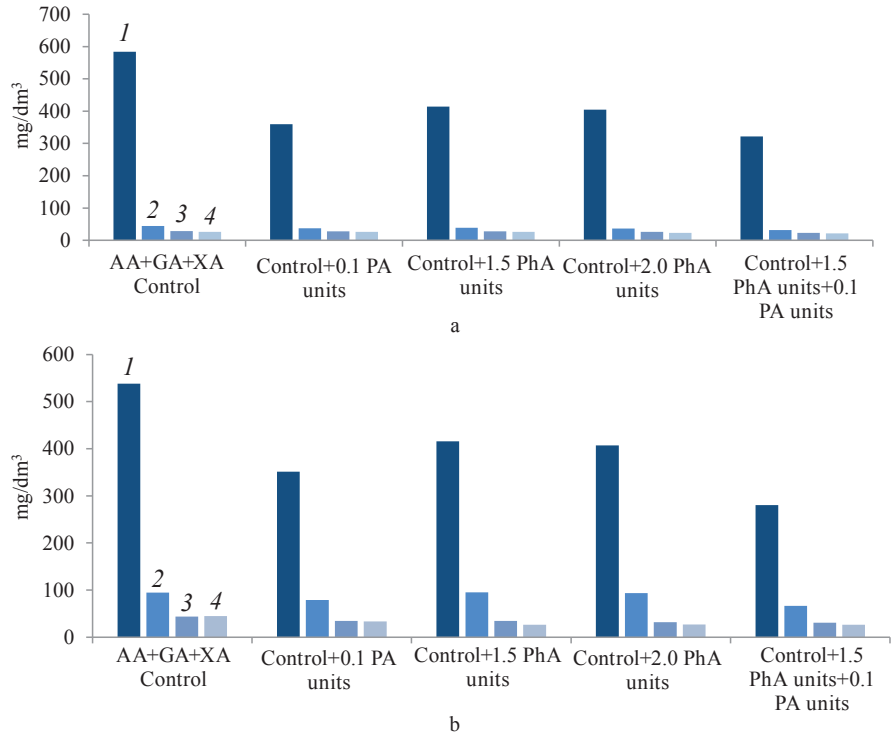
Thus, our study showed that providing a yeast cell with a balanced nitrogenous and mineral nutrition created conditions for synthesizing ethanol with a reduced amount of fermentation by-products. By regulating yeast metabolism we can improve the quality and sensory properties of the target product.

According to our results, the biochemical changes in the grain wort affected the yield of ethanol, the main fermentation product. The destruction of phytic



AA – amylolytic activity, GA – glucoamylase activity, XA – xylanase activity, PA – proteolytic activity, PhA – phytase activity

Figure 4 Total side metabolites during the cultivation of *Saccharomyces cerevisiae* 985-T yeast on wheat (*1*) and corn (*2*) wort



AA – amylolytic activity, GA – glucoamylase activity, XA – xylanase activity, PA – proteolytic activity, PhA – phytase activity

Figure 5 Concentrations of higher alcohols (*1*), aromatic alcohols (*2*), esters (*3*), and aldehydes (*4*) during yeast cultivation on wheat (a) and corn (b) wort treated with various enzymatic complexes

substances enriched the wort with mineral nutrition and activated the physiological activity of yeast cells. It also led to a slight increase in ethanol yield during the fermentation of the wheat and corn wort (by 2.3–2.4 and 1.5–1.7%, respectively) (Table 4). The ethanol concentration in the mash varied due to a higher starch content in corn (Tables 1 and 4).

The metabolic processes in yeast cells improved on the media treated with amylolytic, xylanase, and proteolytic enzymes, as well as a complex of enzymes with phytases. This improvement contributed to a complete fermentation of carbohydrates and an increase in ethanol yield, with a simultaneous decrease in associated metabolites (Table 4, Figs. 4 and 5).

The highest concentration of ethanol generated in the wort treated with a full complex of enzymes was 11.15% in the wheat mash and 12.74% in the corn mash. The ethanol yield from the fermentation of wheat and corn wort increased by 3.2 and 2.6%, respectively, when treated with proteases and by 4.3 and 3.2%, respectively, when treated with proteases and phytase (Table 4).

The rise in ethanol synthesis in the experimental samples was probably associated with an improved conversion of polymers of grain wort and its enrichment with assimilable amine nitrogen. Another reason was a release of macro- and microelements that was vital for yeast cells. These findings were consistent with a number of previous studies [2, 7, 25–27].

Table 4 Ethanol yield during fermentation of wheat and corn wort treated with various enzymatic complexes

Enzyme composition	Ethanol concentration in mash, % vol.		Ethanol yield, cm^3/100 g starch	
	Wheat	Corn	Wheat	Corn
AA+GA+XA (Control)	9.80 ± 0.04	11.81 ± 0.05	65.6 ± 0.3	64.8 ± 0.4
Control + 0.1 PA units	10.87 ± 0.06	12.57 ± 0.07	67.7 ± 0.2	66.5 ± 0.3
Control + 1.5 PhA units	10.65 ± 0.05	12.20 ± 0.06	67.1 ± 0.1	65.8 ± 0.3
Control + 2.0 PhA units	10.72 ± 0.05	12.21 ± 0.07	67.2 ± 0.3	65.9 ± 0.3
Control + 1.5 PhA units + 0.1 PA	11.15 ± 0.06	12.74 ± 0.07	68.4 ± 0.2	66.9 ± 0.2

AA – amylolytic activity, GA – glucoamylase activity, XA – xylanase activity, PA – proteolytic activity, PhA – phytase activity

CONCLUSION

We found almost no changes in the concentration of phosphates during the fermentation of phytases-treated wort, with a slight increase by the end of the process. It was probably caused by the continuing biocatalytic hydrolysis of phytic substances and the release of phosphorus, as well as autolytic processes in the cell. The control samples (without phytolytic enzymes) had a significantly lower residual content of phosphates in the wheat and corn mash (2.4–2.6 and 4.3–5.1 times, respectively).

Our results confirmed that nitrogen and phosphorus nutrition played a regulatory role in the generation and metabolism of ethanol yeast. The catalytic action of phytases and proteases ensured the accumulation of easily assimilable phosphates, minerals, and amino acids in the wort. Also, it intensified the growth of yeast cells and increased the rate of carbohydrate consumption. Finally, it decreased the formation of side metabolites 1.7–1.9 times, mainly due to higher and aromatic alcohols. At the same time, the *Saccharomyces cerevisiae* 985-T yeast synthesized ethanol, whose yield increased by 1.5–4.3%, depending on the type of grain and enzyme complex. The greatest effect was achieved by a full complex of enzymes (carbohydrase, protease, and phytase).

CONTRIBUTION

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

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Mead fermentation parameters: Optimization by response surface methodology

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Abstract:
Introduction. This article presents the development of mathematical models related to the effect of the initial content of dry matter, yeast, and yeast energizer on the fermentation rate, the alcohol content, and the dry matter content in the finished product – mead.
Study objects and methods. The mathematical models were developed by using the response surface methodology (RSM). The effect of yeast, dry matter, and yeast energizer contents were tested in concentration ranges of 150–600 mg/L, 16.3–24.4%, and 140–500 mg/L, respectively. The starting substrates used were honeydew honey and 10% apple juice. Yeast was rehydrated and added in different amounts to obtain required concentrations. Initial dry matter concentrations were measured by a refractometer. At the end of fermentation, oenological parameters of mead, namely dry matter content, pH, and ethanol yield, were determined according to standard methods.
Results and discussion. The statistical estimation of the developed models and the individual model parameters showed that the initial dry matter content had a significant effect on the content of alcohol and dry matter in the final product. While, the initial content of yeast and yeast energizer did not have a significant effect in the tested concentration ranges. In addition, it was proved that the initial content of dry matter and yeast energizer had a significant effect on the fermentation rate, i.e. on the course of fermentation, which was described by a second-degree polynomial.
Conclusion. We determined the optimum content of dry matter (24.4%), amount of yeast (150 mg/L), and concentration of yeast energizer (140 mg/L) in the initial raw material which provided the maximum alcohol yield at a consistent fermentation rate.

Keywords: Response surface methodology, mathematical models, fermentation, mead, yeast

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INTRODUCTION

Response surface methodology (RSM) is a collection of statistical and mathematical techniques used in order to design experiments for adequate response predictions, fit a hypothesized (empirical) model to experimentally obtained data under the chosen design, as well as to optimize the conditions for the given process, i.e. to ensure the appropriate selection of input variables that lead to the desired response of a dependent variable [1].

There are several different options of the design of experiments within RSM, and the options which are used the most are Central Composite Design (CCD) and Box-Behnken Design (BBD). When the analyzed process requires adjustments to the experiment which cannot be carried out using a standard design, some of custom designs are used. In that regard, a particularly interesting option is the Historical Data design option, which uses data available from the experiments which have already been conducted. Specifically, Historical Data creates a blank design layout to accept component and factor settings and responses from an existing data set [2].

RSM was presented for the first time by Box and Wilson in the 1950s, and this methodology is therefore often called the Box-Wilson methodology. Detailed information on response surface methodology is described in [3]. In general, RSM enables testing effects and interaction between different process

parameters. It is successfully used to optimize or control processes in various areas of production, research, and engineering [4–8]. Some of the examples refer to optimization of the medium composition and process parameters for the control of different bioprocesses, including the mead fermentation process [9–13].

Mead is an alcoholic beverage obtained by fermentation of honey solution. Honey is a natural food produced by honey bees from flower nectar (blossom honey) or plant secretions (honeydew honey). Honey is rich in carbohydrates (mainly glucose and fructose), organic acids, and other components, however concentrations of some components (assimilable nitrogen) can be much lower than those considered optimal for fermentation. High sugar contents and low nitrogen concentration in honey slow down fermentation. It means that the fermentation process requires optimal pH, temperature, and growth conditions. Therefore, various additives, such as pollen, fruit pulps or juices, citric acid, etc., can be used to improve alcohol yields, fermentation rates, sensory characteristics of mead, etc. [14–18].

Fruits and their pulps are rich in carbohydrates, fibers, minerals, vitamin C, carotenoids, as well as phenolic and sulfuric substances. Also, their antioxidant properties can help maintain balance between production and elimination of reactive oxygen forms and other related compounds, thereby attenuating free radical-induced damage to cells [16–19]. Among fruits, apples are a widely consumed, rich source of phytochemicals (quercetin, catechin, phloridzin, chlorogenic acid, etc.), all of which are strong antioxidants [19]. Apples also contain water, sugars, acids, pectin, tannins, dyed and aromatic substances, mineral substances, starch, cellulose, vitamins, as well as phenolic compounds and enzymes. All the components give characteristic features to the fruit.

Available literature has not mentioned optimization of honeydew honey as a substrate for obtaining mead. Therefore, this research aimed to assess effects of the concentration of added yeast, yeast energizer and the dry matter content (independent variables) on the ethanol yield and dry matter content in the final product (dependent variables), with the development of a corresponding mathematical model. The developed mathematical model can enable better control of the process in terms of optimum selection and setting of the process parameters.

STUDY OBJECTS AND METHODS

Chemicals and equipment. All chemicals used in this study were of analytical grade. In our experiments we used scales (H54AR, Mettler-Toledo, Columbus, USA and PFB 1200-2, KERN & SOHN, Balingen, Germany), a magnetic stirrer (ARE, Velp Scientifica, Usmate, Italy), a vortex (ZX3, Velp Scientifica, Usmate, Italy), a spectrophotometer (Spectronic 1201, Milton Roy, Ivyland, USA), a pH meter (HI-2211, Hanna Instruments, Smithfield, USA), a waterbath (Wisecircu, J.P. Selecta, Abrera, Barcelona, Spain), a refractometer (Leica Abbe Mark II, Reichert Technologies, Depew, USA), and a conductivity meter (HA-2315, Hanna Instruments, Smithfield, USA).

Physicochemical analyses of honey. The study object was honeydew honey from the territory of the Republic of Srpska, Bosnia and Herzegovina. The quality characteristics of honeydew honey was assured by testing it for water content (18.5%), diastase activity (47.67), HMF content (5.47 mg/kg), acidity (50.67 mmol/kg), reducing sugars (68.16%), saccharose (2.01%), and electrical conductivity (1.17 ms/cm) as described by Ordinance on methods for control of honey and other bee products (Official Gazette of BiH no 37/2009). The pH was measured with a pH meter (4.33).

Honey must preparation. Honeydew honey was stirred with water in different ratios to obtain required dry matter content (Tables 1 and 2). The resultant must was pasteurized at 65°C for 10 min (with regular stirring and skimming off the scum) and then cooled and poured into fermentation flasks. Apple fruit was pressed through a laboratory press to obtain juice that was further used in the study to correct the acidity (pH values of the must were adjusted to 3.7–4) and as a source of additional nutrition for yeast.

The resultant juice was also pasteurized at 65°C for 10 min, cooled, and poured into fermentation flasks in amount required for this study (10%). A total of 27 samples were prepared (Table 2) for the experiments. Initial dry matter concentrations were measured refractometrically. Different amounts of yeast energizer VitaFerm® Ultra F3 (Erbslöh, Geisenheim, Germany) were added into all the samples (Tables 1 and 2). Next, commercial yeast Fermol® Associées (AEB, Italy) was rehydrated in distilled water at 35–40°C during 30 min and added into the samples in different amounts (Tables 1 and 2).

The process of alcoholic fermentation was conducted at 25°C for 20 days. All fermentations were carried out in duplicate using a system that consisted of 250 mL flasks containing 180 mL of must and fitted with an airlock to release CO_2 produced during fermentation. Dynamics of the fermentation process were controlled by weighing the flasks every 24 h throughout alcoholic fermentation and expressed as the cumulative mass of produced ethanol per hour. The rate of fermentation depends on concentration of such inhibitors as ethanol, acetic acid, fatty acids (hexanoic, octanoic, decanoic acid), proteins (enzymes), furfural, hydroxymethylfurfural, etc. The inhibitors interact synergistically with high osmotic pressure and the increasing concentration of ethanol during fermentation [18].

General oenological parameters. At the end of fermentations, oenological parameters of mead – dry matter content, pH, and ethanol content – were determined according to standard methods [20].

Design of experiments and mathematical modelling. The analysis and processing of previously

Table 1 Coded values of experimental data

Factor	Parameter	Minimum	Maximum	Coded low	Coded high	Mean*	SD
A	Dry matter content, %	16.30	24.40	–1 ↔ 16.30	+1 ↔ 24.40	20.30 (20.20)	3.37
B	Yeast content, mg/L	150.00	600.00	–1 ↔ 150.00	+1 ↔ 600.00	350.00 (300.00)	190.65
C	Yeast energizer, mg/L	140.00	500.00	–1 ↔ 140.00	+1 ↔ 500.00	302.33 (267.00)	151.92

* The specified mean values represent the arithmetic mean of the lowest and the highest values (the actual, i.e. the used mean values of experimental data are given in brackets)

Table 2 Historical Data Experimental Design

	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	
Run	*A*: Dry matter content, %	*B*: Yeast content, mg/L	*C*: Yeast energizer, mg/L	R_1: dry matter after fermentation, %	R_2: Alcohol content, vol.%	R_3: Maximum fermentation rate, g/h	pH
1	16.3	150	140	6.15	8.64	1.16	3.23
2	16.3	150	267	6.10	8.40	1.20	3.23
3	16.3	150	500	6.25	8.15	1.34	3.29
4	16.3	300	140	6.40	8.24	1.27	3.34
5	16.3	300	267	6.55	7.83	1.24	3.34
6	16.3	300	500	6.35	8.40	1.28	3.22
7	16.3	600	140	6.60	8.56	1.03	3.29
8	16.3	600	267	6.50	8.24	1.11	3.33
9	16.3	600	500	6.25	7.51	1.45	3.27
10	20.2	150	140	6.90	10.62	1.33	3.18
11	20.2	150	267	7.85	10.45	2.84	3.36
12	20.2	150	500	7.20	10.62	1.47	3.35
13	20.2	300	140	7.70	10.20	1.20	3.21
14	20.2	300	267	7.60	10.79	2.50	3.37
15	20.2	300	500	7.45	10.71	1.44	3.33
16	20.2	600	140	7.30	11.13	1.17	3.31
17	20.2	600	267	6.70	11.22	2.80	3.41
18	20.2	600	500	7.35	10.96	1.93	3.34
19	24.4	150	140	11.80	10.88	0.83	3.07
20	24.4	150	267	10.45	11.30	1.33	3.10
21	24.4	150	500	10.70	11.39	0.88	3.18
22	24.4	300	140	10.70	12.24	1.03	3.11
23	24.4	300	267	10.00	11.90	1.15	3.14
24	24.4	300	500	10.20	11.56	1.15	3.13
25	24.4	600	140	10.20	11.73	1.03	3.14
26	24.4	600	267	10.45	11.30	1.34	3.14
27	24.4	600	500	10.15	11.64	1.73	3.17

obtained experimental data were carried out using the Design-Expert 11 program (Stat-Ease, Inc. USA) and the Historical Data Design option. The following variables were used as independent variables: the initial content of dry matter (Factor *A*), yeast (Factor *B*), and yeast energizer (Factor *C*). As dependent (modelled) variables we used maximum fermentation rate (R_3), alcohol content (R_2), and dry matter content in the product (R_1). Table 1 shows the actual and coded values of the above-mentioned variables, while Table 2 shows the corresponding design of experiments.

The relation between the independent variables (*A*, *B*, *C*) and the modelled variables (R_1, R_2, R_3) is described by a second-degree polynomial model, by fitting the experimentally obtained data with the sum of squares. The general form of a second-degree polynomial is given using the following equation:

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + +\beta_{12} X_1 X_2 + e \quad (1)$$

where Y_i is the response of interest (R_1, R_2, R_3); X_i refers to independent variables (A, B, C); β_0 is the constant coefficient; β_1, β_2, and β_3 are linear coefficients; β_{12}, β_{13}, and β_{23} are coefficients of interaction between the variables; β_{11}, β_{22}, and β_{33} are quadratic coefficients; and e is the model error.

The statistical analysis of the developed mathematical models, i.e. the determination of their statistical significance, was conducted using the analysis of variance (ANOVA), i.e. the Fisher's exact test (F-test). The analysis of variance determined the significance of the effect of each model parameter on the variance of the outcome, in comparison with the total variance of all the observed model parameters.

Optimization. In order to determine the initial content of dry matter, yeast, and yeast energizer resulting in the maximum alcohol content, with the fermentation rate as consistent as possible, we carried out the numerical optimization of the developed mathematical models using the Design-Expert 11 program (Stat-Ease, Inc.). Prior to the optimization, we selected the objective – the range of numeric values within which we looked for solutions and the level of significance of reaching the set optimization objective, i.e. we selected the corresponding optimization criteria (Table 3).

RESULTS AND DISCUSSION

We studied effects of the analyzed independent variables on the values of dry matter content (R_1) and alcohol yield (R_2) in the finished product – mead, as well as on the maximum fermentation rate (R_3). Apart from the determined design of experiments, Table 2 shows the corresponding numeric values of the response of interest (R_1, R_2, and R_3) and the measured pH values.

The results from Table 2 show that the lowest residual dry matter content was measured in the samples which had the lowest dry matter content before fermentation (samples 1–9), while the samples with the highest dry matter content before fermentation (samples 19–27) had the highest content of residual dry matter after fermentation. That is related to the duration of the fermentation process (20 days for all the samples), which means that the dry matter content could decrease, and the ethanol content could increase if the fermentation process was extended.

According to Pereira *et al*., residual dry matter consists of a high number of different compounds: sucrose, maltose, isomaltose, trisaccharides, tetrasaccharides, glycerol, etc [12]. In the research conducted by Savić *et al*., the dry matter content ranged between 5.2 and 11.85% [21]. In our work, the highest ethanol content was obtained in samples 19–27, which had the highest dry matter content before fermentation, while the lowest ethanol content was in samples 1–9. In the research conducted by Martínez *et al*., the ethanol content was 10.11 vol. % after day 18 day of fermentation, and it was 12.52 vol. % after 26 days [22].

The obtained pH values (Table 2) were lower than those of the honey solution, most probably due to acids produced by yeast during fermentation [23, 24]. The pH value is a very important parameter for alcoholic fermentation, because yeast cannot ferment under acidic conditions. In this research, the lowest pH value of mead was 3.07 (sample 19). A low pH value can slow down or even stop the fermentation process, as well as cause incomplete sugar breakdown due to acetic and succinic acid formation, which cause an increase in the content of undissociated fatty acids [23]. Ammonium ion uptake, which is part of yeast energizer, is associated with the excretion of proton ions into the medium, thereby decreasing extracellular pH [25].

By fitting the data from Table 2 within the regression analysis, the corresponding coefficients were determined in Eq. (1), and the following empirical models were developed:

$$R_1 = 20.84195 - 1.89148 \cdot A + 0.004432 \cdot B - 0.000428 \cdot C - 0.000237 \cdot AB - 0.000128 \cdot \cdot AC + 8.53772 \cdot 10^{-7} BC + 0.062105 \cdot A^2 - 4.5267 \cdot 10^{-7} B^2 + 3.36950 \cdot 10^{-7} \cdot C^2 \quad (2)$$

$$R_2 = -19.62681 + 2.61931 \cdot A + 0.000087 \cdot B - 0.002689 \cdot C + 0.000134 \cdot AB + 0.00012 \cdot \cdot AC - 2.84782 \cdot 10^{-6} BC - 0.056302 \cdot A^2 - 1.99177 \cdot 10^{-6} B^2 + 1.31943 \cdot 10^{-6} \cdot C^2 \quad (3)$$

$$R_3 = -14.85963 + 1.57561 \cdot A - 0.003928 \cdot B + 0.010190 \cdot C + 0.000111 \cdot AB + 0.000014 \cdot \cdot AC + 3.51007 \cdot 10^{-7} BC - 0.039979 \cdot A^2 + 1.29218 \cdot 10^{-6} B^2 - 0.000017 \cdot C^2 \quad (4)$$

Table 3 Optimization criteria

	Optimization objective	Range of numeric values	Level of significance of the objective (from 1 to 5)
Factor *A*	in range	16.3–24.4%	not applicable
Factor *B*	minimize	150–600 mg/L	3
Factor *C*	minimize	140–500 mg/L	4
Response R_1	none		not applicable
Response R_2	maximize	7.51–15.00 vol. %	5
Response R_3	minimize	0.83–2.84	5

Table 4 ANOVA for quadratic models in terms of coded factor (equations 5, 6, and 7)

	R_1, %		R_2, vol. %		R_3, g/h	
	F-value	*P*-value	F-value	*P*-value	F-value	*P*-value
Model	64.67	< 0.0001	44.43	< 0.0001	3.84	0.0082
A-Dry matter content	493.60	< 0.0001	360.36	< 0.0001	0.0592	0.8107
B-Yeast	1.31	0.2676	1.10	0.3089	0.8737	0.3630
C-Yeast energizer	1.19	0.2900	0.9048	0.3548	3.34	0.0852
AB	3.92	0.0640	1.35	0.2615	0.9202	0.3509
AC	0.7242	0.4066	0.6884	0.4182	0.0097	0.9226
BC	0.1031	0.7521	1.24	0.2817	1.87	0.1888
A^2	41.78	< 0.0001	37.02	< 0.0001	18.63	0.0005
B^2	0.0162	0.9003	0.3373	0.5690	0.1417	0.7113
C^2	0.3903	0.5404	0.0645	0.8025	10.73	0.0045

The equations given above are written in the form of the actual values of the factors (*A*, *B*, *C*), and they may be used to predict the corresponding response, simply by inserting the values of *A*, *B*, *C* in the given units. The given equations are normalized and may not be used to determine the significance of the factors *A*, *B*, and *C*. When analyzing equations where +1 and –1 correspond to the largest and least factor values, respectively, the relative effect of individual factors of the process under study may be identified by comparing the coefficients in front of the corresponding factor. Apart from that, equations written in a coded form may be used to predict the response for the given factor level. The above-mentioned equations, written in a coded form, are given below:

$$R_1 = 7.33 + 2.05 \cdot A - 0.1048 \cdot B - 0.1001 \cdot C - 0.2163 \cdot AB - 0.0933 \cdot AC + 0.0346 \cdot \cdot BC + 1.02 \cdot A^2 - 0.0229 \cdot B^2 + 0.1092 \cdot C^2 \quad (5)$$

$$R_2 = 10.85 + 1.69 \cdot A + 0.0924 \cdot B - 0.0840 \cdot C + 0.1222 \cdot AB + 0.0876 \cdot \cdot AC - 0.1153 \cdot BC - 0.9235 \cdot A^2 - 0.1008 \cdot B^2 + 0.0427 \cdot C^2 \quad (6)$$

$$R_3 = +2.21 - 0.0216 \cdot A + 0.0824 \cdot B + 0.1615 \cdot C + 0.1010 \cdot AB + 0.0104 \cdot AC + + 0.1422 \cdot BC - 0.6558 \cdot A^2 + 0.0654 \cdot B^2 - 0.5518 \cdot C^2 \quad (7)$$

The conducted analysis of variance (ANOVA) of the data (Table 2) proved their statistical significance as a whole, as well as the statistical significance of individual members of Eqs. (5)–(7). Table 4 demonstrates the ANOVA values for the developed models related to the effect of the process parameters on the dry matter content after fermentation (Eq. (5)), the alcohol content (Eq. (6)) and the maximum fermentation rate (Eq. (7)). The ANOVA was carried out for the equations written in a coded form. All the conclusions drawn for the equations written in a coded form apply to the equations in an actual form as well.

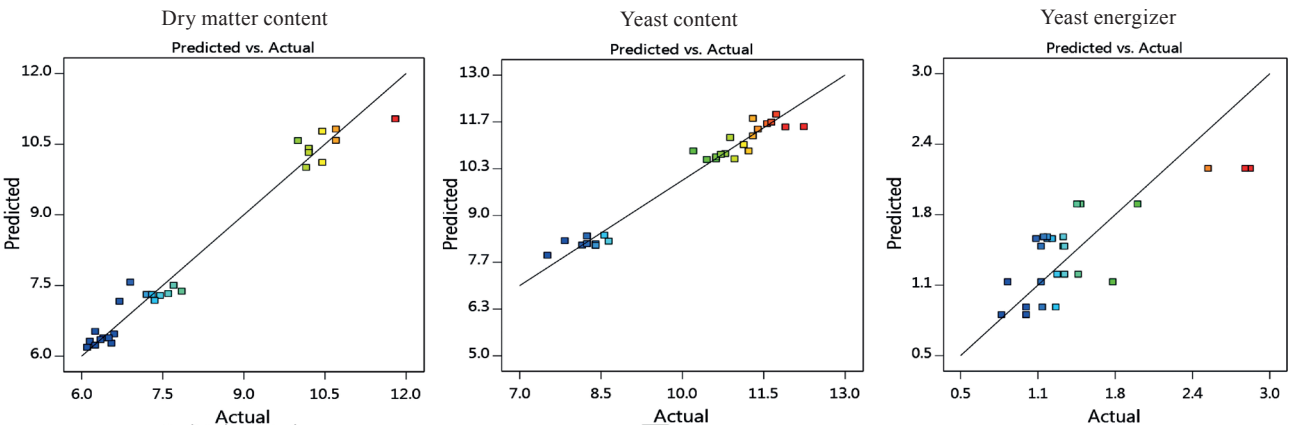

Figure 1 Diagnostics plots

Table 5 Fit statistics

	Dry matter content R_1	Yeast content R_2	Yeast energizer R_3	*Yeast energizer R_3
R^2	0.9716	0.9592	0.6701	0.5988
Adjusted R^2	0.9566	0.9376	0.4955	0.5259
Predicted R^2	0.9299	0.9005	0.2785	0.3957
Adeq Precision	20.6715	17.7830	6.3645	8.3653
*Reduced model				

By analyzing the F-values and the *P*-values for the quadratic equation (5), i.e. the response R_1, it can be concluded that the developed model is statistically significant as a whole taking into consideration that the F-value of the model is 64.47 and that there is only 0.01% of probability for such a high F-value to occur due to noise. The *P*-values below 0.05 indicate that a particular member of the analyzed equation, to which the given value refers, has a statistically significant effect. In the analyzed equation, those are the members *A* and A^2. The *P*-values above 0.1000 indicate that the given member of the equation does not have a statistically significant effect, and in this case, those are *B*, *C*, *AB*, *AC*, *BC*, B^2, and C^2.

The quadratic models related to the effect of the process parameters on the response R_2, i.e. the alcohol content (Eq. (6)), have the F-values of 44.43, and there is only 0.01% of probability for such a high F-value to occur due to noise. Therefore, it can be concluded that the developed model is statistically significant. Like in the previously analyzed equation, the *P*-values of the members *A* and A^2 are below 0.0001, which means that they are statistically significant members of the given model.

The quadratic models related to the effect of the process parameters on R_3, i.e. the maximum fermentation rate (Eq. (7)), have the F-value of 3.84 and the *P*-value of 0.0082, i.e. there is 0.82% of probability for such a high F-value to occur due to noise. Therefore, it can be concluded that the given model is statistically significant. By analyzing the *P*-values of the individual members of Eq. (7), it can be concluded that only the members A^2 and C^2 are statistically significant members of the model, because their *P*-values are below 0.05 (the *P*-value of the member A^2 is 0.0005, and the *P*-value of the member C^2 is 0.0045).

The validation of the developed models was conducted by comparing the experimentally obtained data with the corresponding values obtained by using the model (Fig. 1), and by analyzing the fit statistics from Table 5. First of all, it is necessary to notice that in all the experiments there is a satisfactory relation between the measurement signal (response) and noise, which is expressed by the values of the Adeq Precision parameter above 4 (Table 5).

Figure 1 shows that the actual values in all three cases approximate to the values foreseen by the model, i.e. that the individual values are in the vicinity of the ideal line (y = x), and that they are randomly distributed on both sides of the line y = x. This indicates that there is a correlation between the actual values and the values foreseen by the model. This is verified by the high values of the determination coefficient (R^2), given in Table 5. The table shows that the R^2 values for fitted Eqs. (5) and (6) are higher in comparison with the R^2 values of fitted Eq. (7).

However, since all three R^2 values are above 0.5, only by observing this indicator, it could be concluded that all three models realistically explain the dependence of the observed responses (R_1, R_2, and R_3) on the independent variables (*A*, *B*, *C*). However, that only applies to Eqs. (6) and (7). The further analysis of the fit statistics from Table 5 shows that a reasonable agreement between the adjusted R^2 and the predicted R^2 only exists for the case of fitted Eqs. (5) and (6), while it is not the case for Eq. (7), where there is a significant difference between the two parameters.

Specifically, the predicted R^2 value (0.2785) is not close enough to the adjusted R^2 value (0.4955), i.e. it is higher than 0.2. This indicates the possibility of occurrence of a blocking effect as a result of the conduct of experiments in several blocks (a group of experimental conditions) or a possible problem with the model itself and/or individual data. Given the fact that ANOVA showed for this empirical model that only the members A^2 and C^2 are statistically significant, it is assumed that the presence of the other members in the model contributes to the above-mentioned problem, and the equation is therefore reduced by excluding the member *B*, and the members of the interaction *AB*, *AC* and *BC*. The repeated fitting of data from Table 2 resulted in the following equation written in the actual and in the coded form respectively:

$$R_3 = -16.13759 + 1.61878 \cdot A + 0.011709 \cdot \\ \cdot C - 0.039979 \cdot A^2 - 0.000017 \cdot C^2 \quad (8)$$

$$R_3 = -16.13759 + 1.61878 \cdot A + 0.011709 \cdot \\ \cdot C - 0.039979 \cdot A^2 - 0.000017 \cdot C^2 \quad (9)$$

The ANOVA values for the fitted equation (9) in the coded form show that the equation reduced in such a manner is also statistically significant as a whole, because the F-value of the model is 8.21, and there is only 0.03% of probability that such a high value is a result of noise. Apart from that, the members A^2 and C^2 are also statistically significant with the *P*-values

of 0.0002 and 0.0027, respectively. The comparison between the experimentally obtained values of the maximum fermentation rate and the values obtained by using the empirical model described by Eq. (8) or (9) gives the value of the determination coefficient R^2 of 0.5988, which means that there is a correlation between the values obtained in such a manner. Apart from that, the values of adjusted R^2 of 0.5259 and predicted R^2 of 0.3957, which differ by less than 0.2, indicate the presence of the given correlation. All this indicates that the model reduced in such a manner may be used for determining the maximum fermentation rate (R_3) in the given designed space (the tested range of the change of values of the independent variables). The reduction of the other two models, i.e. fitted Eqs. (2) and (3), and Eqs. (5) and (6), was not carried out, because the given equations have satisfactory values of all the statistical parameters tested (Fig. 1, Tables 4 and 5).

Figure 2 shows the response surface plots which enable an insight into the behavior of the observed dependent variables (responses R_1, R_2, and R_3) to change the independent variables and their possible interaction. The plot A (Fig. 2) shows the effect of different combinations of the initial content of dry matter and yeast on the dry matter content after fermentation at the fixed value of yeast energizer of 320 mg/L. The dry matter content after fermentation increased from 6.4 to 10.1% with an increase in the initial dry matter content from 16.3 to 24.4% at the value of the yeast content of 600 mg/L.

An almost identical increase in the dry matter content after fermentation from 6.2 to 10.7% with the same amount of increase in the dry matter content in the initial raw material was noticed at the value of the yeast content of 150 mg/L. Therefore, it can be concluded that the effect of the yeast content in the initial raw material on the dry matter content after fermentation was negligible in comparison with the dominant effect of the dry matter content in the initial raw material, in the tested range of values of the independent variables.

The plot B (Fig. 2) shows the effect of different combinations of the dry matter content and yeast energizer on the dry matter content after fermentation at the fixed value of the yeast content of 375 mg/L. The dry matter content after fermentation increased from 6.4 to 10.3% with an increase in the initial dry matter content from 16.3 to 24.4% at the value of yeast energizer of 500 mg/L. A similar increase in the dry matter content after fermentation from 6.4 to 10.7% with the same amount of increase in the dry matter content in the initial raw material was noticed at the value of yeast energizer of 140 mg/L. Therefore, it is clear that the effect of yeast energizer in the initial raw material on the dry matter content after fermentation was negligible in comparison with the dominant effect of the dry matter content in the initial raw material, in the tested range of values of the independent variables.

Taking into consideration the previous conclusions on the negligible effect of the content of yeast and yeast energizer in the initial raw material on the dry matter content after fermentation, it is expected that different combinations of the two given independent variables do not have an effect on the value of the observed response. This is confirmed by the plot F (Fig. 2), which shows that there is almost no change in the dry matter content after fermentation at different combinations of the given independent variables and at the fixed dry matter content in the initial raw material of 20.35%.

The plot C (Fig. 2) demonstrates the effect of different combinations of the content of dry matter and yeast on the alcohol content after fermentation at the fixed value of yeast energizer of 320 mg/L. The alcohol content after fermentation increased from 8.14 to 11.73% with an increase in the dry matter content from 16.3 to 24.4% at the value of the yeast content of 600 mg/L in the initial raw material. An almost identical increase in the dry matter content after fermentation from 8.21 to 11.31% with the same amount of increase in the dry matter content in the initial raw material was noticed at the value of the yeast content of 150 mg/L. Therefore, it can be concluded that the effect of the yeast content in the initial raw material on the alcohol content after fermentation was negligible in comparison with the dominant effect of the dry matter content in the initial raw material, in the tested range of values of the independent variables.

The plot D (Fig. 2) shows the effect of different combinations of the content of dry matter and yeast energizer on the alcohol content after fermentation at the fixed value of the yeast content of 375 mg/L. The alcohol content after fermentation increased from 8.13 to 11.66% with an increase in the dry matter content from 16.3 to 24.4% at the value of yeast energizer of 500 mg/L in the initial raw material. A similar increase in the alcohol content after fermentation from 8.47 to 11.66% with the same amount of increase in the dry matter content in the initial raw material was noticed at the value of yeast energizer of 140 mg/L. Therefore, it can be concluded that the effect of yeast energizer in the initial raw material on the alcohol content after fermentation was negligible in comparison with the dominant effect of the dry matter content in the initial raw material. Taking into consideration this conclusion, as well as the conclusion drawn from the analysis of the plot C, it can be concluded that different combinations of the content of yeast and yeast energizer do not have a significant effect on the alcohol content after fermentation either, similar to the effect on the dry matter content after fermentation as shown in the plot F. To ensure visibility of the work, the corresponding plot is not given in Fig. 2.

The plot E (Fig. 2) shows the effect of different combinations of the yeast content and the dry matter content in the initial raw material on the maximum fermentation rate at the fixed value of yeast energizer of 375 mg/L. Unlike the previous plots, the effect of both observed independent variables can be clearly noticed

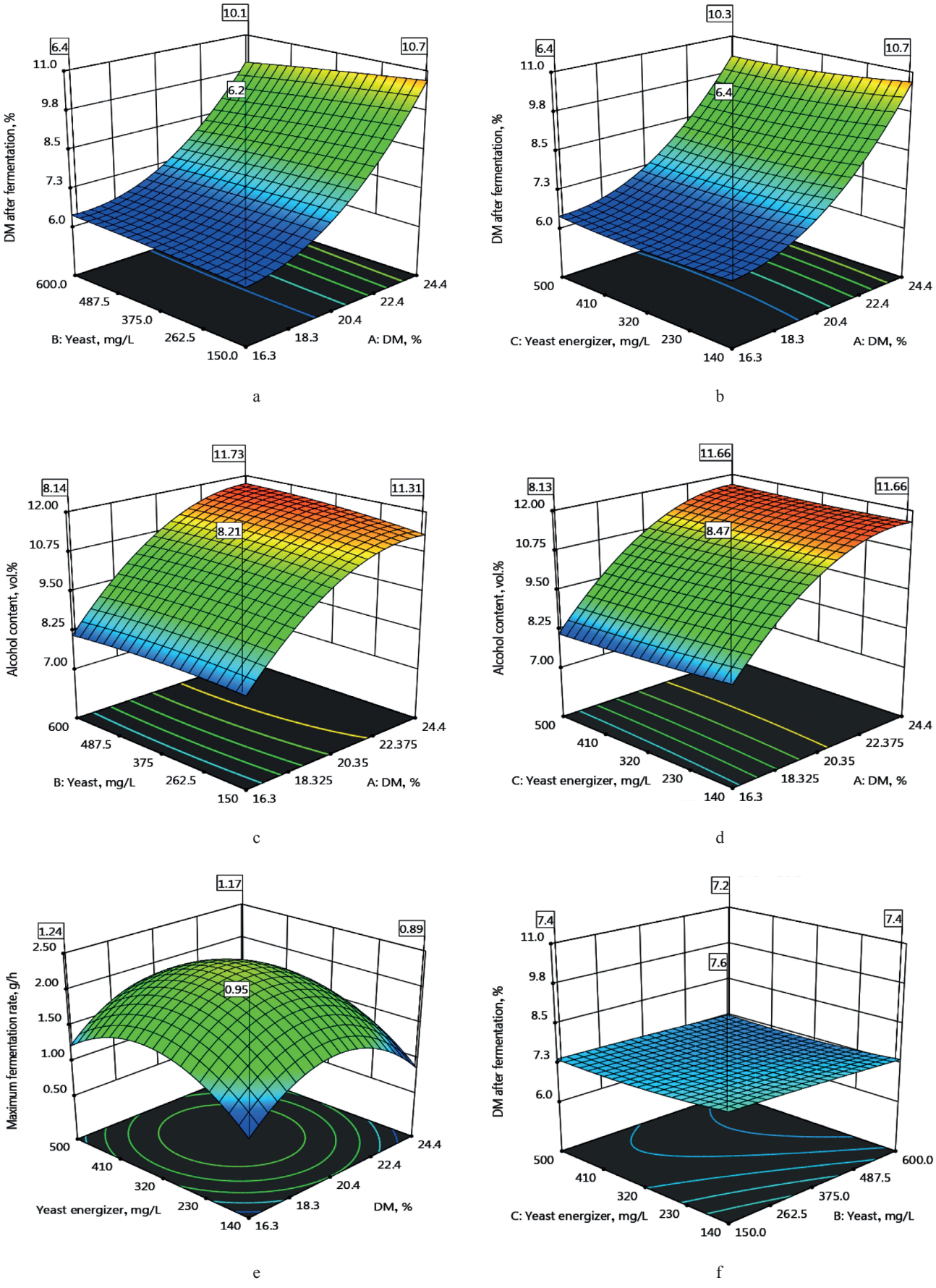

Figure 2 Response surface plots for dry matter content after fermentation (plots A, B, F), Alcohol content (plots C, D) and maximum fermentation rate (plot E). Plots A and C at the fixed content of yeast energizer of 320 mg/L. Plots B, D, and E at the fixed content of yeast energizer of 375 mg/L. Plot F at the fixed dry matter (DM) content in the initial raw material of 20.35%

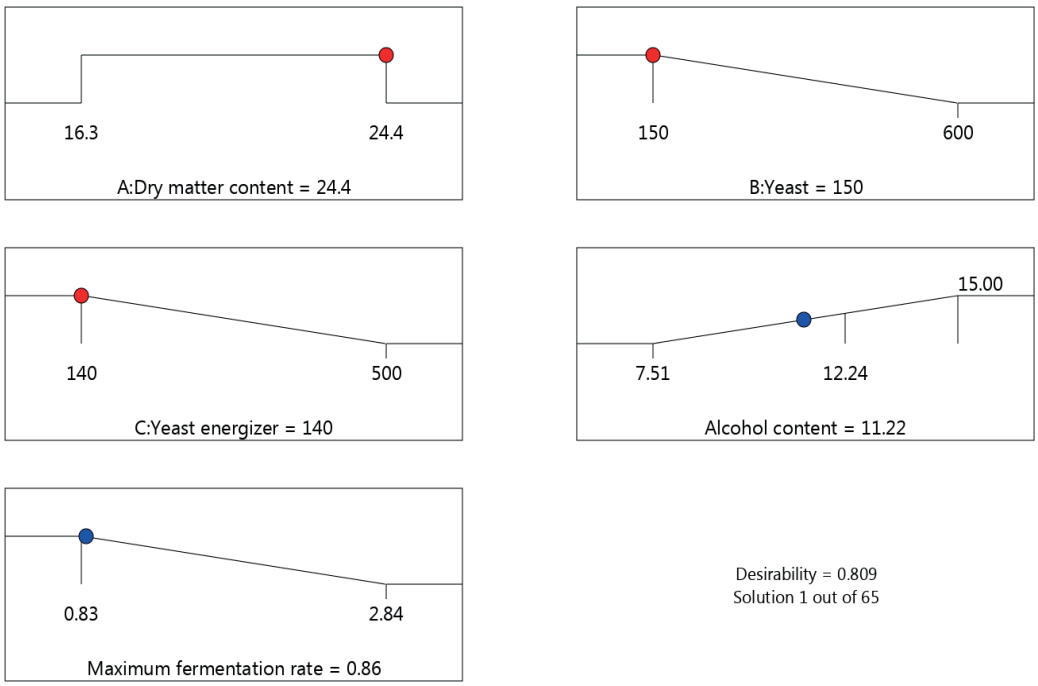

Figure 3 Optimum conditions and the corresponding responses

here, which was in accordance with the developed model (Eq. (8)), which had two quadratic members. The maximum fermentation rate increased, went through the maximum, and then decreased, at a particular value of yeast energizer in the initial raw material.

A similar trend of a change in the maximum fermentation rate was noticed with a change in the value of yeast energizer in the initial raw material, at a particular value of the dry matter content in the initial raw material. It is obvious that it is possible to select particular combinations of the content of dry matter and yeast energizer in the initial raw material, which would give the maximum alcohol content at the corresponding, i.e. desired values of the fermentation rate and the dry matter content, which was the subject of the optimization study.

Figure 3 shows the results of numerical optimization of the developed mathematical models. According to the defined optimization criteria (Table 3), the optimum conditions were the dry matter content of 24.4%, the content of yeast of 150 mg/L, and yeast energizer of 140 mg/L in the initial raw material. Under such conditions, the alcohol content obtained after fermentation was 11.22% with a moderate fermentation rate of 0.86 g/h.

The above-mentioned solution had the highest level of desirability (0.809) among a total of 65 offered solutions. That means that it is possible to select a series of combinations of the minimum content of yeast and yeast energizer in the initial raw material which would enable the maximum yield of alcohol at a moderate fermentation rate, with the dry matter content within the range of the analyzed numeric values.

CONCLUSION

Response surface methodology allowed us to develop empirical mathematical models in the form of second-degree polynomials. The models describe the effect of the initial content of dry matter, yeast, and yeast energizer on the maximum fermentation rate, the alcohol yield, and the dry matter content in the finished product – mead.

The statistical analysis has proved that the initial dry matter content had the statistically significant effect on the content of alcohol and dry mater in the final product. The initial content of yeast and yeast energizer in the tested range of values of the given variable was negligible. The developed mathematical models were used to select optimum fermentation conditions: the dry matter content of 24.4%, the yeast content of 150 mg/L, and the content of yeast energizer of 140 mg/L, in the initial raw material. Under such conditions, the alcohol yield obtained after 20 days of fermentation was 11.22% at a moderate fermentation rate of 0.86 g/h.

CONTRIBUTION

Saša Papuga, Igor Pećanac, Maja Stojković, Aleksandar Savić, and Ana Velemir conceived and designed the experiments; performed the experiments; contributed reagents, materials, and analytical tools; and wrote the paper. Saša Papuga analyzed the data, developed mathematical models, and performed parameter optimisation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Biostability of binder-free wood and plant plastics protected with antiseptics

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Abstract:

Introduction. Agriculture produces a lot of plant and food waste that is highly biodegradable. In order to recycle this waste and use it in the production of new materials, we need to find effective ways to increase their resistance to biodegradation. We aimed to study the biostability of binder-free wood and plant plastics, as well as to find an optimal method of their antiseptic protection.

Study objects and methods. Our objects of study were binder-free plastics based on sawdust, wheat and millet husks. To determine their biostability, we exposed them in active soil for 21 days and analyzed their physical and mechanical properties. Also, we examined the effects of several methods of antiseptic treatment on the samples' strength, water resistance, and biodegradation.

Results and discussion. All the wood- and plant-based samples showed low biostability. Exposure in active soil caused significant morphological and structural changes, as well as impaired the samples' physical and mechanical properties, especially those of the plant-based plastics. Their resistance to biodegradation was significantly determined by the type of filler or antiseptic, as well as by the method of antiseptic administration. Whether added to the press mixture or applied to the surface, the antiseptics changed the samples' physical and mechanical properties. Among the antiseptics used, copper sulfate showed the best effect when introduced directly into the sawdust press mixture. It ensured the lowest decrease in flexural strength, but increased hardness, water absorption, and swelling. The wheat- and millet-based plastics protected with copper sulfate showed an increase in strength indicators, but lower water resistance.

Conclusion. The antiseptic protection of binder-free wood and plant plastics affects a number of their physical and mechanical properties and therefore should take into account the expected conditions for their performance.

Keywords: Binder-free plant plastics, binder-free wood plastics, bioplastics, wheat husks, millet husks, biostability, biodegradation, antiseptic protection

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INTRODUCTION

In the spotlight of current research and development are new formulations and technologies for producing plastics based on plant fibers and fillers, polymer composites combining products of traditional petrochemistry and biotechnology, biodegradable plastics, and biopolymers [1]. We can see new trends in the development of these technologies. Many of them become commercialized and acquire a wide applied significance in addition to their scientific value [2].

Recent years have witnessed a growing interest in biocomposites and bioplastics filled and reinforced with natural fibers and plant components, as well as in plant bioplastics that do not contain any products of large-scale petrochemistry [3–5]. Over the last few years, the global market of biodiversity-based plastics has had an average annual growth of 40% [3]. Largely stimulated by consumer demand, the development of bioplastics aims to improve the performance, availability, and environmental sustainability of materials and products [6]. The problem of microplastic pollution has also attracted a lot of attention recently.

The requirements for biodegradable polymers are changing: the decomposition of a polymer matrix

into macro- and microscopic particles is no longer an indicator of satisfactory destruction [7]. Although actively developing abroad for several decades, bioplastic technologies are a relatively new field of research in Russia. Its President, Vladimir Putin, declared 2017 the year of ecology, which stimulated a search for low-waste and less resource-intensive production methods, as well as for recycling and waste disposal technologies to make industrial enterprises more environmentally friendly [8]. In line with this concept is the processing of wood and plant waste such as sawdust and husks of wheat, oats, buckwheat, and other crops into environmentally friendly and practical materials. Quite promising is the production of wood and plant plastics without binders [9].

Russia yearly produces significant amounts of waste suitable for recycling and processing, such as husks, oilcakes, fibers, etc. However, this waste is not widely used to produce new materials. The reasons are a lack of effective processing technologies and equipment, financial and economic aspects, and a low market interest [10].

To produce binder-free composite bioplastics based on polymers and plant fillers, wood and plant bioplastics, we need to prove their high performance properties. For example, materials with a high rate of biodegradation can be used for mulching or to make agrotechnical films, as well as disposable containers for seedlings and soil [11]. However, structural and finishing products, or reusable packaging, need to be highly resistant to various environmental factors.

For this, composites are often used whose matrix contains recycled polyethylene or polypropylene with the addition of plant components (fibers, husks, and flour). The properties of such composites are well studied [12]. For example, the resistance of wood and plant plastics is known to be determined by the biostability of the press material (its main components) and the absence of molecules that are a substrate or nutrient for soil, saprophytic micro- and macroorganisms [13, 14].

Polymer molecules can be destroyed physicochemically, through hydrolysis, under the action of acidic or alkaline media, or under the action of enzymes from fungal and bacterial cultures. Both ways of biodegradation are possible with binder-free plant and wood plastics [10]. They are mainly damaged by fungi and, to a lesser extent, by bacteria that cause rot and destroy lignin [13].

The shelf-life of household products made of binder-free wood bioplastics is estimated at 7.5 years if used at room temperature and moderate humidity [15]. Antiseptic protection is needed to maintain and improve their performance characteristics. However, materials treated with antiseptic agents change their physical, mechanical, and operational properties [16, 17]. Thus, to fully use agricultural plant waste in recycling and production of new materials, we need to find the most effective methods to increase their resistance to biodegradation.

In this regard, it seems relevant to study the biostability of binder-free wood and plant plastics based on sawdust, wheat and millet husks, as well as to find an optimal way of their antiseptic protection. Our aim was to study the biodegradation of wood (based on sawdust) and plant (based on millet or wheat husks) plastics produced without binders and treated with antiseptics.

For this, we analyzed the biostability of the samples of binder-free wood plastics and binder-free plant plastics, assessed the effect of antiseptics on their physical and mechanical characteristics, and analyzed the biostability of the samples antiseptically protected by different methods.

STUDY OBJECTS AND METHODS

Our study objects were the antiseptically treated samples of binder-free wood plastic (BF-WP) based on sawdust and binder-free plant plastic (BF-PP) based on wheat and millet husks. The samples were 2–4-mm-thick discs, 90 mm in diameter, made by pressing from raw materials containing the plant component (sawdust, wheat or millet husks). The weight of the press material was 10 g per disk. The pressing time was 10 min, pressure 124 MPa, cooling time under pressure 10 min. Some of the samples were treated with antiseptic compounds by adding them to the press material or by applying them to the finished sample after conditioning. We used a water repellent (1 g/disc), 12% $CuSO_4$ (0.6 kg/100 m^2), and a Forwood antiseptic (Raduga Coating Works, Novosibirsk) (2 g/disc). The amounts of antiseptics were based on the previous studies.

Before assessing biostability, we analyzed the physical and mechanical properties of the samples. In particular, we determined the density, flexural strength, hardness, elasticity number, compression modulus, flexural modulus, breaking stress, yield stress, water absorption, and swelling in thickness after 24 h. Then, the samples were kept in active soil for 21 days to study biostability.

The soil was prepared in accordance with State Standard 9.060-75. At the beginning of the tests, the soil extract had a pH of 7.0 and a biological activity coefficient of 0.8. The soil's microbiocenosis contained native field strains of microorganisms. After the soil exposure, the samples were analyzed for macro- and microvisual signs of biodegradation (splitting, swelling, loosening, cavities, morphological changes in the plant particles, changes in color, colonies of microorganisms, hyphae, fungal fruit bodies inside or on the surface of the sample, sliming of the surface). Then, we examined the physicomechanical parameters of those samples which were not damaged by the exposure in active soil.

RESULTS AND DISCUSSION

First, we analyzed the key physical and mechanical properties of the control samples, namely binder-free wood plastic based on sawdust (BF-WP) and binder-free plant plastics based on wheat husks (BF-PP-wheat) and millet husks (BF-PP-millet). We found that the

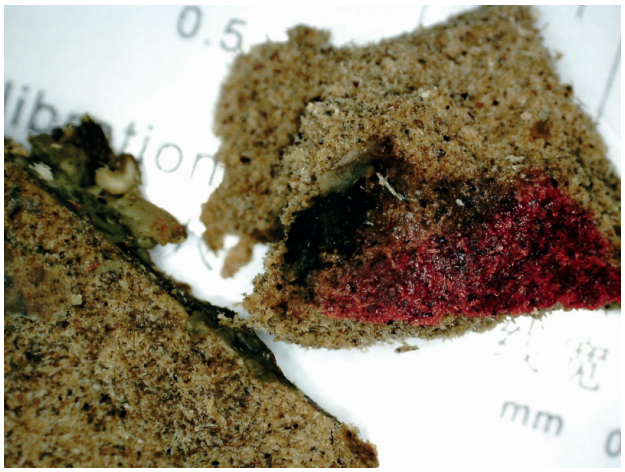

Figure 1 Pigmented colonies of microorganisms in binder-free wheat-based plant plastic after 21 days of exposure in active soil

exposure in active soil caused significant visual changes in both wood and plant samples. On average, 60% of BF-PP-millet, 58% of BF-PP-wheat, and 47% of BF-WP samples showed pronounced longitudinal and transverse splitting, edge swelling, and loosening in thickness. They also had micro- and macrocavities, especially along the edges and in the splitting areas. The defects varied from 1.5 to 5.5 mm.

All the samples featured microscopic signs of morphological changes in the plant particles: edge fibrillation, fragmentation and destruction of individual husk and sawdust particles, focal darkening, and microcavities of different size between the particles. Surface sliming and signs of mold growth were also found in all of the samples. In particular, multiple large colonies of mold fungi in different stages of maturity were present in 74% of BF-PP-millet, 85% of BF-PP-wheat, and 62% of BF-WP samples (Fig. 1).

On the whole, the visual signs of biological degradation were more pronounced in the plant-based samples. The sawdust-based samples had mainly edge and surface changes that hardly affected the middle.

The exposure in active soil had a negative effect on the physical and mechanical properties of the control samples, which were not treated with antiseptic compounds. The sawdust-based samples showed a decrease in hardness by 66%, elasticity number by 43%, compression elasticity modulus by 76%, breaking stress by 64%, and yield stress by 64% (Fig. 2).

The plant plastics based on wheat and millet husks had similar changes, namely a decrease in hardness by 62 and 70%, elasticity number by 46 and 47%, compression elasticity modulus by 73 and 80%, breaking stress by 60 and 68%, and yield stress by 60 and 68%, respectively.

The highest average of flexural strength was in the sawdust BF-WP samples (4 MPa) and the lowest was in the wheat BF-PP samples (1 MPa). Water absorption and swelling had the lowest values in the millet BF-PP samples (85%) and the highest values in the BF-WP and wheat BF-PP samples (94 and 96%, respectively).

Biostability tests showed a high biodegradability potential of all the samples. Biostability can be increased by changing the process parameters (pressing temperature, pressure, and time) [18]. However, antiseptic treatment is the main way to reduce biodegradation. An antiseptic component can be either added to the raw mixture or applied to the finished product. Thus, antiseptic treatment is a prerequisite for using binder-free wood and plant plastics in highly bioactive conditions, i.e., in an aggressive microbial destructive environment.

At the next stage, we treated the experimental plastics with antiseptics by adding them to the press material or applying to the surface to protect the

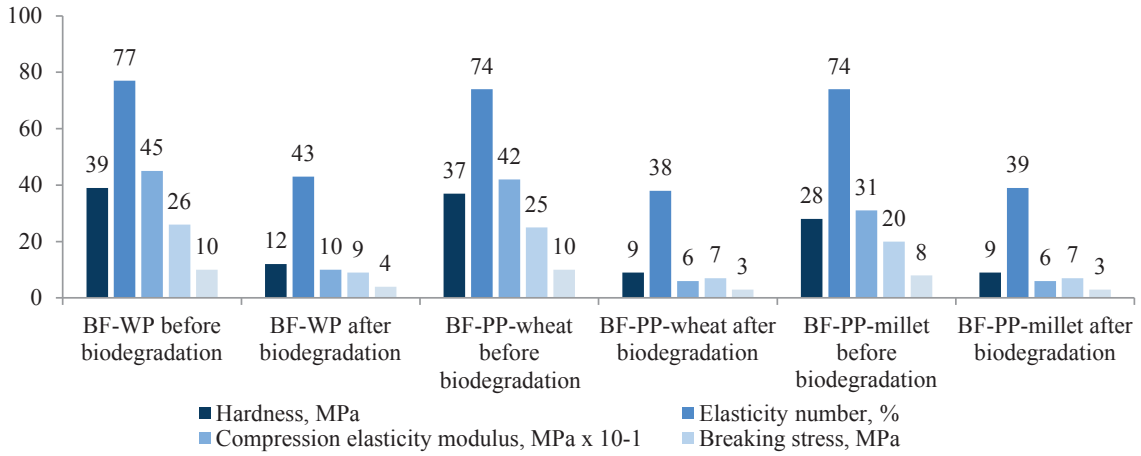

Figure 2 Changes in physical and mechanical properties of binder-free wood and plant plastics BF-WP and before and after biodegradation in active soil

Table 1 Physical and mechanical properties of binder-free wood plastics protected with antiseptic coating (biostability tests)

Physical and mechanical properties	Control			Antiseptic coating								
				Water repellent			Copper sulfate			Forwood antiseptic		
	Week											
	1	2	3	1	2	3	1	2	3	1	2	3
Flexural strength, MPa	3.4	2.6	2.0	3.5	2.8	2.6	3.2	2.8	2.7	3.2	2.3	2.2
Hardness, MPa	8.6	8.6	8.6	17.2	9.1	9.0	9.0	8.8	8.9	9.2	9.0	8.9
Elasticity number, %	40	39	39	65	38	37	41	41	41	51	42	37
Compression elasticity modulus, MPa	62	58	58	156	64	63	63	61	61	64	62	62
Breaking stress, MPa	6.4	6.4	6.4	12.3	6.8	6.7	6.7	6.6	6.7	6.8	6.7	6.7
Yield stress, MPa	2.6	2.6	2.6	4.9	2.7	2.7	2.7	2.6	2.7	2.7	2.7	2.7
Water absorption in 24 h, %	82	82	95	49	55	56	67	72	71	54	76	84
Swelling in thickness in 24 h, %	6.1	6.8	8.4	3.7	6.5	7.5	5.5	7.0	7.4	5.6	7.6	8.1

material from biodegradation, improve its biostability, and reduce its biodegradation potential. The samples' physical and mechanical properties were analyzed before and after biostability tests. We found that these properties were affected by the type and method of antiseptic administration.

The BF-WP samples had the worst indicators when a water repellent was introduced directly into the press material. In particular, there was an average decrease in flexural strength by 49%, hardness by 14%, water absorption by 30% (after 24 h), and swelling in thickness by 1.5% (after 24 h).

This might be explained by the disturbed formation of supramolecular bonds between the mixture particles during pressing. Lignin was present in the liquid phase of the mixture and the water repellent distributed it on the surface of the particles, providing them with hydrophobic properties. Thus, this modification became a structural and mechanical factor that interfered into the formation of bonds between the particles. However, when applied to the surface of the BF-WP samples, the water repellent improved their physical and mechanical properties by an average of 1–10%.

The best indicators were found in those BF-WP samples which were protected with copper sulfate introduced into the press material. They had the highest values of hardness, compression elasticity modulus, and breaking stress compared to all the other experimental (protected) and control (unprotected) samples.

A similar picture was observed in the binder-free plant plastics. Introduced into the press mixture, the water repellant caused a sharp deterioration in flexural strength and water absorption (by 10 and 11%, respectively). When applied to the surface, it improved these properties by 10 and 14%, respectively. Copper sulfate that was introduced directly into the press mixture increased the strength indicators (flexural strength by 14%, hardness by 49%), but reduced water resistance (water absorption rose by 23% and swelling by 28%). These effects must be taken into account when formulating binder-free, antiseptically protected plastics based on wood and plant materials.

The experimental BF-WP and BF-PP samples were exposed in active soil for 21 days and then tested for biostability. Our analysis of the physical and mechanical properties of the control (unprotected) and experimental BF-WP samples showed a significant decrease in strength indicators. The greatest decrease in flexural strength (by 39%) was found in the controls. This indicator fell by 29% in the samples treated with

Table 2 Physical and mechanical properties of binder-free wood plastics protected with an antiseptic introduced into the press mixture (biostability tests)

Physical and mechanical properties	Control			Antiseptic introduced into the press mixture					
				Water repellent			Copper sulfate		
	Week								
	1	2	3	1	2	3	1	2	3
Flexural strength, MPa	3.4	2.6	2.0	1.1	1.1	0.8	4.7	3.9	3.7
Hardness, MPa	8.6	8.6	8.6	8.9	8.4	8.4	16.2	14.1	10.3
Elasticity number, %	40	39	39	50	45	41	48	34	33
Compression elasticity modulus, MPa	62	58	58	61	57	57	146	121	76
Breaking stress, MPa	6.4	6.4	6.4	6.6	6.3	6.3	11.5	10.1	7.6
Yield stress, MPa	2.6	2.6	2.6	2.7	2.5	2.5	4.6	4.1	3.1
Water absorption in 24 h, %	82	82	95	110	115	115	44	47	51
Swelling in thickness in 24 h, %	6.1	6.8	8.4	7.5	7.7	9.8	4.2	4.5	4.5

Table 3 Physical and mechanical properties of binder-free plant plastics protected with antiseptic coating (biostability tests)

Physical and mechanical properties	Control			Antiseptic coating								
				Water repellent			Copper sulfate			Commercial antiseptic		
	Week											
	1	2	3	1	2	3	1	2	3	1	2	3
Flexural strength, MPa	2.0	1.8	1.3	2.5	1.7	1.1	2.1	2.0	1.6	1.6	1.2	1.0
Hardness, MPa	8.7	8.7	8.3	9.1	8.7	8.7	8.8	8.6	8.5	8.9	8.7	8.4
Elasticity number, %	36	36	41	33	38	39	39	40	40	36	38	40
Compression elasticity modulus, MPa	60	59	56	64	60	59	60	59	58	62	60	57
Breaking stress, MPa	6.5	6.4	6.2	6.8	6.5	6.5	6.6	6.4	6.4	6.7	6.5	6.3
Yield stress, MPa	2.6	2.6	2.5	2.7	2.6	2.6	2.6	2.6	2.6	2.7	2.6	2.5
Water absorption in 24 h, %	89	113	158	90	121	151	103	119	151	88	101	122
Swelling in thickness in 24 h, %	7.5	7.9	9.6	5.2	5.7	7.8	5.2	6.0	7.5	7.4	7.5	7.9

Table 4 Physical and mechanical properties of binder-free plant plastics protected with an antiseptic introduced into the press mixture (biostability tests)

Physical and mechanical properties	Control			Antiseptic introduced into the press mixture					
				Water repellent			Copper sulfate		
	Week								
	1	2	3	1	2	3	1	2	3
Flexural strength, MPa	2.0	1.8	1.3	0.4	0.3	0.3	2.0	0.6	0.3
Hardness, MPa	8.7	8.7	8.3	8.9	8.9	8.9	8.9	8.8	8.7
Elasticity number, %	36	36	41	37	38	38	34	38	38
Compression elasticity modulus, MPa	60	59	56	62	61	61	62	60	60
Breaking stress, MPa	6.5	6.4	6.2	6.7	6.6	6.6	6.7	6.6	6.5
Yield stress, MPa	2.6	2.6	2.5	2.7	2.7	2.7	2.7	2.6	2.6
Water absorption in 24 h, %	89	113	158	161	203	228	135	135	171
Swelling in thickness in 24 h, %	7.5	7.9	9.6	5.1	5.2	5.3	5.3	5.4	5.6

the Forwood antiseptic and by 26% in the samples with a water repellent introduced into the press mixture (Tables 1 and 2).

Flexural strength had the smallest losses in the samples protected with copper sulfate, namely 15% for the coated sample and 21% for the sample with a modified press mixture.

Hardness was the highest in the wood plastics treated with the water repellent and those with the copper sulfate-modified press mixture, namely 17.2 and 16.2 MPa, respectively, on the eighth day of exposure in active soil. However, it was these samples that had the greatest loss of hardness by the end of the biostability test, by 48 and 36%, respectively. Yet, this indicator remained the highest in the samples with added copper sulfate (10.3 MPa).

Water absorption had the highest values in the samples with an added water repellant, averaging 115% after three weeks of exposure. The lowest values were in the samples with added copper sulfate, namely 51% by the end of the tests (a loss of 16%).

The plant plastics also showed changes in their physical and mechanical parameters. The smallest loss (24%) of flexural strength over three weeks of exposure in active soil was found in the samples with an added water repellent, although they had one of the lowest values (0.4 MPa) in the first week. On the eighth day, this indicator was the highest in the samples coated with a water repellent (2.5 MPa), decreasing by 53% to 1.1 MPa by the end of the test (Table 3). The hardness indicator decreased in all the plant samples within three weeks of exposure in the range of 1–6%.

Daily water absorption was the highest in the samples with an added water repellent, amounting to 228% after three weeks of exposure in active soil, with a 42% decrease of water absorption. By the end of the biostability tests, the lowest water absorption was in the samples coated with the Forwood antiseptic (122%).

CONCLUSION

Our study showed high biodegradation and low biostability of the binder-free wood and plant plastics based on sawdust, and wheat and millet husks. Therefore, antiseptic protection is required to improve their performance. Their exposure in a bioactive environment caused some morphological and structural changes, as well as affected their physical and mechanical properties.

We found that the plant-based plastics underwent a more pronounced degradation in active soil than the sawdust-based plastics. According to our results, the samples' resistance to biodegradation was determined by such process parameters as the type of filler

and antiseptic, as well as the method of antiseptic administration.

We treated the plastics with three types of antiseptic (water repellent, copper sulfate, and Forwood) by adding them to the press mixture or applying them to the surface. Both methods changed the initial properties of the samples. When used as a coating, the water repellent improved the samples' physical and mechanical properties. When added to the press mixture, however, it significantly impaired their strength and water resistance.

Copper sulfate showed the best effect among those antiseptics introduced into the press mixture. It decreased the flexural strength of the sawdust-based samples by 5% and increased their hardness, water absorption, and swelling. The plant-based samples with added copper sulfate showed better strength indicators, but lower water resistance. Thus, the antiseptic treatment of binder-free plastics based on wood or plants affects a number of their key physical and mechanical properties and should be administered with regard to expected performance conditions.

CONTRIBUTION

All the authors were equally involved in developing the research concept, obtaining and analyzing the data, and writing the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Fermented oat milk as a base for lactose-free sauce

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Abstract:

Introduction. We studied the use of fermented oat milk to produce sauce and evaluated its properties. The research was motivated by the current demand for so called "plant milk" commonly perceived as an alternative to cow's milk.

Study objects and methods. The experimental samples were produced from oats-based drinks (1.5 and 3.2% fat) fermented with starter cultures of lactic acid microorganisms following the guidelines for yoghurt production. Apple pectin was used as a thickener. Rheological studies were performed using an RM-1 rotational viscometer and a CT-2 texture analyzer according to the standard methods. Sensory evaluation was based on a scoring scale. Physicochemical parameters were determined according to generally accepted methods.

Results and discussion. Oat milk was fermented to produce a sauce base. Acid accumulation increased throughout fermentation up to 135–137°T. Apple pectin (3%) was added to stabilize the structure and ensure the desired consistency. Higher concentrations of pectin increased the hardness and adhesive strength of the samples from both 1.5 and 3.2% oat milk. The 1.5% sauce scored highest in the sensory evaluation. Its physicochemical indicators met the standard requirements for related fermented milk products. We found the best consistency indicators at a pectin concentration of 3%.

Conclusion. The new fermented sauce based on low fat oat milk (1.5% fat) had high consumer appeal as well as physicochemical, sensory, and rheological characteristics. The sauce can be used by people with lactose intolerance and vegetarians.

Keywords: Oats, plant milk, sauce, fermentation, apple pectin, rheology, lactose-free

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INTRODUCTION

The relationship between nutrition and disease is in the spotlight of modern agenda [1, 2]. Half of global health risks identified by the World Health Organization are related to diet, including increased sodium and cholesterol intake, iron and zinc deficiency, etc. Therefore, new food products should be designed not only to satisfy hunger and provide the body with basic nutrients, but also to prevent nutrition-related diseases and improve our physical and emotional well-being [3].

On the one hand, consumers are becoming better aware of the relationship between food composition and health. These are both young people with an active lifestyle and older people who want to maintain and improve their quality of life. On the other hand, the need for special or alternative foods may be associated with a physiological inability to assimilate some vital food components. For example, people with allergies, whose number is on the rise, should avoid gluten, casein, lactose, and other components found in most balanced and affordable ingredients and products (flour, bread, milk, etc.). All this speaks to the relevance of recent research in this field [4–7].

The rising demand for healthy foods and eco-friendly bio-products has an immediate effect on the food market [8, 9]. New types of products are being developed that are enriched with individual components and multifunctional additives [10, 11].

Plant-based milk is a good example of this trend. Made from nuts or grains, it has a long history in both Eastern and Western cultures. Soy milk is the most popular type, although the demand is also growing for almond, coconut, rice, and oat milk. European countries, especially Spain, boast a 20% rise in the annual sales of soy milk and other non-dairy beverages. In 2011, the USA sold $1.3 billion worth of milk from soy, almond, rice, and other plants, and their sales have been growing ever since [12, 13].

Table 1 Oat milk profile*

Sample	Protein, %	Carbo-hydrates, %	Energy, kcal	Ingredients
Oat milk, 1.5% fat	1.0	6.5	45	Water, oat flour, rapeseed oil, calcium (tricalcium phosphate), vitamins D_2, and B_2
Oat milk, 3.2% fat	1.0	6.5	60	

Note: manufacturer's information (www. https://ne-moloko.ru)

Plant-based milk is currently marketed in a wide range of formulations: flavored, sweet, low fat, fortified, etc. It is a good alternative for people with a cow's milk allergy or lactose intolerance. In addition, this product can attract people who do not consume food of animal origin for religious or other reasons, for example, vegans and vegetarians.

Noteworthily, researchers are also searching for ways to improve plant milk, using biotechnology or fortifying it with additional components [14–23].

Following the global trend, the Russian food market is becoming more active in satisfying the new consumer demands. Along with the traditional criteria of consumer choice (price, taste, and quality), new criteria are emerging to indicate new models of consumer behavior. They include an increased interest in one's health and lifestyle, product safety, and others. A healthy lifestyle is gradually ceasing to be a fashionable trend and becoming a matter of routine.

Taking into account the demand for these products on the food market and a lack of information on them, we aimed to expand on the prospects of using plant materials to produce new types of foods. We selected oatmeal or oat flakes as the most suitable material. Our choice was based on its availability, chemical composition, nutritional and biological potential, and common use by the Russian population.

Oatmeal is obtained from processing oats. It contains vitamins A, E, K, and group B, as well as potassium, calcium, magnesium, phosphorus, sodium, and zinc. Oatmeal is rich in fiber and high-quality plant protein that helps construct body cells. Its protein is easily digestible and has a balanced amino acid composition. Also, oats-based products protect the walls of the stomach and reduce the acidity of gastric juice, which is important for people with gastrointestinal diseases: gastritis, peptic ulcer, or flatulence [24].

Historically, oatmeal was used in Russia to prepare not only hot dishes, but also kissel and other medicinal beverages. Therefore, oats-based products are still highly appealing. In addition, people like their pleasant smell, color, and a sweet taste.

Currently, we see a growing range of food products with a scientifically grounded composition and target properties. Particular attention is paid to products that have a positive effect on the human body. However, there is a lack of new formulations for foods with a wide range of applications, such as sauces. Sauces can improve the chemical composition and sensory properties of dishes, improve their digestion, etc. [25–27]. In addition, they are becoming an integral part of our daily diet, accompanying almost every dish.

Mayonnaise and ketchup are still the most popular sauces, both in public catering and at home. However, there is a new trend towards reducing the production and consumption of high-fat mayonnaise motivated by the modern food culture and the promotion of a healthy lifestyle [28].

Low-fat salad dressings, which appeared in the 1930s as an alternative to mayonnaise, only became popular at the end of the 20th century, especially in Western countries. In Russia, their use is limited to restaurants with foreign cuisine. Most Russians still know very little about this product.

Therefore, there is a need to formulate new sauces using various ingredients, including plant-based materials, and methods of treatment, including biotechnology. In our work, we aimed to formulate sauce based on oat milk and analyzed its sensory, physicochemical, and rheological parameters.

STUDY OBJECTS AND METHODS

The experimental samples were based on oat milk (1.5 and 3.2% fat, Nemoloko, Russia), with a commercial apple pectin (Pektowin, Poland) used as a thickener (Table 1).

The samples were fermented using the yoghurt technology [29]. The starter culture contained *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *Bulgaricus*, *Lactobacillus acidophilus*, *Bifidobacterium lactis*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus paracasei*, and *Bifidobacterium infantis*. It was introduced in line with the manufacturer's recommendations (https://vivostarters.com). The samples were fermented for 12 h at 38–40°C and then refrigerated for 24 h for curd to form.

The control sample was obtained by fermenting oat milk. The experimental samples were fermented oat milk samples with 2, 3, and 4% of pectin. The physicochemical analysis of the materials and the fermented samples was in line with the guidelines [30]. The rheological studies were carried out using an RM-1 rotational viscometer and a CT-2 texture analyzer according to the generally accepted methods [31, 32]. The sensory evaluation used the weight coefficients and the methods described by Glebova *et al.* and Khrundin *et al.* [33, 34].

All the experiments were performed in at least three repetitions, followed by statistical processing.

RESULTS AND DISCUSSION

To determine the degree of fermentation, we measured active and titratable acidities in 1.5% (Fig. 1a) and in 3.2% oat milk (Fig. 1b).

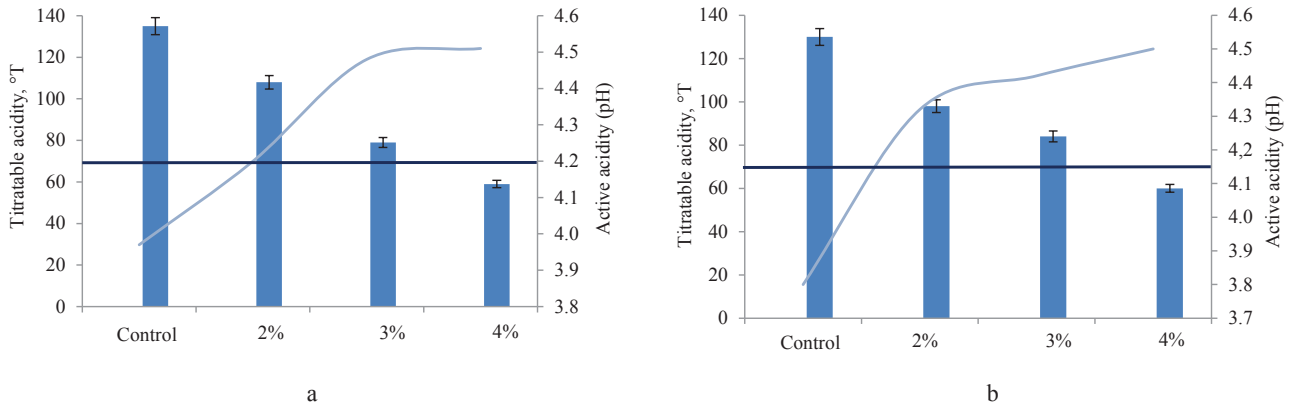

Figure 1 Acidity of control (pectin-free) and experimental (with 2, 3, and 4% of pectin) samples obtained from 1.5% oat milk (a) and 3.2% oat milk (b)

Both the 1.5 and 3.2% samples showed active acid accumulation (60–135 °T), which was within the standard values for similar fermented milk products (Fig. 1). Two of the experimental samples (with 2 and 3% of pectin) reached the threshold acidity of 75 °T, apart from the control. However, the sample with 4% of pectin also had certain features of a fermented product (characteristic smell, taste, and the presence of curd). Therefore, taking into account the product's composition, we can make correction for acidity. We found an increase in titratable acidity and a decrease in pH in both the 1.5 and 3.2% samples.

The physicochemical indicators of the control samples (Table 2) showed their similarity to the traditional fermented milk products.

Earlier studies showed layering and unstable curding in the oat milk samples, despite adequate fermentation [34].

When the samples stabilized following the introduction of pectin, we analyzed their physicochemical and sensory parameters, as well as structural and mechanical properties.

The physicochemical parameters of the experimental oat milk samples are shown in Table 3, respectively.

Table 2 Physicochemical properties of the control samples

Sample	Fat, %	Protein, %	Dry matter, %	Density, kg/m^3	Titratableacidity, °T	Active acidity (pH)
1.5% milk	2.01	2.79	8.43	1024.69	137	4.58
3.2% milk	2.89	2.32	9.37	1021.68	135	4.55

Table 3 Physicochemical properties of samples from 1.5 and 3.2% oat milk

Sample	Fat, %	Protein, %	Dry matter, %	Density, kg/m^3
1.5% oat milk				
Control (pectin-free)	2.01	2.79	8.43	1024.69
2% of pectin	1.81	3.18	8.66	1027.73
3% of pectin	1.88	3.85	8.16	1031.12
4% of pectin	1.97	3.70	9.06	1031.34
3.2% oat milk				
Control (pectin-free)	2.89	2.32	9.37	1021.68
2% of pectin	2.95	2.78	9.70	1025.16
3% of pectin	3.01	3.04	10.00	1027.06
4% of pectin	2.97	3.24	10.00	1028.58

Table 4 Sensory evaluation of oat milk samples

Parameter	Description
Appearance and consistency	Uniform, not viscous enough
Taste and smell	Pure and typical of fermented milk, with a pronounced taste and smell of a plant
Color	Uniformly white

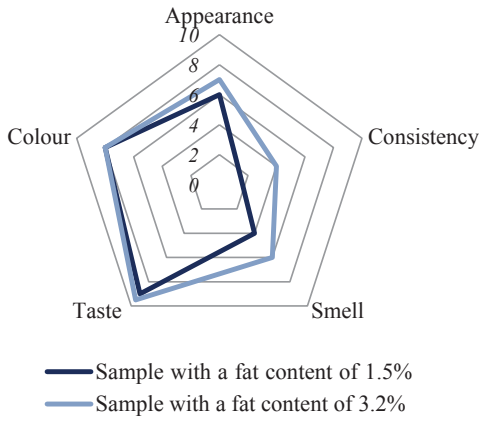

Figure 2 Sensory profile of experimental oat milk samples

Table 5 Effective viscosity of samples from 1.5 and 3.2% oat milk

Sample	Time of measurement, s					
	0	30	60	90	120	180
1.5% oat milk						
Control (pectin-free)	240	30	30	30	30	30
2% of pectin	280	160	150	150	150	150
3% of pectin	380	410	400	400	390	390
4% of pectin	860	880	870	830	830	830
3.2% oat milk						
Control (pectin-free)	120	20	20	20	20	20
2% of pectin	110	70	60	60	60	60
3% of pectin	140	130	130	120	120	120
4% of pectin	780	760	760	760	740	740

As we can wee, the physicochemical parameters of the samples were generally satisfactory and corresponded to those of the raw materials they were made from. We found a slight increase in protein in the experimental samples, which is associated with a higher content of pectin in them.

The sensory evaluation (Table. 4, Fig. 2) revealed a positive evaluation of all the parameters, except for consistency. It was marked as an indistinct liquid, which is not typical for this kind of products.

Fermented products are structured systems whose disperse phase particles interact with each other to form a network and give the system more or less pronounced properties of a solid. The resulting curd (gel) has certain mechanical properties: viscosity, plasticity, elasticity, and strength.

The textural and structural properties of food products are important indicators of the manufacturing process, the quality of the end product, its shelf life, etc. [35, 36]. We found it worthwhile to study the rheological characteristics of the fermented oat milk samples since there has been very little research in this area.

We performed preliminary measurements of shear stress to select optimal parameters for a better interpretation of experimental data. As a result, we chose to use rotor No. 3 at a spindle speed of 60 min^{-1} in the range from 0 to 180 s. Effective viscosity measurements are shown in Tables 5.

Table 6 Rheological characteristics of oat milk samples

Consistency indicators	Pectin concentration,%			
	Control (pectin-free)	2% of pectin	3% of pectin	4% of pectin
1.5% oat milk				
Hardness, g (*Fmax*, g)	17.0	18.3	20.0	20.7
Adhesive strength, g	3.4	3.9	4.2	5.5
General deformation, mm (h_{gen}, mm)	7	7	7	7
Elastic deformation, mm (h_{el}, mm)	6.461	6.454	6.219	4.844
Plastic deformation, mm (h_{pl}, mm)	0.539	0.546	0.781	2.156
Elasticity (Δh)	0.92	0.92	0.89	0.69
Relaxation depth, g	0.7	0.7	1.6	3.8
3.2% oat milk				
Hardness, g (*Fmax*, g)	17.4	17.2	19.1	31.8
Adhesive strength, g	3.9	4.1	4.5	8.9
General deformation, mm (h_{gen}, mm)	7	7	7	7
Elastic deformation, mm (h_{el}, mm)	6.547	6.602	6.586	2.648
Plastic deformation, mm (h_{pl}, mm)	0.453	0.398	0.414	4.352
Elasticity (Δh)	0.94	0.94	0.94	0.38
Relaxation depth, g	0.70	0.70	1.10	12.60

*h_{gen}, h_{el}, h_{pl} – general, elastic, and plastic deformations were determined by a texture analyzer

Table 7 Quality indicators of fermented oat milk

Quality indicator	Description and content
Consistency and appearance	Homogeneous, moderately viscous
Taste and smell	Pure, fermented milk-like, sweetish, with a pronounced taste and smell of oats
Color	Creamy white, uniform
Titratable acidity, °T	85–90
Active acidity, pH	4.2–4.5
Viable microflora cells, CFU/cm^3	3.1–3.4×10^7
Escherichia coli bacteria in 50 g	n.d.
Yeast per 1 g	n.d.
Mesophilic anaerobic bacteria spores in 1 g	n.d.

n.d. – not detected

We found that the fermented oat milk samples acted in a similar way to traditional fermented milk products and could be classified as abnormally viscous liquids. Initially, they displayed predominantly pseudoplastic properties which over time became more characteristic of Newtonian fluids.

Then, we analyzed the samples' hardness, adhesive strength, deformation indexes, and relaxation depth as the most important structural and mechanical characteristics of the product's internal structure and consistency (Table 6).

As can be seen in Table 7, higher pectin concentrations increased the hardness and adhesive strength of both 1.5 and 3.2% oat milk samples. This might be caused by the interaction of pectin and oat milk components, primarily fat, resulting in a well-developed spatial gel-like structure. The samples with a higher fat content (3.2%) showed a doubled adhesive strength due to pectin's ability to form stable emulsions with strong interfacial boundaries.

Table 8 Experimental formulation of the sauce based on fermented oat milk

Ingredient	Contents of ingredients for sauce formulations, %	
	Experimental	Control
1.5% fermented base	50.0	–
1.5% natural yoghurt	–	50.0
Olive oil	–	10.0
Dill	15.0	15.0
Cucumber	32.0	22.0
Salt	2.0	2.0
Black pepper	1.0	1.0
Total	100.0	100.0

Elasticity is the product's ability to deform under load, while relaxation depth indicates its ability to recover after load removal. These indicators are shown in Figs. 3 and 4.

As we can see in Fig. 3, increasing the pectin concentration to 3% hardly changed the samples' elasticity due to stress. However, its value sharply decreased at a 4% pectin content, especially in the 3.2% samples. This was probably because pectin thickened the emulsion and caused a denser curd to form. Changes in the relaxation depth indexes confirmed that finding (Fig. 4). We found that higher concentrations of pectin improved the samples' ability to restore their original structure after the load was removed. Noteworthily, all the textural indicators had maximum values at a pectin concentration of 4%.

Thus, our experiments confirmed that fermented oat milk could be used to develop new food products.

Further, we aimed to design a sauce based on fermented oat milk. In line with the current trend towards lower fat intake, we chose to use 1.5% milk.

The quality indicators of the fermented oat milk-based sauce are presented in Table 7.

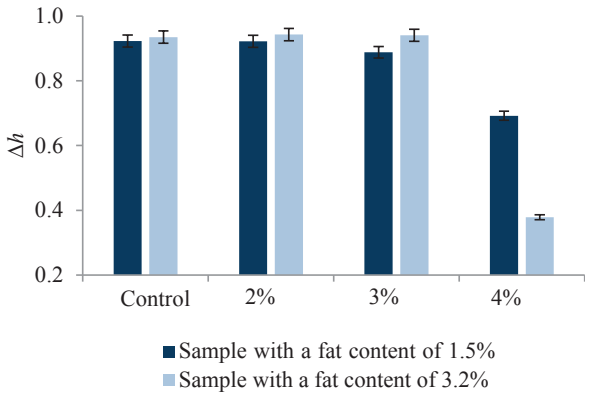

Figure 3 Elasticity of the control (pectin-free) and experimental (2, 3, and 4% of pectin) samples

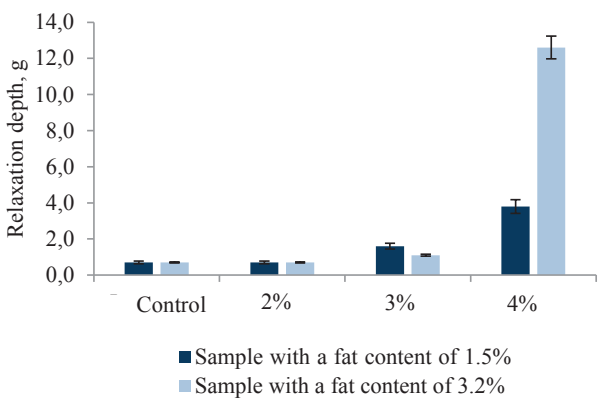

Figure 4 Relaxation depth of the control (pectin-free) and experimental (2, 3, and 4% of pectin) samples

Table 9 Sensory scores for the sauce based on fermented oat milk, points

Indicator	Sauce samples	
	Experimental	Control
Appearance	0.43	0.50
Color	0.5	0.5
Consistency	1.30	1.50
Smell	0.65	0.71
Taste and aftertaste	1.12	1.15

We formulated an experimental sauce using literature data [37–39]. Traditional (or Greek) yogurt sauce was used as a control. The formulations and ingredient ratios for the control and experimental sauces are presented in Table 8.

Sensory evaluation is key to quality control for this type of products. Sauces are currently evaluated according to State Standard 31986-2012. First, a sauce is poured in a thin stream to analyze its consistency and taste. It is followed by the evaluation of its color, the shape of slices, the consistency of fillers, as well as smell and taste. However, this evaluation is rather vague since it does not provide any quality gradation or objective quantitative analysis. Nor does it determine the product's quality level or identify its defects. Therefore, we applied the categorization method. In particular, we categorized the samples based on their score in a single or group sensory indicators (Table 9).

The use of fermented oat milk instead of cow's milk did not affect the sauce's sensory properties. Its consistency and appearance scored even higher than the control.

The quality indicators of the sauce based on fermented oat milk are presented in Table 10. As we can see, the sauce appealed to the consumers and showed stability during storage. In addition, it has a low energy value and therefore can be used in a low-fat diet.

Table 10 Quality indicators of the sauce based on fermented oat milk

Indicator	Description and content
Appearance, consistency	Homogeneous, sour cream-like, slightly viscous, jelly-like consistency with inclusions
Taste and smell	Slightly pungent and sour, typical of flavoring additives
Color	From white to creamy green, homogeneous or affected by the additives
Fat, %	0.8 ± 0.1
Protein, %	2.0 ± 0.1
Emulsion stability (% of unbroken emulsion), at least	97
Energy value, kcal	70

CONCLUSION

Our work showed a possibility of obtaining a new fermented product from plant-based materials. We studied the fermentation of, and acid accumulation in, oat milk. Then, we analyzed the samples' sensory, physicochemical, and rheological characteristics.

As a result of our experiments, we designed a sauce based on fermented oat milk, with 3% pectin used as a thickener. Various combinations of the ingredients in our formulation can create new food systems with high consumer appeal and thus expand the range of food products.

Further research is needed to get a clearer picture of the curds obtained. For example, in dishes with sauces, culinary treatment methods are just as important as the sauce's consistency and stability during storage. This will be the focus of our future study.

CONTRIBUTION

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

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Bovine serum albumin with gallic acid: Molecular modeling and physicochemical profiling

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Abstract:
Introduction. Gallic acid is a biologically active natural compound with strong antioxidant properties. Gallic acid is highly soluble and stable. It is known to increase the thermal stability of protein. However, its bioavailability is low, but interaction with proteins can solve this problem. Bovine serum albumin can bind various ligands, including polyphenols. The resulting complex of gallic acid and bovine serum albumin can become a promising functional food additive. *Study objects and methods.* This research featured in silico molecular modeling of gallic acid and bovine serum albumin using the HyperChem program. The methods of infrared spectrometry, potentiometry, and sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) made it possible to describe the physicochemical profile of the complex. *Results and discussion.* The molecular modeling confirmed that hydrophobic interactions were responsible for the chemical bond between gallic acid and bovine serum albumin. The SDS-PAGE test showed that the protein molecule remained intact. The reducing properties of the complex grew as the concentration of gallic acid increased. At 100 mg/L of gallic acid, the reducing properties were 7.8 ± 1.3 mg/L equivalent of gallic acid. At 200 and 300 mg/L, the values reached 15.90 ± 2.65 and 23.30 ± 5.05 mg/L, respectively. The IR spectrometry revealed a significant difference between the samples with different concentrations of gallic acid. *Conclusion.* The research managed to predict the properties of the complex of bovine serum albumin and gallic acid during its formation. The resulting complex had the highest reducing properties at 0.69 g of bovine serum albumin and 300 mg of gallic acid. The obtained parameters can be used in the food industry to develop new food additives.

Keywords: Serum proteins, SDS-PAGE, IR spectroscopy, amperometric detector, polyphenols

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INTRODUCTION

Bovine serum albumin is a small and stable protein. Its molecular weight is 66.4 kDa. This protein predominates in bovine blood, where it is responsible for maintaining blood oncotic pressure and transporting various substances [1]. In milk, the amount of serum proteins is about 0.7–1.3% of the total mass [2]. The food industry obtains bovine serum albumin from animal blood and uses it to control such technological aspects of production as foam formation and gelation, emulsification, and encapsulation [3–7]. Bovine serum albumin has a number of important functional properties, e.g. an extensive profile of essential amino acids [8]. It is also known to inhibit lipid peroxidation in in vivo studies. In addition, it can reversibly bind most ligands [9, 10]. When it binds to polyphenols, bovine serum albumin changes its thermal stability, light absorption in the UV and visible range, the number of free amino groups and thiol groups, as well as secondary structure [11, 12].

Polyphenols are secondary metabolites of plant origin. They are found in plant materials and serve as

quality markers of fruits and vegetables [13]. Phenolic compounds proved beneficial in human diet [14]. They prevent obesity and reduce the risk of diabetes and coronary heart disease [15–17]. Phenolic compounds inhibit oxidative stress and free radicals while increasing molecular thermal stability [18, 19]. When binding, polyphenol molecules form complexes with oppositely charged biopolymers [20]. This property can protect proteins from oxidation by occupying free amino acid residues in the protein, thus solving the problem of antioxidant delivery [21].

Gallic acid is a hydrolyzed tannin found in many fruits and vegetables. Green and black teas are especially rich in gallic acid, where it is represented by gallates, e.g. epigallocatechin-3-gallate, epicatechin-3-gallate, epigallocatechin, etc. Gallic acid has antimicrobial, anti-inflammatory, antitumor, and antioxidant properties [22, 23].

The final reaction product of gallic acid and bovine serum albumin can exhibit different properties of protein and polyphenol, which makes it a promising food additive in complex food systems. The food industry demonstrates a growing interest in this research area, but the interaction patterns between gallic acid and bovine serum albumin still remain understudied.

The research objective was to establish the interaction patterns between bovine serum albumin and gallic acid using molecular modeling, as well as to identify how gallic acid affects the protein.

STUDY OBJECTS AND METHODS

Molecular modeling. Molecular modeling (HyperChem) helped predict the chemical bonds and energy state of the complex of gallic acid and bovine serum albumin. It featured the conformational structures typical of proteins in polypeptide chain sequences and active centers. To study the properties of gallic acid, we applied the AMBER method, while the Polak-Ribière method was used to simulate the potential energy of the system [24, 25]. Bovine serum albumin was represented by a hydrated 4F5S molecule from the Protein Data Bank with pre-calculated minimum-energy conformer, and a three-dimensional electrostatic potential map [26]. The PockDrug open web server was used to detect the binding sites [27]. To predict the interaction pattern, the AutoDock Vina calculated the charges in the molecule by the Gasteiger method [28, 29].

Bovine serum albumin molecule depends on the pH of the medium and exists in several isomeric forms of conformers E, N, F, and B. The E-form (unfolded) is the main isomer at low pH (< 4.0). As pH reaches 4.0–4.5, the F-form (fast) of bovine serum albumin appears as a result of the rapid N→F transition. The transition occurs as domain III unfolds. It has leveled α-helical structures. B-isomer appears at pH = 7.0–9.0 and subsequently causes A-isomer to appear, which is an aggregated form of bovine serum albumin [30]. The experiment featured neutral pH (7.4) because it is in this environment that bovine serum albumin is found in blood plasma in its stable B-form [26].

Formation of the complex of bovine serum albumin and gallic acid. Gallic acid belongs to food phenolic compounds with antioxidant effects and has low toxicity [31]. The amount of gallic acid to be added was determined according to the recommended intake of food and biologically active substances: 100 mg/L is adequate for human consumption, 300 mg/L being the upper limit [32].

The experiment included three concentrations of gallic acid (100, 200, and 300 mg/L), bovine serum albumin (0.69 g/L), neutral pH, and room temperature. Commercial bovine serum albumin (Diaem) was lyophilized and had a purity of ≥ 99.0%, while anhydrous gallic acid (Diaem) had a purity of ≥ 98.0%. Solutions included deionized water (Smart2Pure, Thermo Scientific, USA). Tris-HCl buffer (pH 7.4) was used as a solvent for bovine serum albumin. All solutions were kept in a refrigerator at 4°C for 12 h before the experiment.

Physicochemical methods for studying the complex of bovine serum albumin and gallic acid. The experiment involved sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), liquid chromatography with amperometric detection, and Fourier-transform infrared spectroscopy (FTIR).

SDS-PAGE. The test was based on Laemmli disk electrophoresis technique with denaturing agents [32]. Polymerization of the acrylamide gel featured a Bio-Rad vertical electrophoresis chamber (glass size = 10×10 cm, gel thickness = 1 mm). The concentration of the separating gel was 12.7%. The protein content was measured spectrophotometrically using the calibration curve of standard solutions of bovine serum albumin.

The experiment involved a bromophenol blue solution, a separating buffer, a dissociating mix based on β-mercaptoethanol, and a sample with a protein content of 10 μg. They were poured into tubes in equal measures. The resulting mix was stirred and incubated in water bath for 10 min. The electrophoresis occurred at 8–24 mA until the band of the leading dye reached the bottom of the gel. After the electrophoresis, the gel was placed in 30% trichloroacetic acid to fix proteins, followed by staining in Coomassie G-250 solution. The electrophoregrams were processed using the Gel Doc EZ gel (Image Lab, BioRad).

Amperometric method. The experiment involved a TsvetYauza 01-AA amperometric detector (Russia) with chromatogram registration. A solution of gallic acid was used as standard to evaluate the ability of the complex to enter into an oxidation (reduction) reaction at the electrode [34]. Gallic acid has stronger reducing properties than bovine serum albumin. As a result, gallic acid equivalent was used to measure the gallic acid interaction with the protein and to define the total antioxidant activity of the complex compared to gallic acid. Another experiment was conducted to

separate the complex from unreacted gallic acid. It involved a laboratory dialysis bag with a cut-off mass of 12–14 kDa.

FTIR spectroscopy. The transformation of chemical bonds was carried out in liquid samples using a Nicolet iS50 FTIR spectrometer (Thermo Scientific, USA) in an attenuated total internal reflector Smart iTR ATR (ZnSe) (Thermo Scientific, USA). Infrared radiation between two substances with different refractive indices creates a damped wave, which gradually penetrates the sample with each subsequent internal reflection. This technique analyzes objects in different states of aggregation and gives access to the structure and composition of the sample.

IR spectrometry is based on the ability of substances to absorb infrared radiation. Absorption occurs at the wavelengths with the energy that corresponds to the excitation energies of vibrational motions in molecules. Absorption bands indicate certain bonds in the compound under analysis. Absorption bands of amide groups I and II indicate the main conformations of a molecule, which marks the noncovalent addition reaction [35].

Statistical data analysis employed STATISTICA 10 software (StatSoft, Inc.).

RESULTS AND DISCUSSION

Molecular modeling. Molecular modeling determined interaction patterns between bovine serum albumin and gallic acid, as well as their binding sites. The energy of the gallic acid molecule was 1.984 kcal/mol; the energy gradient was 0.0997 kcal/mol. Figure 1 shows a three-dimensional electrostatic density map of bovine serum albumin that was constructed to determine the binding sites.

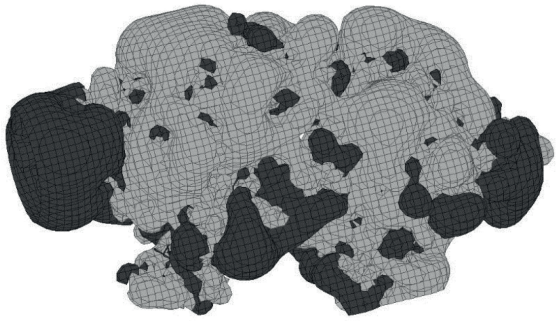

Figure 1 Electrostatic density map of bovine serum albumin: light gray – negatively charged area, dark gray – positively charged area

According to scientific publications, the molecule of gallic acid was to react along the negatively charged region, which was more favorable as far as energy was concerned. PockDrug data were used to confirm the hypothesis [27]. P23 appeared to be the suitable locus in bovine serum albumin. The ligand attached to a pocket of 530.57 angstroms. The hydrophobicity index was 2.28 on the Kyte-Dullittle scale, which means that the number of hydrophobic interactions was higher by the same number of times in this area, and the conformation was likely to stabilize [36]. The binding site was located deep inside the protein structure in a hydrophobic gap lined with the following amino acids: Lys 421, Glu 186, Asp 561, Gln 521, Asp 517, Arg 458, and Glu 424.

The binding energy was –5.6, –5.5, –5.4, –5.4, –5.3, –5.3, and –5.2 kcal/mol, respectively. The ligand was also located within this locus, thus stabilizing the conformation. The proposed interaction between gallic acid and bovine serum albumin was hydrophobic (Fig. 2). Such interactions increase the entropy of the system. As the temperature rises, their

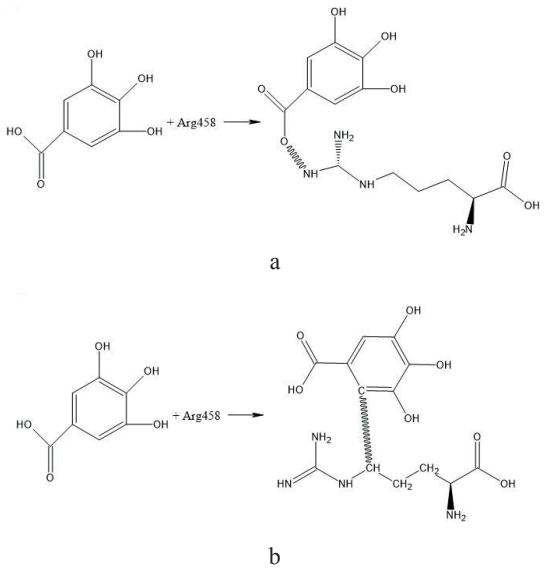

Figure 2 Modeled reaction between gallic acid and bovine serum albumin: (a) ionic reaction, (b) hydrophobic reaction

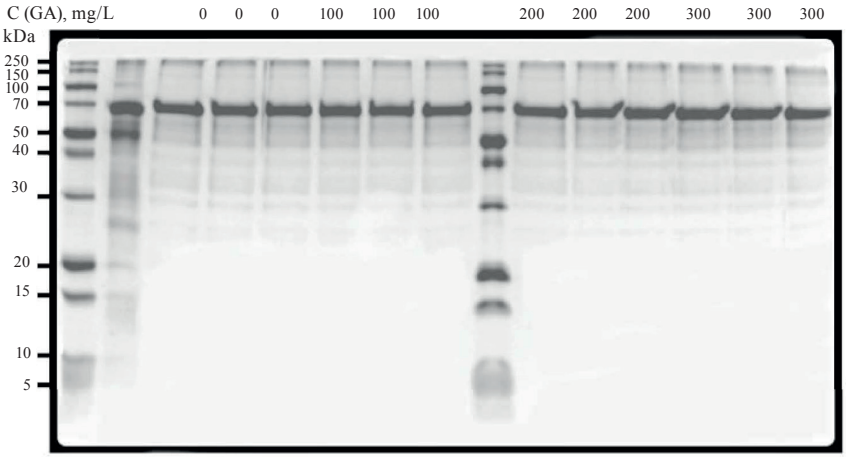

Figure 3 SDS-PAGE of the complex of bovine serum albumin and gallic acid in concentration of 100–300 mg/L (lanes 6–8, 10–12, and 13–15, respectively). Lanes 3–5 show bovine serum albumin standard (0.69 g/L)

number increases. In aromatic chains, such interactions reach their energy maximum already at 42°C, in aliphatic chains – at 60°C. Thus, temperature rise can lead to hydrophobic flocculation of the resulting complex and its subsequent precipitation. Therefore, the experiment was conducted at room temperature, and SDS-PAGE was used to monitor the state of the complex.

In protein, all carbon atoms not bound to polar atoms are hydrophobic, as are sulfur atoms in cysteine and methionine. This arrangement also opened up the possibility of other reactions because several ionic and polar residues were in the immediate vicinity of the binding site between gallic acid and bovine serum albumin.

The model confirmed the chemical bond between molecules of gallic acid and bovine serum albumin due to the polar regions of the protein with negatively charged regions and the hydrophobic regions of the protein. The bonds in the pocket can change the molecular bonds of the protein and reduce the reducing properties of gallic acid. The model showed changes in the secondary structure of the protein in the complex with gallic acid. Some gallic acid molecules react along the hydrophobic sites of bovine serum albumin, while others react along the polar regions. Therefore, gallic acid can be found in reaction solutions with a polar solvent, e.g. water.

Gallic acid is known to bind to bovine serum albumin in an amount of 45–65% of the ligand [37]. The bond formation of the polyphenol + bovine serum albumin complex depends on the size of the ligand: the larger the ligand, the more pronounced the binding to the protein, e.g. various catechins and bovine serum albumin [37]. Binding constant K increased in the series catechin-epigallocatechin-epigallocatechin-3-gallate up to 2.7×10^5, 2.9×10^5, and 3.2×10^5 mol/L, respectively. Based on these data, the reaction mix had an excess of gallic acid.

SDS-PAGE. Figure 3 shows the gel electrophoresis of samples denatured with sodium dodecyl sulfate. From left to right, the concentration of gallic acid in the complex increases. The first lane on the left illustrates the marker of protein molecular weight. The next lane shows the bovine serum albumin standard. The control solution of bovine serum albumin usually has a band of about 70 kDa and coincides with the band of the experimental solution without gallic acid. According to the gel electrophoresis, the gallic acid molecule did not affect the molecular weight of the protein and did not promote the formation of protein conglomerates.

The attachment of a gallic acid molecule to bovine serum albumin did not affect the charge and mass of the entire complex because the molecular mass of bovine serum albumin (68 kDa) significantly exceeded the mass of gallic acid (170 Da), and the charge was concentrated in the pocket of bovine serum albumin. An increase in the concentration of gallic acid resulted in no additional bands, which means no additional protein complexes with a different molecular weight and minor protein components. The samples with the complex showed no additional bands in the range of 68–5 kDa that usually designate the destruction of bovine serum albumin molecule. Thus, the SDS-PAGE analysis detected neither significant changes in the molecular weight nor violations of the integrity of the protein molecule.

Amperometric assessment method with gallic acid standard. According to the amperometric measurement of a standard bovine serum albumin solution by gallic acid equivalent, the indicator was 5 ± 1 μg/L of gallic acid, which corresponded to the low reducing properties of the protein in the amperometric detector (Fig. 4).

When the concentration of gallic acid was 100 mg/L, the bovine serum albumin + gallic acid values corresponded to 7.8 ± 1.3 mg/L in gallic acid equivalent. The result indicated a decrease in the reducing properties of gallic acid. When the concentration of gallic acid reached 200 and 300 mg/L, the reducing properties of the complex increased ($P < 0.05$) in terms

of the gallic acid equivalent and reached 15.9 ± 2.65 and 23.3 ± 5.05 mg/L gallic acid, respectively.

The recorded parameters in the experimental solution decreased in comparison with the level of gallic acid added to the reaction mix. This change indicated the reaction with bovine serum albumin. The chromatogram showed that the indicators of the complex changed proportionally as more gallic acid entered the reaction. Thus, the binding to bovine serum albumin increased as the concentration grew. The molecular modeling suggested a partial reaction between gallic acid and bovine serum albumin.

Gallic acid was removed from the reaction mix with a concentration of 300 mg/L using dialysis membranes with a cut-off mass of 12–14 kDa. The reducing properties of the concentrate of the complex were 63% of the initial level.

The most favorable thermodynamic conditions for the development of H-bonds were likely to form when a part of the unreacted gallic acid was in the solution. This result corresponded to the data obtained about the binding of bovine serum albumin to polyphenols, which was in t he range of 45–65% [37].

FTIR spectroscopy. Figure 5 shows the spectral data. The main parameters evaluated were the changes in the amide I region. During the formation of the complex, the concentration of gallic acid in the bovine serum albumin solution was gradually increased (from 100 to 300 mg/L).

FTIR spectroscopy made it possible to estimate the concentration of gallic acid that caused the greatest changes in the properties of the protein. The data can facilitate further practical studies. The molecular modeling and amperometric parameters showed that not all gallic acid bound to bovine serum albumin. Another experiment made it possible to determine the sample with the maximal change in the spectrum intensities.

The experiment evaluated the changes in the intensities and shifts of the amide I and amide II bands of the protein. Amide I had the following wavelengths:

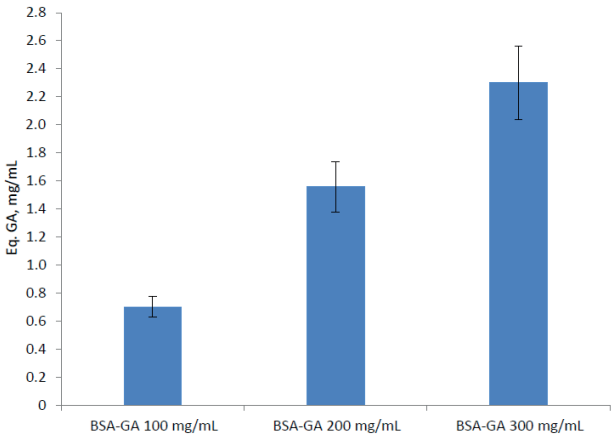

Figure 4 Changes in gallic acid equivalent during amperometric testing of the bovine serum albumin and gallic acid complex at 100, 200, and 300 mg/L of gallic acid

1652 (α-helix), 1618 (β-sheet), 1675 (the molecule unfolds), 1694 (β-antiparallel sheet), and 1633 cm^{-1} (random turns) [35]. These absorption bands are characteristic of all proteins, and they can be used to evaluate the changes in the secondary structure that occurs when gallic acid incorporates into the bovine serum albumin molecule. The ongoing reaction was expected to affect amide II in the region of 1546 cm^{-1} (stretching the C-N bond in combination with the N-H bond). It was also expected to change the spectral line at 1390 cm^{-1}, which is typical of bovine serum albumin and causes its protonation. Another characteristic band of gallic acid (1539 cm^{-1}), also showed an increase in the concentration of gallic acid in the solution.

Figure 5 shows changes in the absorption band at 1652–1653 cm^{-1}. They indicate a gradual decrease in the number of α-helices, which means that amino acids are losing the possibility of hydrogen bond formation when the concentration of gallic acid is high [37]. The number of hydrogen bonds might have decreased following the increase in the concentration of gallic acid involved. The characteristic band at 1539 cm^{-1} grew together with the concentration in the solution. The absorption band at 1652 cm^{-1} demonstrated a short-wave shift to the blue region by 1653.16 cm^{-1}, which also marked a certain change in peptide bonds in this region of the protein.

The initial IR spectra contained various broad peaks in the ranges in question, which complicated the analysis. In such cases, it is recommended to use the first- and second-order derivatives of the original spectra. This research involved the Savitzky-Golay method to perform differentiation in all three ranges [4].

To make the assessment more accurate, the second-order derivative of the original spectra was used in the regions of the bovine serum albumin peptide bond and the characteristic gallic acid band. The spectral line at 1546 cm^{-1} grew less intensive as the concentration of gallic acid decreased. Consequently, valence and deformation stretching of the C-N and N-H bonds occurred in the bovine serum albumin molecule, following the decrease in the number of peptide bonds. The spectral line at 1390 cm^{-1} grew more intensive as the concentration of gallic acid increased, which means that the number of protons in the system increased when gallic acid was added. These changes indicated that hydrogen bonds between bovine serum albumin and gallic acid could develop by attaching polyphenol hydroxyl residues to protein peptide bonds [38].

The spectral line at 1618 cm^{-1} showed changes in the β-sheet. These changes were opposite to the changes in the α-helix. In addition, the number of antiparallel structures increased at 1694 cm^{-1}. Bovine serum albumin unfolded at 1675 cm^{-1}, with a slight shift to the short-wavelength region. The unfolding might have been a consequence of the decreasing number of α-helices in the structure. It confirmed the changes in the region of 1652 cm^{-1}. The shape of random coils did not change when the gallic acid concentration changed (1633 cm^{-1}).

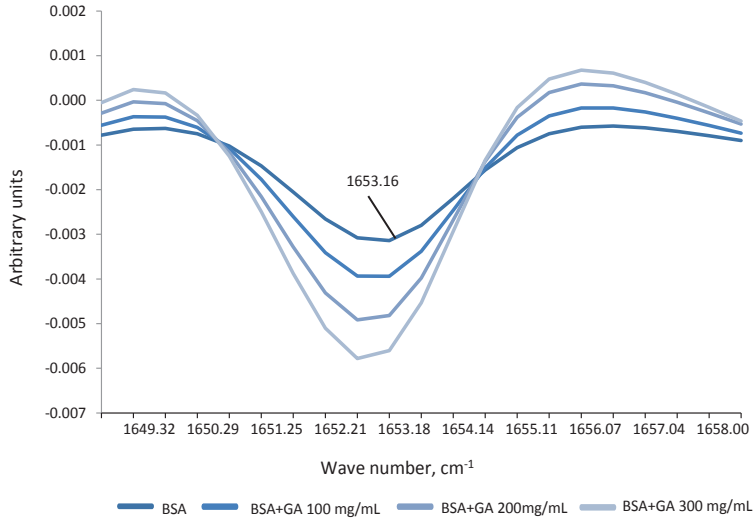

Figure 5 Derivatives of spectral lines. BSA – standard solution of bovine serum albumin. GA – gallic acid (in different concentrations)

The IR spectra of the bovine serum albumin molecule showed a slight unfolding. Protonation resulted from the decrease in the number of peptide bonds. The falling number of α-helices indicated the replacement of free hydrogen bonds. All these changes indirectly confirmed the formation of the bovine serum albumin + gallic acid complex. The sample with 300 mg/L of gallic acid had the strongest effect on bovine serum albumin. This ratio can be recommended to develop new food additives.

CONCLUSION

The present research featured molecular modeling of the reaction between bovine serum albumin and gallic acid. The experiment confirmed that chemical bonding developed between the molecules in the binding pocket of the protein. The bonding occurred mainly through hydrophobic interactions. It involved polar regions with negatively charged areas of the protein molecule. Presumably, some changes in chemical bonds affected the secondary structure of the protein. In a polar solvent, the bonds of gallic acid with the protein molecule might be partially unstable.

According to SDS-PAGE, electrophoresis of gallic acid did not violate the integrity of the protein molecule and did not produce minor proteins. When the concentration of gallic acid increased during the reaction with bovine serum albumin, it did not lead to the formation of protein complexes with a molecular weight higher than that of bovine serum albumin.

The chromatogram of the bovine serum albumin + gallic acid complex revealed amperometric changes that corresponded with the increase in the concentration of gallic acid introduced into the reaction. Therefore, free gallic acid in the solution provided the most favorable thermodynamic conditions for chemical binding. The obtained data confirmed the molecular modeling about the duality of gallic acid binding. When gallic acid was removed from the reaction, the reducing properties of the complex fell down to 63%, following the proportion of binding in the pocket with bovine serum albumin.

The FTIR data confirmed the molecular modeling. The number of peptide bonds in the bovine serum albumin molecule decreased due to valence and deformation stretching of the C-N and N-H bonds. As the amount of gallic acid grew, the number of protons in the system increased, as did the possibility of hydrogen bonding between bovine serum albumin and gallic acid. As a result, bovine serum albumin unfolded, and the secondary structure of the protein changed. The highest binding occurred when the concentration of gallic acid was 300 mg/L, which corresponded to the reaction mix with an excess of gallic acid. This concentration can be recommended for further use in the food industry as part of new food additives.

The present research was limited to a laboratory experiment and requires further study by thermodynamic calculations.

CONTRIBUTION

R.O. Budkevich designed and supervised the project. N.M. Fedortsov, E.V. Budkevich, I.A. Evdokimov, and S.A. Ryabtseva performed the experiments and discussed the results.

CONFLICT OF INTEREST

The authors declare no conflict of interest regarding the publication of this article.

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Zeboid cow milk: physicochemical quality indicators

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Abstract:
Introduction. A herd of zeboid cattle was created by the Snegiri Scientific and Experimental Farm (Moscow region, Russia) as a result of long-term selection and crossbreeding zebu (*Bos indicus* L.) with cattle (*Bos taurus* L.). These hybrid cows have good physiological parameters, high resistance to diseases, and a significant adaptive potential. The quality of milk produced by zebu cows at different lactation and milking times has not been studied as well as their milking capacity. Therefore, we aimed to assess the variability of specific physicochemical indicators of milk produced by Snegiri's zeboid dairy herd.
Study objects and methods. The milk of 193 zeboid cows (6–12% of zebu blood) from the Snegiri Farm was analyzed by standard methods for quality indicators such as fat, nonfat milk solids, density, bound water, freezing point, protein, and lactose. Then, we determined how these indicators changed depending on the lactation number and the time of milking (morning/evening). Statistical analysis was applied to process the data.
Results and discussion. Such indicators as nonfat milk solids, density, bound water, freezing point, protein, and lactose of zeboid cow milk were consistent with the normal indicators for raw cow's milk. Only its fat content (4.39%) exceeded the norm. We found no correlation between the quality of milk and the number of lactations. However, the evening milk was more concentrated, with a significant increase in nonfat milk solids and density, as well as with a lower freezing point.
Conclusion. Zeboid cows, which can be bred in suboptimal conditions, produce milk suitable for dairy products since it has a high fat content regardless of lactation and milking time.

Keywords: Zeboid cattle, milk, quality indicators, fat, nonfat milk solids, density, freezing point

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INTRODUCTION

Crossbreeding zebu (*Bos indicus* L.) with cattle (*Bos taurus* L.) has produced hybrids that are well adapted to different natural and climatic conditions [1–3].

Although zebu cows are less prolific and have lower milk productivity than B. *taurus* breeds, they are better adapted to the environment and more resistant to a number of diseases. Zebu milk has a very high content of fat (5–6%) and protein (3.7–4.2%) [4]. Therefore, zebus are crossbred to produce hybrids with high-fat milk [5]. Like zebu, zeboid cows produce milk that is suitable for dairy products (butter, cheese, cream, cottage cheese, etc.). In addition, high-fat milk production is more cost-effective. Since one liter of 3.5% milk contains 30% less fat than one liter of 5% milk, farmers need more low-fat milk to produce, cool, store, transport, and process, which increases the cost of a dairy product [6].

This field has been so important that the Soviet Union established a special authority, the Council for

Breeding Zebu and Zeboid cattle, to provide guidance to its farms. As a result, Azerbaijan created a new breed of dairy cattle – the Azerbaijani Brown – by crossing zebu with the Brown Swiss and Brown Carpathian breeds. The new breed produced high-fat milk [7].

In 1967, Uzbekistan created the Bushuyev breed by crossing local zeboid cattle with the Dutch and Swiss bulls [5]. This breed was made up of 5 main lines, with the Mota TE-10 line producing the highest-fat milk (4.14%) [7]. The farms in the Vakhsh Valley, Tajikistan, crossed local zeboid cattle with the Brown Swiss to create the Tajik intra-breed type of the Swiss zeboid cattle. The cows of this type yielded 3000 kg of 4% fat milk [7]. Turkmenistan crossed zeboid cattle with the Red Steppe bulls to produce the Red zeboid cattle with a milk yield of 2000–2500 kg and a fat content of 3.8–4,0 % [5].

In 1956, the Snegiri Scientific and Experimental Farm of the Main Botanical Garden (Moscow region) became the first institution in the European part of Russia to experimentally cross the Azerbaijani zebu with the Black Pied breed [10]. The farm developed a unique breed of dairy zeboid cattle that was highly productive in a temperate climate zone with an average annual temperature of +4°C. Subsequently, new hybrids were created by crossing this unique breed with the Cuban and New Zealand zebu, as well as the Punjabi Sahiwal zebu. Then, the Snegiri Farm developed schemes to cross their bulls with other breeds, including the Black Pied, Jersey, Ayrshire, Kholmogory, Aulie-Ata, Simmental, Red Steppe, and Brown Latvian breeds. In 1999, they began to use Holstein bulls in crossbreeding to increase milk yield and improve the shape of the udder in hybrid cows [4].

The resulting crossbreeds were resistant to tuberculosis, brucellosis, leukemia, and other diseases. They inherited high fat and protein contents from zebu, had a good physiological capacity for milking, and increased milk yield in better feeding and maintenance conditions [11-13]. Among Snegiri's zeboid cattle, the Elite-Record class crossbreeds produce maximum milk yield (over 5000 kg per lactation), with an average fat content of 4.64% [4].

Although the factors of milk production by zeboid cows have been studied quite well, the milk's quality indicators deserve more attention [4, 10]. Therefore, we aimed to study individual physicochemical indicators of milk produced by Snegiri's zeboid cattle depending on the number of lactations and the time of milking (morning or evening).

STUDY OBJECTS AND METHODS

We studied the milk of 193 zeboid cows (6–12% of zebu blood) bred by the Snegiri Farm. In particular, we determined milk quality indicators such as fat, nonfat milk solids, density, bound water, freezing point, protein, and lactose. Then, we analyzed how they changed depending on the lactation number and milking time (morning/evening).

The above quality indicators were determined by the following methods: fat content by the Gerber method (volumetrically); nonfat milk solids – by calculation;

Table 1 Quality indicators ($M \pm \sigma$) of zeboid cattle milk against lactation number (Snegiri Farm, Moscow region)

Number of cows (n)	Quantitative and qualitative indicators of milk						
	Fat, %	Nonfat milk solids, %	Density, °A	Bound water, %	Freezing point, (–10^{-2}°C)	Protein, %	Lactose, %
				First lactation			
85	4.45 ± 0.760	8.25 ± 0.338	26.51 ± 1.367	3.13 ± 0.899	54.18 ± 2.036	3.08 ± 1.229	4.69 ± 0.185
				Second lactation			
15	4.34 ± 0.856	8.39 ± 0.301	26.14 ± 1.433	2.20 ± 0.148	54.99 ± 1.835	2.99 ± 0.110	4.77 ± 0.167
				Third lactation			
26	4.45 ± 0.931	8.34 ± 0.324	26.87 ± 1.405	2.51 ± 0.621	54.68 ± 1.979	2.98 ± 0.122	4.74 ± 0.176
				Fourth lactation			
32	4.54 ± 1.205	8.09 ± 0.783	26.32 ± 1.877	3.13 ± 0.119	54.01 ± 2.242	2.94 ± 0.136	4.60 ± 0.444
				Fifth lactation			
13	4.46 ± 0.707	8.17 ± 0.467	26.17 ± 1.593	3.93 ± 0.517	53.58 ± 2.822	2.92 ± 0.169	4.64 ± 0.253
				Sixth lactation			
7	4.01 ± 1.037	8.29 ± 0.299	27.03 ± 1.203	2.82 ± 0.513	54.38 ± 1.784	2.95 ± 0.113	4.72 ± 0.164
				Seventh lactation			
6	3.83 ± 0.955	8.25 ± 0.264	27.03 ± 1.006	2.98 ± 0.414	54.28 ± 1.510	2.92 ± 0.104	4.64 ± 0.163
				Eighth lactation			
9	3.77 ± 1.087*	8.30 ± 0.278	27.28 ± 1.927	2.51 ± 0.379	54.62 ± 1.759	2.96 ± 0.094	4.73 ± 0.163
				Mean values for all lactations			
193	4.39 ± 0.911	8.25 ± 0.445	26.62 ± 1.510	3.08 ± 0.929	54.27 ± 2.069	3.01 ± 0.821	4.69 ± 0.249

Note: * $P < 0.05$

density – on a lactodensimeter; bound water – in a RD-8 dryer (Funke Gerber); freezing point – cryoscopically; protein content – by the Kjeldahl method; and lactose content – by the refractometric method.

The standard deviation (σ) indicated the variability of the mean value (*M*). Primary data grouping and biometric calculations were performed in Excel Microsoft and STATISTICA.

Randomly selected data were statistically analyzed by the Student's t-test, with the normality of distribution preliminarily determined by the Kolmogorov-Smirnov and Shapiro-Wilk tests. The nonparametric Mann-Whitney U-test was used in case the populations from which the data were selected for comparison were not distributed normally.

RESULTS AND DISCUSSION

The analysis of milk quality against lactation number (Table 1) showed that only the eighth lactation cows had a significant decrease in milk fat compared with the first lactation cows (3.77 and 4.45%, respectively) ($P < 0.05$). We noticed that this indicator became more variable with the age of the cows (σ = 0.760 for the 1st lactation and σ = 1.087 for the 8th lactation).

As we can see in Table 1, the mean fat content (4.39%) in the zeboid cattle milk was significantly higher than the standard content (3.4%) in Russia. The mean protein content (3.01%) was consistent with the Russian norm (3%). The freezing point (–0.543°C) of the milk samples was in line with the Russian Standard for raw cow's milk (R 52054-2003) and the European Standard for the extra grade milk [14]. The contents of bound (adsorption-bound) water (3.08%), lactose (4.69%), and nonfat milk solids (8.25%) were consistent with the standard indicators of cattle milk (2–3.5, 3.6–5.5, and > 8.2%, respectively) [15–17].

The mean density of the zeboid cattle milk (26.62°A) corresponded to the minimum norm for high-quality milk, 1.027 g/cm^3 (27°A). The low density of zebu milk is probably due to its high fat content, since studies show a decrease in density with an increase in fat [18].

Table 2. Quality indicators ($M \pm \sigma$) of zeboid cattle milk against lactation number and milking time (Snegiri Farm, Moscow region)

Milking time	Quantitative and qualitative indicators of milk						
	Fat, %	Nonfat milk solids, %	Density, °A	Bound water, %	Freezing point, (-10^{-2}°C)	Protein, %	Lactose, %
First lactation, n = 85							
Morning	5.09 ± 0.633	8.16 ± 0.380	26.10 ± 1.709	3.83 ± 0.577	53.57 ± 2.181	3.18 ± 2.475	4.64 ± 0.210
Evening	4.39 ± 0.974	8.35 ± 0.348**	26.92 ± 1.566**	2.42 ± 0.774*	54.80 ± 2.370	2.98 ± 0.129	4.74 ± 0.193**
Second lactation, n = 15							
Morning	4.58 ± 1.408	8.26 ± 0.271	26.42 ± 1.810	2.94 ± 0.580	54.15 ± 1.709	2.95 ± 0.096	4.70 ± 0.154
Evening	4.11 ± 0.861	8.51 ± 0.386*	27.86 ± 1.949*	1.46 ± 0.390	55.83 ± 2.409*	3.04 ± 0.138	4.84 ± 0.218*
Third lactation, n = 26							
Morning	4.67 ± 1.171	8.26 ± 0.327	26.34 ± 1.811	3.10 ± 0.960	54.17 ± 2.039	2.95 ± 0.118	4.69 ± 0.185
Evening	4.22 ± 1.394	8.42 ± 0.398	27.13 ± 1.936*	1.90 ± 0.825	55.23 ± 2.515	3.01 ± 0.148	4.70 ± 0.217
Fourth lactation, n = 32							
Morning	4.76 ± 1.433	8.16 ± 0.299	25.86 ± 1.770	3.90 ± 0.892	53.50 ± 1.759	2.92 ± 0.109	4.63 ± 0.169
Evening	4.31 ± 1.652	8.02 ± 1.546	26.78 ± 2.945	3.55 ± 0.953	54.52 ± 3.184	2.96 ± 0.180	4.56 ± 0.880
Fifth lactation, n = 13							
Morning	4.40 ± 0.448	8.13 ± 0.448	26.06 ± 1.548	3.98 ± 0.120	53.35 ± 2.850	2.90 ± 0.169	4.63 ± 0.242
Evening	4.52 ± 0.721	8.22 ± 0.527	26.28 ± 1.848	3.88 ± 0.004	55.81 ± 3.018	2.93 ± 0.197	4.67 ± 0.286
Sixth lactation, n = 7							
Morning	3.57 ± 0.701	8.24 ± 0.271	27.24 ± 0.866	2.94 ± 0.332	54.17 ± 1.614	2.93 ± 0.104	4.70 ± 0.146
Evening	4.45 ± 1.668	8.33 ± 0.390	26.81 ± 2.209	2.71 ± 0.212	54.59 ± 2.400	2.98 ± 0.143	4.74 ± 0.220
Seventh lactation, n = 6							
Morning	3.77 ± 1.242	8.27 ± 0.312	27.18 ± 1.612	2.87 ± 0.378	54.33 ± 1.881	2.90 ± 0.164	4.60 ± 0.340
Evening	3.90 ± 1.167	8.23 ± 0.303	26.88 ± 1.552	3.09 ± 0.067	54.22 ± 1.777	2.93 ± 0.114	4.68 ± 0.164
Eighth lactation, n = 9							
Morning	3.92 ± 0.897	8.26 ± 0.241	27.68 ± 2.130	2.68 ± 0.130	54.52 ± 1.543	2.94 ± 0.086	4.70 ± 0.137
Evening	3.63 ± 1.022	8.33 ± 0.451	27.54 ± 3.463	2.34 ± 0.886	54.71 ± 2.763	2.97 ± 0.147	4.75 ± 0.272
Mean values for all lactations, n = 193							
Morning	4.74 ± 1.853	8.19 ± 0.345	26.23 ± 1.706	3.57 ± 0.303	54.02 ± 3.970	3.04 ± 1.644	4.66 ± 0.198
Evening	4.29 ± 1.235	8.30 ± 0.724*	27.01 ± 2.075***	2.59 ± 0.162**	54.79 ± 2.583*	2.98 ± 0.147	4.72 ± 0.410

Note: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.00$

Then, we analyzed the milk quality indicators in relation to the milking time (morning/evening) of the zeboid cattle of different lactations (Table 2). We found that fat and protein contents in the morning and evening milk changed randomly within 3.57–5.09 and 2.90–3.18%, respectively. However, nonfat milk solids levels in the milk from the first and second lactations, as well as the mean value for all lactations, were significantly higher in the evening. The same trend was observed for the density of milk in the first three lactations and the mean values. The lactose content increased significantly by the evening milking only in the first and second lactations. However, the freezing point in the second lactation and the amount of bound water in the first and second lactations, as well as on average for all lactations, significantly decreased in the evening milking.

On the whole, the evening milk (Table 2) showed a significant decrease in bound water (from 3.57 to 2.59%) with a simultaneous increase in nonfat milk solids (from 8.19 to 8.23%) and a rise in milk density (from 26.23 to 27.01°A). The high density of the evening milk might be due to an increased amount of dissolved minerals, since the mean contents of protein and lactose did not change significantly in the entire herd. This was evidenced by the decrease in the freezing point of the evening milk from 54.02×10^{-2} to -54.79×10^{-2}°C.

The changes in the chemical and physical indicators of milk quality were primarily caused by the milk produced in the first three lactations. Besides, the number of cows in the first, second, and third lactations prevailed in the zeboid cattle herd.

Noteworthily, the crossing of zebu (*Bos indicus* L.) with cattle (*Bos taurus* L.) results in a pronounced relative heterosis, or the superiority of hybrids over the worst of the parental forms, in terms of milk quality, especially its fat content. Various authors have reported the following fat contents in the milk of the breeds that were used to create the zeboid cattle at the Snegiri Farm: 3.39 (Black Pied), 5.87 (Jersey), 4 (Ayrshire), 3.68 (Kholmogory), 3.85 (Aulie-Ata), 3.89 (Simmental), 3.82 (Red Steppe), 4.01 (Brown Latvian), and 3.6% (Holstein) [19–22]. The zebu (Azerbaijani, Cuban, New Zealand, and Sahiwal) produced 5.1–6.0% fat milk and the zeboid cattle in our study, 4.39% fat milk [7]. These data revealed an advantage of zeboid cattle over traditional breeds, since water metabolism in cows producing higher-fat milk puts less pressure on their body and is more economical in terms of energy and feed.

CONCLUSION

As a result of the study, we made the following conclusions.

1. The physicochemical indicators of zeboid cattle milk quality (fat, nonfat milk soilds, density, bound water, freezing point, protein, lactose) were consistent with the Russian standards for raw cow's milk, except for the fat content (4.39%), which significantly exceeded the norm.

2. The changes in the physicochemical indicators did not depend on the number of lactations. Only the eighth lactation cows showed a decrease in milk fat with age.

3. The evening milk was more concentrated, which manifested in an increased amount of NONFAT MILK SOLIDS, higher density, and a lower freezing point.

4. Given the high fat content, zeboid cattle milk is suitable to produce dairy products, regardless of lactation number and milking time. Besides, zeboid cattle can be bred effectively under suboptimal maintenance conditions.

CONTRIBUTION

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

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Bioassay of oxidative properties and toxic side effects of apple juice

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Abstract:

Introduction. Apple juice owes its beneficial properties to various biologically active compounds, e.g. antioxidants. Therefore, food science needs effective methods that would cover all the mechanisms of their effect on human metabolism. However, fruit juice production raises certain safety issues that are associated not only with production risks, but also with some natural components in the raw material. The *Allium* cepa test seems to be an effective solution to the problem. This plant bioassay has a good correlation tested on mammalian cell cultures.

Study objects and methods. Onion roots (*A. cepa)* were treated with aqueous solutions of juices and sorbic acid to assess their antioxidant profile. The toxic effects on root tissues were described according to biomass growth, malondialdehyde (MDA) concentration, and proliferative and cytogenetic disorders.

Results and discussion. The study revealed the optimal conditions for the *A. cepa* assay of the antioxidant properties of apple juice. The antioxidant activity was at its highest when the juice was diluted with water 1:9 and the onion roots were treated with sorbic acid. The lipid oxidation of the *A. cepa* roots decreased by 43%. A comparative analysis of three different juice brands showed that the difference in their antioxidant profiles was ≤ 3%. As for toxic side effects, the chromosome aberrations increased by six times in all samples.

Conclusion. The research offers a new *in vivo* method for determining the antioxidant profile of apple juice. Three juice brands proved to have irreversible cytotoxic and genotoxic effects.

Keywords: Apple juice, bioassay, antioxidant activity, side effects, *Allium cepa* test, biologically active substances

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INTRODUCTION

Apple juice is one of the most popular fruit juices in Russia. Therefore, domestic food industry needs reliable methods for its nutritional value and risk assessment. The beneficial properties of apple juice are associated with various biologically active compounds. Recent antioxidant studies show that apple juice is rich in such antioxidants as polyphenols, e.g. quercetin, phloretin, chlorogenic acid, and epicatechin. A fruit and vegetable diet reduces oxidative stress, thus preventing chronic diseases and slowing down aging. Apples and apple products are known to reduce the risk of cancer, cardiovascular diseases, asthma, and type II diabetes [1]. The chemical composition of juices depends on the variety of apples, their ripeness, climate, cultivation method, etc. Apple juice production involves a wide variety of apple cultivars but gives preference to winter and autumn varieties because they are juicy, firm-fleshed, and rich in aromatic and phenolic substances.

Consumers see apple juice as a source of biologically active compounds that are beneficial to human health. As a result, the volume of its industrial production keeps increasing. Food processing determines the nutritional value of the finished product [2]. Crushing, heat treatment, fermentation, and clarification of apples affect the phytochemical composition of apple juice. These processes decrease the amount of phenolic compounds. After heat treatment and direct extraction, fruit juice had 10% of the antioxidant properties of fresh fruits. After pulp fermentation, this figure was 3%. Pulp fermentation decreased the content of phloridzin,

chlorogenic acid, and catechin by 31, 44, and 58%, respectively. Most of the active compounds remained in apple pomace [3].

Another study compared polyphenols in apple juice after heat and high pressure treatments [4]. The phenolic profile of the resulting apple juice changed significantly. The epicatechin concentration was 0.42 mg/100 mL in the raw juice; it decreased to 0.31 mg/100 mL at 25°C and increased to 0.39 mg/mL at 65°C. Heat treatment increased the amount of catechin and chlorogenic acid, while pressure treatment decreased the amount of polyphenols. The authors linked this phenomenon to structural destruction because the rapid release of carbon dioxide led to pressure gradient.

Various plant assays of antioxidants properties receive more and more scientific attention each year. Unfortunately, different antioxidant tests use different terms and measurements [5]. Moreover, antioxidants may respond differently to different radicals or their sources. Phytochemical compounds are present in numerous products and possess numerous mechanisms of action on metabolic processes. Thus, the food industry has a wide choice of adequate antioxidant assessment methods [6]. Therefore, an objective analysis of data on bioactive compounds needs specifically tailored markers. Finally, the bioactivity of plant food products depends on a whole complex of phytochemical compounds. Lipid peroxidation is measured by the levels of malondialdehyde (MDA), β-carotene, and diene conjugates [6].

Other methods determine the total antioxidant potential according to the concentration of free radicals, e.g. 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2-diphenyl-1-picrylhydrazy[l] (DPPH) assay, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, ferric reducing/antioxidant power (FRAP) assay, ferrous oxidation-xylenol orange (FOX) assay, ferric thiocyanate (FTC) assay, and aldehyde/carboxylic acid (ACA), etc.

These approaches make it possible to analyze the level of antioxidant activity both in food products and in living organisms after consumption. However, bioassays seem to be the most informative and accurate methods, since all nutritionally valuable substances are bioavailable and bioactive. Testing food matrices on laboratory animals or human cell lines is expensive and labor-consuming. Therefore, plant assays are more preferable.

Scientists compared the level of lipid peroxidation in onion roots after their treatment with apple juice and a model aqueous solution of fructose, glucose, sucrose, D-sorbitol, and malic acid. After incubation, the content of MDA in root tissues was 1.7 times higher in the model solution than in the apple juice [7]. Such results proved that the juice possessed some antioxidant activity, which lowered the carbohydrate-induced lipid oxidation almost to the control values, i.e. those of water.

Domestic regulations ban synthetic additives from juice production. Unfortunately, these measures fail to eliminate juice-related safety risks. Therefore, food producers have to check raw materials for various contaminants, such as heavy metals, pesticides, and herbicides, as well as to monitor the safety of technological production means, e.g. detergents, lubricants, packaging material, etc. Moreover, technological methods of juice processing require exposure to high temperatures during pasteurization, sterilization, etc., which can result in accumulation of toxic compounds and adducts. For example, some phytochemical compounds of plant products are known to react with cellular macromolecules during storage, thus causing cellular toxicity or even genotoxicity if they react with DNA [7, 8].

Almost all higher plants contain such natural mutagens as pyrolizidine alkaloids and some flavonoids [9]. In fact, recent studies linked the consumption of fruits and juices to cancer and asthma in children [10–13]. Finally, juices are rich in carbohydrates, and fructose and sucrose produce adverse metabolic effects on human health [14, 15]. Food scientists have developed numerous physicochemical assay methods for these toxic agents. However, bioassays seem to be the only method that gives an integrated assessment of their synergetic effect.

In this regard, the *Allium cepa* test is especially promising. This test is recommended by WHO experts as a standard for cytogenetic monitoring. The *A. cepa* assay is a popular method to define the bioindicator of cyto- and genotoxicity of xenobiotics in food products and their components [16]. The *A. cepa* test provides a prompt comparative analysis of individual compounds and their combinations. *A. cepa* cells share metabolic mechanisms with all eukaryotes, but unlike animal and human cell lines, they are not subject to transformation and can be useful in detoxification modeling. This test can screen biomarkers that determine the negative potential of food matrix toxicants for metabolic processes in onion root tissues [17].

Taking into account these indicators and the data on antioxidant activity, plant bioassays can logically be applied to various brands of apple juice [7]. However, research databases seem to contain no publications on the *Allium*-based comparative evaluation of various domestic brands of apple juice. The present research objective was to compare the antioxidant activity, cytotoxicity, and genotoxicity of various domestic apple juice brands.

STUDY OBJECTS AND METHODS

Preparation of bioassay solutions. The research featured samples of processed and clarified apple juices from four producers. The juices were purchased from a retail chain and marked as A, B, C, and D. The juices were within the expiration date, with intact packaging. The juices were diluted with bottled water in ratios 1:5, 1:9, and 1:20. Sorbic acid (Thermo Fisher Scientific, USA) simulated oxidative stress. Solutions of sorbic acid (100 and 50 mg/L) included bottled water and

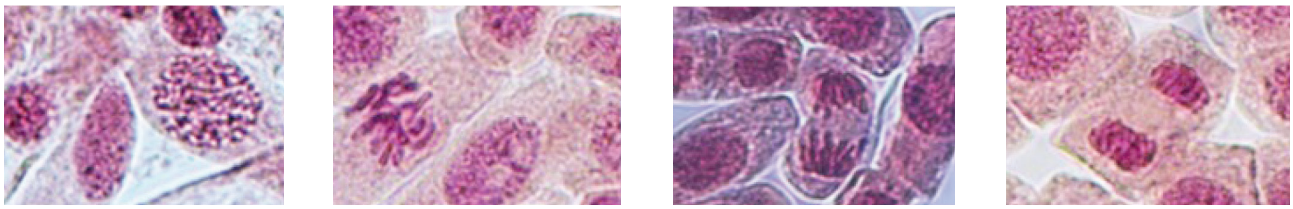


Figure 1 Mitosis phases, from left to right: prophase, metaphase, anaphase, and telophase

were prepared in a water-bath by heating to 78°C with constant stirring.

Bioassay. The bioassay featured peeled onion bulbs of the same weight (5–7 g) and diameter (≥ 3 cm). The onions were placed in 2-mL test tubes with bottled water and left for two or three days, depending on the experimental conditions, in a thermostat (24 ± 1°C) in total darkness. After two days of preliminary germination, the onions with a root length of ≥ 1 cm were placed in experimental solutions with apple juice, sorbic acid, or their mix. They were incubated in the thermostat for the next 24 or 48 h. Bottled water was used as a negative control. Ten onions were selected from each group of experimental and control samples. After preliminary three days of germination and two days of treatment with solutions of different juices, some onions were thoroughly washed and then incubated in bottled water for another 48 h at 25 °C to be tested for recovery treatment. After the experiment, all roots were cut off, dried with filter paper, and weighed. The weight gain was determined as the arithmetic mean for each solution.

Staining and microscopy. A 2% solution of acetoorcein was used to stain the preparations of onion apical root cells. The solution included 1 g of orcein dye per 50 mL of 45% acetic acid. A 70% solution of ethyl alcohol facilitated the long-term storage in the refrigerator. The experiment involved the instant pressure method. A root end of 2–4 mm in length was cut off from the root and washed in distilled water. The piece was placed in a drop of 45% acetic acid and crushed with a glass spatula under a coverslip. The cells were observed in interphase, prophase, metaphase, anaphase, and telophase in an Axioskop 40 (Zeiss) light microscope under 40× magnification (Fig. 1).

Cytogenetic indicators. The mitotic index, %, was calculated by the following formula:

$$\text{Mitotic index} = \frac{\text{cells in mitosis}}{\text{total cell count}} \quad (1)$$

The chromosomal aberration analysis revealed disorganization, adhesion, overlap, lagging, colchicine mitosis, and a small percentage of bridging and micronuclei formation (Fig. 2).

For a quantitative description, the index of chromosome aberrations, %, was calculated as follows:

$$\text{Chromosome aberrations} = \frac{\text{chromosome aberrations}}{\text{total cell count}}$$

The cytogenetic studies revealed on average 10 000 cells per variant.

Concentration of malondialdehyde in the onion root cells. The lipid peroxidation in root tissues was determined by the amount of malonic dialdehyde (MDA) interacting with 2-thiobarbituric acid (MDA in fresh mass) [18]. During the experiment, 0.2–0.9 g of onion roots were placed into a polymer 15-cm^3 tube (weighing error ± 0.0001 g). After that, 1 cm^3 of trichloroacetic acid (Merck, Germany) with a mass concentration of 200 g/dm^3 was added to the sample. The mix was stirred and diluted with 3 cm^3 of the same trichloroacetic acid solution. The tubes were centrifuged for 15 min at 1000×g at 4°C. Then, 1 cm^3 of the upper liquid layer was transferred to another tube. After that, 4 cm^3 of a thiobarbituric acid solution (0.5 g of thiobarbituric acid (Diam, Russia)) was poured into 100 cm^3 of trichloroacetic acid solution (200 g/dm^3). The tubes were placed in a 95°C water-bath for 30 min followed by an ice bath. Next, the tubes were placed in a centrifuge for 10 min at 1000×g at 20°C. The resulting solutions were subjected to spectrophotometry in a Cary WinUV 100 spectrophotometer (Varian, USA) at wavelengths of 600 and 532 nm.

Statistical analysis. Statistical processing involved Microsoft Excel 2016 and Statistica 12 software. The root mass indicator was calculated using the nonparametric Mann-Whitney test to compare two means ($P \leq 0.05$). Fisher's test ($P \leq 0.05$) quantified the differences in data with a binomial distribution, i.e. mitotic index and frequency of chromosome aberrations.

RESULTS AND DISCUSSION

The research tested the antioxidant effect of water-diluted apple juice on *Allium cepa* roots after sorbic acid-induced oxidative stress. Antioxidants of plant origin could delay or prevent lipid oxidation because they inhibited the development and accumulation of free radicals [19]. However, sorbic acid is known to trigger the dose-dependent development of oxidative stress and increase the malonic dialdehyde (MDA) content in root tissues [20]. Concentrated solutions of apple juice activated lipid oxidation during the *A. cepa* test [7]. Therefore, the initial task was to select the optimal concentrations of sorbic acid and juice to obtain the maximal antioxidant effect. The onion samples spent 48 h incubating in solutions of sorbic acid and apple juice: 100 mg/L of sorbic acid was diluted with brand A apple juice as 1:2, 1:5, and 1:9. After the incubation, the

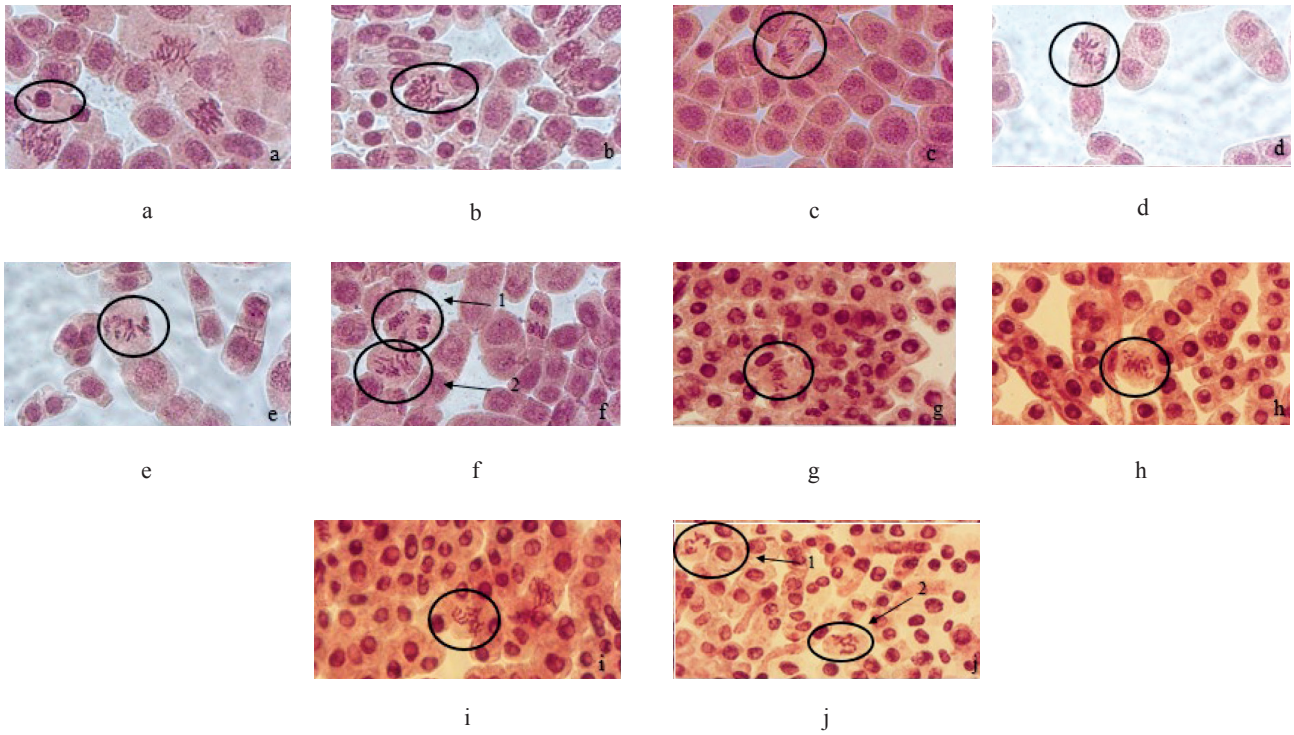
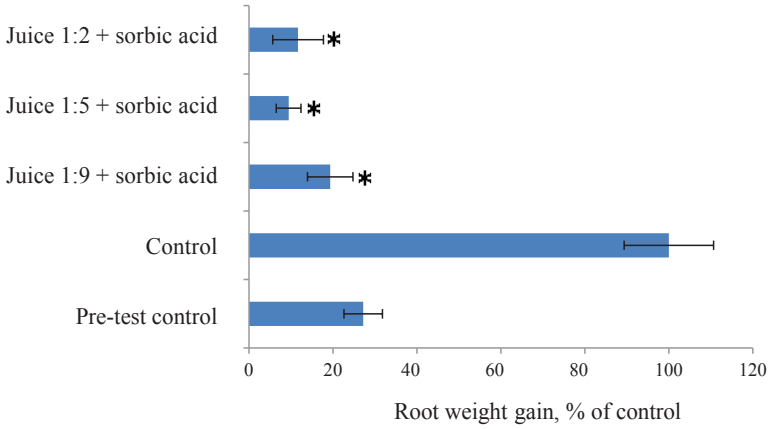


Figure 2 Chromosome aberrations: a) lagging in telophase; b) metafhase with chromosome loss; c) lagging in anaphase; d), e), and i) disorganization in metaphase; f) multipolar mitosis and disorganization in metaphase; g) disorganization in metaphase; h) metafhase with chromosome loss; j) mitosis или colchicine mitosis



* statistically significant difference from control ($P < 0.05$); error bars determine the value of the standard deviation

Figure 3 Decrease in weight gain of onion roots after treatment with brand A juice and sorbic acid (100 mg/L)

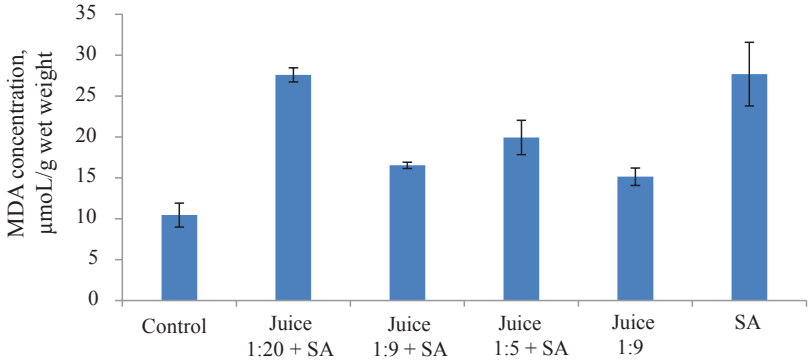


Figure 4 MDA in the roots treated brand A apple juice and sorbic acid (SA, 50 mg/L)

mass of the roots remained the same. In fact, they turned yellow and mucous, which meant that the doses had an acute toxic effect (Fig. 3).

In the next experiment, the treatment time and the acid concentration were halved, and the juice samples were diluted as 1:20, 1:9, and 1:5. Figure 4 shows that the 1:9 juice solution provided the maximal protective effect under oxidative stress caused by a 50 mg/mL solution of sorbic acid. In these samples, the level of MDA was lower by 43% than in the samples with the same concentration of sorbic acid.

The obtained data confirmed the results described in [21], where apple juice in rats' diet decreased the level of MDA in their blood plasma. The phenolic compounds and dietary fiber of apple juice proved to reduce the lipid oxidation in humans as well [1, 22, 23].

The dose-dependent decrease in MDA was revealed only in the first two, more diluted juice solutions (Fig. 4). In 1:5 juice samples, this indicator increased again. This effect was associated with carbohydrates, which are known to have prooxidant properties at this concentration during the *A. cepa* test [7]. Therefore, these data also confirmed that the maximal antioxidant activity of apple juice depended not only on its biologically active compounds, but also on the concentration of carbohydrates.

Some recent research featured the effect of fructose on the redox balance in the organs of the central nervous system. Rat studies revealed an increase in lipid oxidation of brain tissues after both short-term and long-term intake of this carbohydrate [24]. These animal models showed the same results as the abovementioned plant bioassays for the prooxidant properties of apple juice carbohydrates. Therefore, the *A. cepa* test proved to be a reliable research method for the molecular mechanisms of antioxidant and prooxidant properties of apple juice.

Growth indicators demonstrated no significant differences after the onions were treated with solutions of juice and sorbic acid (Table 1). However, previous research revealed that the increase in juice concentration had an adverse effect on onion root cell proliferation [7]. However, the decrease in the mitotic index against the increase in the juice proportion was not dose-dependent (Table 1). Both juice concentrations, 1:20 and 1:9, had the same values of this indicator. Probably, the maximal antioxidant status of the samples diluted 1:9 had protected the proliferative processes by reducing the effects of oxidative stress.

Similar conclusions were reported in a publication about the effect of antioxidants on bisphenol-induced oxidative stress in mouse spermatozoa [25]. Antioxidants preserved the motility of these germ cells, improved the fertilization process, and prevented premature development of the resulting fetus.

The low values of the mitotic index meant a low proportion of dividing cells in the experimental samples with mixes of juice and sorbic acid (Table 1). Therefore, no comparative analysis of chromosomal aberrations was necessary.

The next stage featured the antioxidant potential of various juice brands diluted 1:9 after 24 h of sorbic acid-induced oxidative stress. Juice brands A, B, and C in the mix reduced the level of MDA by 23, 26, and 26%, respectively (Fig. 2). Juice brands B and C also revealed some antioxidant activity; however, the differences between the experimental samples in MDA values were insignificant (3%). In the experimental mixes, the root masses were very similar and minimal, while the values of the mitotic index showed some statistically significant differences (Table 2).

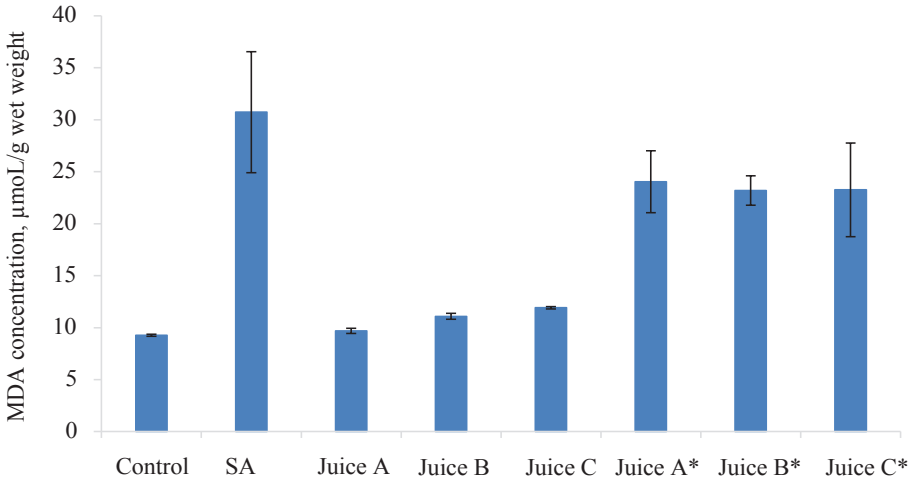
Phenolic compounds are mainly to be found in apple peel and pulp cell walls [1, 26]. Therefore, the processed and clarified juices had some residual differences in antioxidant activity in relation to lipid oxidation. Nevertheless, the bioassay was able to register a rather high antioxidant activity even in these non-pulp juices. The similar MDA values could also be explained by the absence of the pulp as the main source of phenolic compounds.

The results indicated an acute toxic effect (Fig. 3) and an increase in the level of lipid oxidation (Fig. 5) in the mixes of various juices and sorbic acid at concentrations of 100 and 50 mg/L, respectively. For some juice-containing drinks, domestic regulatory documents state much greater permissible concentrations of this preservative, ≥ 1 g/kg. Therefore, sorbic acid can reduce the initial antioxidant potential of these products, but not the content of phytochemical compounds. These data are important if the production

Table 1 Root weight gain, mitotic activity, and frequency of chromosome aberrations in onion root meristem cells after incubation in solutions of brand A juice, sorbic acid (SA), and their mixes

Experiment	Root weight gain, g/onion, mean ± SE*	Mitotic index, %, mean ± SE	Chromosome aberrations per total cells, %, mean ± SE
Control	0.296 ± 0.048^{a}**	8.70 ± 0.24^{a}	0.26 ± 0.04^{a}
Juice 1:20 + SA, 50 mg/L	0.189 ± 0.034^{ab}	1.08 ± 0.11^{b}	0.02 ± 0.02^{b}
Juice 1:9 + SA, 50 mg/L	0.162 ± 0.029^{bc}	1.01 ± 0.08^{b}	0.08 ± 0.02^{c}
Juice 1:5 + SA, 50 mg/L	0.138 ± 0.032^{bcd}	0.40 ± 0.06^{c}	0.04 ± 0.02^{bc}
Juice 1:9	0.109 ± 0.012^{bcd}	1.40 ± 0.09^{d}	0.21 ± 0.03^{ad}
SA, 50 mg/L	0.243 ± 0.021^{ab}	5.86 ± 0.21^{e}	0.18 ± 0.04^{d}

*SE – standard error, ** – values marked by the same letter have no significant statistic difference ($P < 0.05$)



Note: Vertical error bars indicate the value of the standard deviation; * marks the incubation experiments with mixes of juices and sorbic acid (SA, 50 mg/L)

Figure 5 MDA in roots treated with various apple juice brands

Table 2 Root weight gain, mitotic activity, and frequency of chromosome aberrations in onion root meristem cells after incubation in solutions of juices and their mixes with sorbic acid (SA)

Experiment	Root weight gain, g/onion, mean ± SE*	Mitotic index, %, mean ± SE	Chromosome aberrations per total cells, %, mean ± SE
Control	0.236 ± 0.030^{a}**	8.75 ± 0.24^{a}	0.29 ± 0.05^{a}
SA, 50 mg/L	0.211 ± 0.034^{ab}	5.17 ± 0.19^{b}	0.18 ± 0.04^{b}
Juice A	0.139 ± 0.016^{bc}	2.55 ± 0.13^{c}	0.05 ± 0.02^{c}
Juice B	0.146 ± 0.016^{bcd}	1.10 ± 0.09^{d}	0.11 ± 0.03^{b}
Juice C	0.184 ± 0.024^{abcde}	0.99 ± 0.08^{d}	0.01 ± 0.01^{d}
Juice A + SA, 50 mg/L	0.155 ± 0.021^{bcdef}	0.48 ± 0.05^{e}	0.020 ± 0.001^{cd}
Juice B + SA, 50 mg/L	0.143 ± 0.030^{bcdefg}	0.58 ± 0.07^{ef}	0.02 ± 0.01^{e}
Juice C + SA, 50 mg/L	0.171 ± 0.012^{abcdefg}	0.56 ± 0.07^{ef}	0.04 ± 0.02^{e}

* SE – standard error, ** – values marked by the same letter have no significant statistic difference ($P < 0.05$)

Table 3 Root weight gain, mitotic activity, and frequency of chromosome aberrations in onion root meristem cells before and after recovery treatment in juice solutions

Experiment	Root weight gain, g/onion, mean ± SE*	Mitotic index, %, mean ± SE	Chromosome aberrations per total cells, %, mean ± SE
		Before recovery treatment	
Control	0.799 ± 0.089^{a}**	8.52 ± 0.27^{a}	0.02 ± 0.01^{a}
Juice A	0.561 ± 0.056^{ab}	3.06 ± 0.19^{b}	0.12 ± 0.04^{b}
Juice C	0.540 ± 0.048^{bc}	3.64 ± 0.18^{c}	0.08 ± 0.03^{bc}
Juice D	0.597 ± 0.060^{abc}	3.95 ± 0.20^{c}	0.15 ± 0.04^{abc}
		After recovery treatment	
Control	1.060 ± 0.082^{a}	7.82 ± 0.28^{a}	0.16 ± 0.04^{a}
Juice A	0.791 ± 0.088^{ab}	7.99 ± 0.25^{ab}	0.43 ± 0.06^{b}
Juice C	0.827 ± 0.094^{abc}	8.08 ± 0.27^{ab}	0.99 ± 0.10^{c}
Juice D	0.944 ± 0.095^{abc}	6.93 ± 0.25^{c}	0.41 ± 0.06^{b}

technology provides for this preservative. However, only bioassay can determine how these effects interact.

Our experiments on the toxic potential of different juice brands were aimed at a comparative assessment of their side effects on the growth and the cytological, cytogenetic, and biochemical parameters of onion roots. We found no scientific publications that featured the *A. cepa* test as a means of researching the toxic effect of apple juice. The main task was to obtain data on possible irreversible violations of these processes. In case of complete or partial irreversibility after the juice treatment, the detoxification systems of the plant organism failed to cope with the load, and these negative phenomena might progress in the future.

The previous experiments had a high toxic load because of sorbic acid (Table 2). In this experiment,

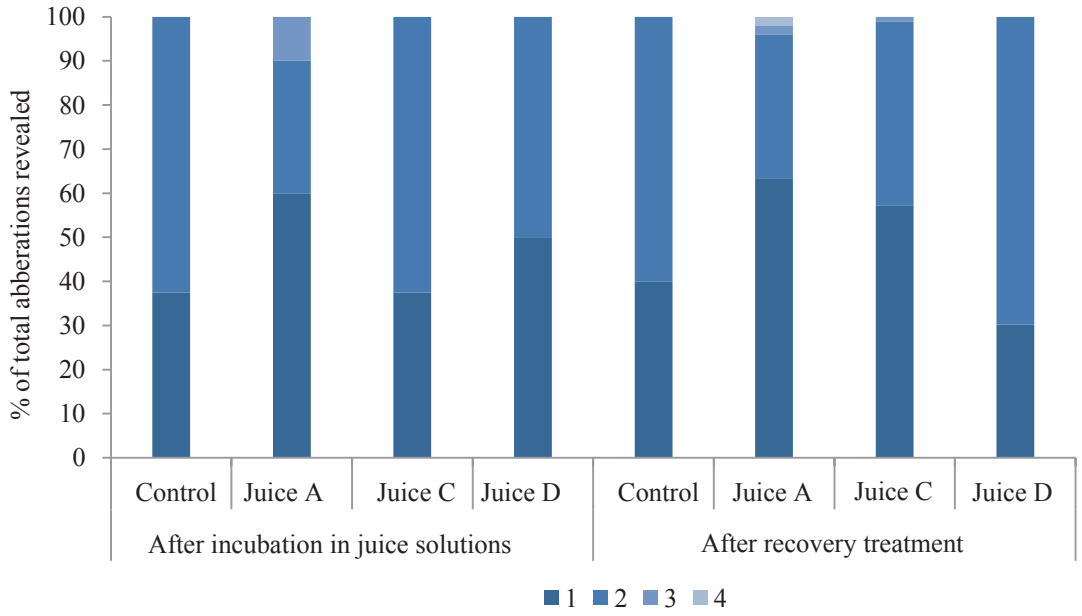
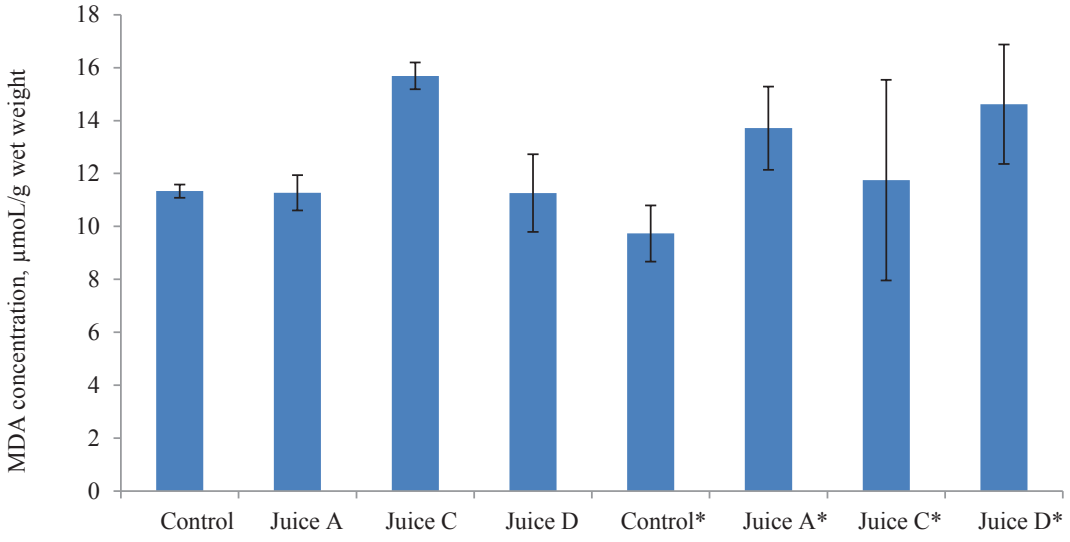


Figure 6 Chromosomal aberrations in meristem cells of onion roots before and after recovery treatment in juice solutions: 1) disorders of chromosome segregation (overlap, lag); 2) anomalies of mitotic apparatus (adhesion, multipolar mitosis); 3) aberrations of clastogenic character (bridges, fragments); 4) miscellaneous (fragmentation, agglutination, pulverization)



Note: Vertical error bars indicate the value of the standard deviation, * – marks incubation in acid solutions followed by germination in bottled water

Figure 7 MDA in roots treated with aqueous solutions of juices

the treatment time with juice solutions reached 48 h. After recovery treatment, the average weight of the roots was by 11–25% lower than that in the control samples, but this difference was not significant (Table 3). Mitotic index had the same trend, except for the brand D juice samples, although this indicator differed from the control by only 11%. However, the cytogenetic analysis showed a significant increase in chromosomal aberrations in all the experimental groups, while the maximal growth by more than six times was recorded in the brand C juice samples.

Figure 6 shows the chromosome aberrations found in the apical meristem of the onion roots after incubation in juice solutions, as well as after incubation and subsequent regeneration in bottled water. No statistically significant differences ($P \leq 0.05$) in chromosomal disorders were revealed before or after recovery treatment in bottled water. However, juice A samples demonstrated all kinds of aberrations after recovery treatment

Thus, all the experimental samples revealed irreversible significant genotoxic effects (Table 3), represented mostly by chromosome disorganization

in metaphase, lagging in anaphase, metafhase with chromosome loss, and lagging in telophase (Fig. 6). Disorganization of chromosomes in metaphase, for instance, was a typical irreversible side effect of benzoic acid on onion roots [20].

After recovery treatment, MDA content was higher in all the experimental variants by 21–51% compared with the control values (Fig. 7). This indicator also demonstrated the irreversible nature of the identified adverse effects after exposure to juice solutions.

Thus, the maximal negative effects after recovery treatment were recorded when analyzing the values of the mitotic index and MDA in the D juice samples and the level of chromosome aberrations in the C juice samples. If the first two indicators differed from the control only by tens of percent, the latter differed by several times in all the experimental variants. In the juice C samples, the level of cytogenetic disorders was two times higher compared to samples A and D. This biomarker requires more attention when assessing the genotoxic potential of this product, both phytochemical and technological.

CONCLUSION

The research featured a new bioassay method for determining the antioxidant potential of processed apple juice. The juice reduced the lipid oxidation in onion roots to 40% after oxidative stress induced by sorbic acid. The antioxidant potential in juice solutions depended on the ratio of biologically active compounds and carbohydrates.

The research included a comparative analysis of three juice brands. Sorbic acid had a possible negative effect on the quality of juice-containing products: even 50 mg/L reduced the antioxidant profile of the finished product. When the concentration of sorbic acid reached 100 mg/L, its effect became toxic, and onion roots died. No side toxic subchronic effects on the weight gain were registered after onion roots were treated with three juice brands. However, one of the three juices demonstrated an irreversible decrease in the proliferative index by 11%.

The cytogenetic analysis of the root meristem revealed the maximal adverse side effect: chromosomal aberrations increased in all experimental groups. For one brand, these disorders increased by more than six times. In general, the *Allium cepa* bioassay of toxic subchronic effects provided reliable results for side effects in apple juice production.

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 regarding the publication of this article.

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Effects of high-protein feed supplements on lamb productivity

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Abstract:
Introduction. Today's feed market offers a variety of new products of plant and animal origin that increases the productivity of young sheep. Using feed supplements can help farmers to fully realize the genetic potential of wool-and-meat genotype sheep.
Study objects and methods. We studied the effect of a whole milk replacer (skimmed powdered milk) and an ORGANIC high-protein feed supplement on the growth of young sheep and the quality of their meat. In particular, we determined the effect of starter feeds on the biochemical and morphological parameters of sheep blood at the Vtoraya Pyatiletka Breeding Farm, Stavropol Krai.
Results and discussion. Substituting starter feeds with a whole milk replacer and an ORGANIC supplement for the standard feed in the diet of sheep aged 0–4 months increased metabolic energy (by 12.5%), crude protein (by 22.4 and 25.5%, respectively), lysine (by 24.8 and 21.4%, respectively), and methionine + cystine (by 31.0%). The starter feeds also led to higher live weight (by 29.6 and 33.7% ($P \leq 0.001$)), absolute and average daily gain (by 24.6 and 29.1% ($P \leq 0.001$)), slaughter weight (by 36.5 and 42.1% ($P \leq 0.001$)), slaughter yield (by 2.50 and 2.96 abs.% ($P \leq 0.05$)), and meat marbling (by 3.6 and 11.7%). The number of muscle fibers increased by 2.1 and 3.3%, respectively. Additional profits rose from 1761.5 to 2091.5 rubles per head and the product profitability reached 50.5–57.9%.
Conclusion. The starter feeds containing a milk replacer and an ORGANIC feed supplement proved effective for sheep aged of 0–4 months in the suckling period, ensuring live weight of 39–40 kg and improving meat quality and productivity.

Keywords: Young sheep, milk replacer, ORGANIC feed supplement, slaughter and meat qualities, economic efficiency

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INTRODUCTION

Modern market conditions call for highly competitive sheep breeding, which requires specialized meat production of high quality. Sheep meat is the most expensive meat in developed countries. Its consumption depends on cultural factors and tends to increase with growing population and incomes. Major exporting countries see a decline in their sheep stocks caused by the shrinking wool market. Sheep meat production is expected to be developed by small and medium-sized farms close to their markets [1].

Today, there is an urgent need for highly productive young sheep and safe lamb meat of high-quality. The world demand for lamb is very high, both in nutritional and commercial terms. Therefore, lamb production is a priority in Russia and abroad [2].

Many studies have shown that lamb aged 0–7 months is the best type of sheep meat. Russia consumes 1.0 kg of lamb per capita, compared to 1.29 kg worldwide. Private farms account for 88.6–89.2% of sheep meat production in Russia [3].

Nutrition is one of the main factors of meat productivity alongside good maintenance conditions [4]. Selective breeding of farm animals is also gaining ground. It aims to produce new genotypes of wool-and-meat sheep for manufacturers of livestock products.

To fully realize the genetic potential of wool-and-meat sheep genotypes, we need to improve their feeding systems by adding new supplements of plant and

animal origin to enhance their productivity. Adequate nutrition is the main principle of animal feeding that contributes to higher productivity. Diets should be differentiated according to production schemes and planned productivity. Rationed feeding is one of the most important indicators of nutrition that ensures normal physiological state, high productivity, good reproductive qualities, and production profitability. Nutrition enhanced with feed supplements rich in bioactive substances is the most rational way to obtain low-cost and high-quality products [5]. Radzhabov *et al.* provide a good coverage of intensive sheep raising methods based on adequate feeding [6].

By-products of starch, dairy, meat, and other industries are increasingly used as feed supplements in animal diets. However, it is important to study their composition, nutritional benefits, and possible side effects for the animal organism [7, 8]. Most changes caused by feed supplements occur at an early age, during intensive growth and development of lambs. This is a period of greatest assimilation of feed nutrients leading to the maximum increase in live weight. Feed supplements improve animal productivity, activate digestive metabolic processes, and ultimately make sheep breeding cost-effective [9, 10].

The ORGANIC supplement (patented in Russia) is a high-protein feed supplement obtained from collagen-containing solid waste of leather production. It is used to enrich feed for all types of productive animals, fish, and poultry, with a highly digestible, "protected" protein. The product has stable quality indicators, namely 82–85% of crude protein and a complete composition of essential and non-essential amino acids. It facilitates digestion and gastrointestinal functioning, which improves the digestibility and absorption of feed nutrients, as well as increases the natural resistance of the animal's body [11].

We aimed to study the effect of the ORGANIC feed supplement on the growth and development of sheep, meat productivity, and economic efficiency.

To achieve the aim, we set a number of objectives, namely to:

– formulate new starter feeds for wool-and-meat lambs aged 0–4 months based on their maintenance and feeding conditions;
– evaluate the effect of starter feeds on the growth of young sheep, as well as meat productivity and quality;
– assess the effect of starter feeds on the biochemical and morphological parameters of blood and general health of young sheep; and
– calculate feed efficiency and economic efficiency of using starter feeds in young sheep breeding.

STUDY OBJECTS AND METHODS

We aimed to test new starter feeds for wool-and-meat lambs aged 0–4 months of the Russian Meat Merino breed. Experimental studies were conducted at the Vtoraya Pyatiletka Breeding Farm (Stavropol Krai, Russia).

Its sheep breeding technology involves stall and pasture maintenance; weaning (at the age of 120 days); shearing (May – June); grazing (on pastures with kosher basic feeding technology); artificial insemination (September – October); and lambing (February – March). When in the stalls, sheep receive coarse, juicy, and concentrated feeds.

Diets for young sheep were determined by such factors as age, live weight, and productivity [9]. We used three diets, namely: standard feed (control), starter feed with 5% of skimmed powered milk as a whole milk replacer (experiment), and starter feed with 3% of the ORGANIC supplement (experiment). The composition of starter feeds and their nutritional value are shown in Table 1.

Compared to standard feed (Formula 1), starter feed with the whole milk replacer (MR) (Formula 2), and starter feed with the ORGANIC supplement (OS) (Formula 3) had higher values of total nutritional value (by 4.7 and 6.6%, respectively), crude protein (by 19.8 and 27.3%, respectively), digestible protein (by 7.6 and 15.1%, respectively), lysine (by 23.7 and 18.6%, respectively), methionine + cystine (by 32.7%), threonine (by 23.1 and 21.2%, respectively), and crude fat (by 83.4 and 82.7%, respectively) (Table 1).

The formulated starter feeds (Table 1) were tested on three groups of ewes (12 heads in each) with single ram lambs (aged from 2 days to 4 months) based on the analogous pair principle (Table 2).

As we can see in Table 2, the control ram lambs (Group I) received alfalfa hay and standard feed (Formula 1) based on the formulation of the All-Russian Research Institute of Animal Husbandry. For the rams in the experimental groups, the standard feed was replaced with starter feed with the milk replacer and starter feed with the ORGANIC supplement, respectively.

Feed samples were analyzed at the Feed and Metabolism Laboratory, Stavropol State Agrarian University. Total nitrogen (crude protein) was determined on a UDK-142 protein (nitrogen) analyzer, crude fat – on a SER-148 fat analyzer, macro- and microelements – on a Spectroscan MAX GV universal analyzer, vitamins – on a LCMS-10 EV liquid chromatograph, crude fiber – on a FIVE fiber analyzer, and total feed moisture – on an AD-4714 A moisture analyzer.

The guidelines by Dmitrik *et al.* were used to determine the morphological composition of lamb carcasses and conduct a microstructural analysis of the *Longissimus dorsi* muscle [12]. The animals' internal homeostasis was assessed on the basis of morphological and biochemical blood analyses [13, 14]. The counting of erythrocytes and leukocytes was performed in the Goryaev chamber. The leukocyte formula was based on a blood smear stained using the Romanowsky-Giemsa technique. The results were biometrically processed using statistical methods (Microsoft Excel).

Table 1 Feed formulations for lambs aged 0–4 months

Indicator	Feed formulations		
	Formula 1*	Formula 2**	Formula 3***
Feed composition, %			
Barley	29.00	14.00	15.00
Oats	28.0	–	–
Wheat	10.00	14.00	15.93
Wheat Bran	10.00	11.33	15. 0
Corn	–	20.00	15.00
Peas	10.0	–	–
Sunflower cake (crude protein 34%, crude fiber 20)	10.00	19.97	20.00
Alfalfa flour (crude protein 14.0 %)	–	10.00	10.00
ORGANIC feed supplement	–	–	3.00
Skimmed milk powder	–	5.00	–
Feed yeast (crude protein 42%)	–	3.00	3.00
Feed chalk	–	0.58	0.35
Monocalcium phosphate	1.00	0.50	1.03
P61-1 premix for calves	1.00	0.80	0.80
Table salt	0.70	0.82	0.89
Components			
Energy feed units	1.06	1.11	1.13
Metabolic energy, MJ	10.6	11.1	11.3
Dry matter, g	880.0	891.8	890.6
Crude protein, g	149.3	178.8	190.0
Digestible protein, g	131.5	141.5	151.4
Lysine, g	5.9	7.3	7.0
Methionine + cystine, g	4.9	6.5	6.5
Threonine, g	5.2	6.4	6.3
Crude fat, g	28.3	51.9	51.7
Crude fiber, g	79.7	90.1	93.8
Sugar, g	37.7	35.6	33.4
Ca, g	3.26	8.0	8.6
P, g	7.21	6.7	6.9
Mg, g	2.6	2.3	2.5
Table salt, g	1.4	10.0	10.0
Fe, mg	78	20.0	20.0
Cu, mg	5.2	4.0	4.0
Zn, mg	31.4	32.0	32.0
Mn, mg	35.5	40.0	40.0
Co, mg	2.0	2.0	2.0
I, mg	1.0	1.2	1.2
Se, mg	0.11	0.16	0.16
Vitamins			
A, thousand IU	4.8	16.0	16.0
D_3, thousand IU	0.01	3.20	3.20
E, mg	12.5	56.0	56.0
B_1, mg	3.5	2.4	2.4
B_2, mg	1.1	8.0	8.0
B_3, mg	9.3	16.0	16.0
B_5, mg	13.0	8.0	8.0
B_{12}, mg	–	0.016	0.016

*Formula 1 – standard feed used at the Vtoraya Pyatiletka Breeding Farm

** Formula 2 – starter feed with 5% of milk replacer (New Compound Feeds Company)

*** Formula 3 – starter feed with 3% of ORGANIC feed supplement (New Compound Feeds Company)

Table 2 Diets of suckling lambs aged 0–4 months (n = 12)

Group	Diet description
I (control)	Normal diet (ewe milk, alfalfa hay, mineral feed) + standard farm feed (Formula 1)
II (experimental)	Normal diet + starter feed with whole milk replacer (Formula 2)
III (experimental)	Normal diet + starter feed with ORGANIC supplement (Formula 3)

Table 3 Actual feed intake by sheep aged 0–4 months

Indicator	Group		
	I (control)	II (experimental)	III (experimental)
Alfalfa hay	0.30	0.36	0.36
Standard farm feed (Formula 1)	0.41	–	–
Starter feed with the whole milk replacer (Formula 2)	–	0.42	–
Starter feed with the ORGANIC supplement (Formula 3)	–	–	0.42
Table salt, g	6.0	6.0	6.0
Felucen mineral salt, g	5.0	5.0	5.0
Disodium phosphate, g	4.9	4.9	4.9
Components			
Energy feed units	0.64	0.72	0.72
Metabolic energy, MJ	6.4	7.2	7.2
Dry matter, g	0.60	0.70	0.70
Crude protein, g	98	120	123
Digestible protein, g	79	90	93
Lysine, g	4.20	5.24	5.10
Methionine + cystine, g	2.90	3.80	3.80
Crude fiber, g	112	134	134
Sugar, g	23	23	23
Ca, g	5.2	8.0	7.9
P, g	5.8	4.6	5.1
Mg, g	1.51	1.51	1.57
NaCl, g	6.7	6.3	6.3
Fe, mg	82.3	69.2	68.4
Cu, mg	5.80	5.71	5.66
Zn, mg	22.0	24.6	24.3
Mn, mg	35.7	42.4	41.9
Co, mg	0.9	1.0	0.9
I, mg	0.41	0.52	0.52
Vitamins			
A, mg	12	34	34
D_3, thousand IU	31	1376	1360
E, mg	37	62	61

Table 4 Feed consumption by sheep during the suckling period

Group	Diet			
	Ewe milk	Alfalfa and legume hay	Feed	Total
Standard farm feed (control):				
– feed weight, kg	136.0	10.8	24.4	171.2
– energy feed units	40.80	4.97	31.70	77.47
– digestible protein, g	4488	443	3684	8615
Starter feed with whole milk replacer:				
– feed weight, kg	136.0	11.2	25.8	173.0
– energy feed units	40.80	5.15	33.50	79.45
– digestible protein, g	4488	459	3947	8894
Starter feed with ORGANIC supplement:				
– feed weight, kg	136.0	10.9	25.7	172.6
– energy feed units	40.80	5.00	33.40	79.20
– digestible protein, g	4488	447	5110	10045

RESULTS AND DISCUSSION

Adequate feeding, especially during intensive growth and development, is highly important for the animals' productivity. In our study, the lambs' needs for basic nutrients were determined by their health and growth indicators.

Substituting the standard feed (Formula 1) for starter feeds containing the milk replacer (Formula 2) and the ORGANIC supplement (Formula 3) in the diets of sheep (Table 3) aged 0–4 months increased their metabolic energy (by 12.5%), crude protein (by 22.4 and 25.5%, respectively), lysine (by 24.8 and 21.4%, respectively), methionine + cystine (by 31.0%), and vitamins A, D, E (1.6–44.3 times).

Table 4 shows the total feed consumption by the experimental lambs during the suckling period. Over 120 days of growth, the control group consumed 77.47 energy feed units and 8.62 kg of digestible protein. In the experimental groups, energy feed units were lower by 1.98 and 1.73 and digestible protein by 0.279 and 1.430 kg, respectively. As we know, ewes produce plenty of milk (1.2–1.5 liters) during lactation to meet their lambs' nutritional needs. However, its amount significantly reduces in the months following lactation. For this reason, we formulated starter feeds containing the milk replacer (WMR) and the ORGANIC supplement (OS).

Feeding starter feeds to suckling lambs for four months affected their live weight (Fig. 1). Compared

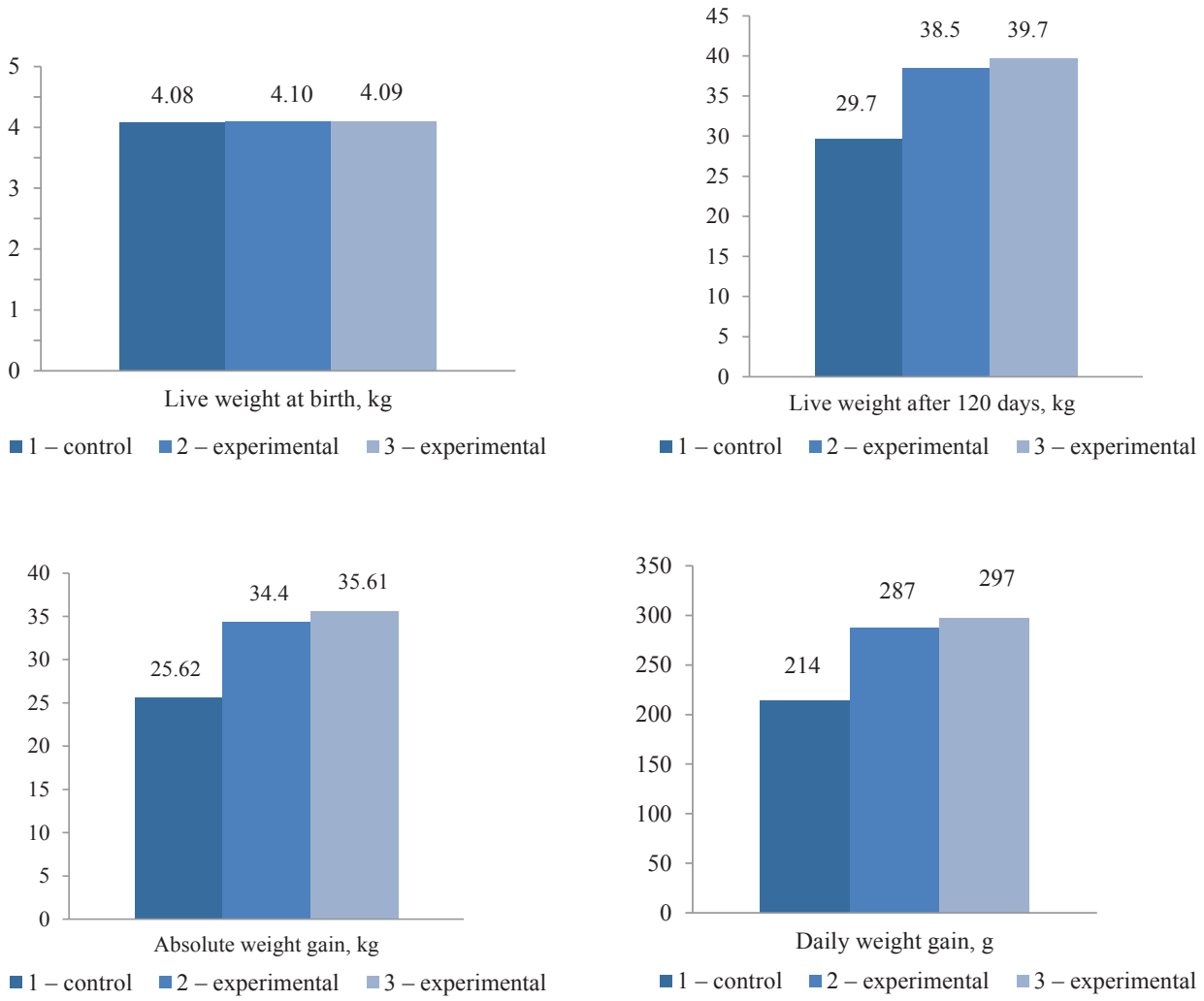


Figure 1 Live weight and live weight gain of young sheep that received: 1 – standard feed (control); 2 – starter feed with the whole milk replacer, 3 – starter feed with the ORGANIC supplement; n = 12

to the control lambs, live weight of the experimental lambs aged 120 days in the groups that received starter feeds with the WMR and OS increased by 8.8 kg, or 29.63% ($P \leq 0.001$), and 10.0 kg, or 33.67% ($P \leq 0.001$), respectively. The average daily gain rose by 73.0 g, or 34.11% ($P \leq 0.001$), and 83 g, or 38.78% ($P \leq 0.001$), respectively, with 100% animal safety.

Therefore, we found that the use of starter feeds with the WMR and the OS instead of standard feed was zootechnically justified. These feeds contributed to a better palatability of the diet feed and a greater intake of nutrients.

Lamb production needs to be increased to enhance the economic efficiency of sheep breeding. The meat from sheep aged up to one year is easily digestible and has a low fat content. For this reason, it is recommended for dietetic diet. Scientists have also established that the most intensive weight gain occurs in the first six months.

For the next stage of our study, we carried out a control slaughter of lambs at the age of four months. This age was due to intensive protein deposition in the first six months. Adipose tissue accumulating more intensively in the later period of life affects the biological value of meat and the economic efficiency of lamb production.

Hematological parameters show a complete picture of the organism's development. We found that the blood parameters of 4-month-old lambs of the experimental and control groups were within the physiological norms (Fig. 2).

The contents of erythrocytes and hemoglobin were within the physiological norms, indicating the absence of anemia (Fig. 2). Compared to the control group, the blood of the experimental lambs that received starter feeds with the MR and the OS had a slight increase in hemoglobin, namely by 2.6 ($P > 0.1$) and 4.7% ($P < 0.05$), respectively, and in erythrocytes, by 5.6 and 11.1% ($P < 0.05$), respectively. This contributed to a more intensive metabolism. The contents of leukocytes were almost identical, with a difference of 2.9 and 5.9%, respectively.

Higher total protein in the serum of the experimental lambs indicated more intensive protein digestion and absorption of amino acids in the intestine, as

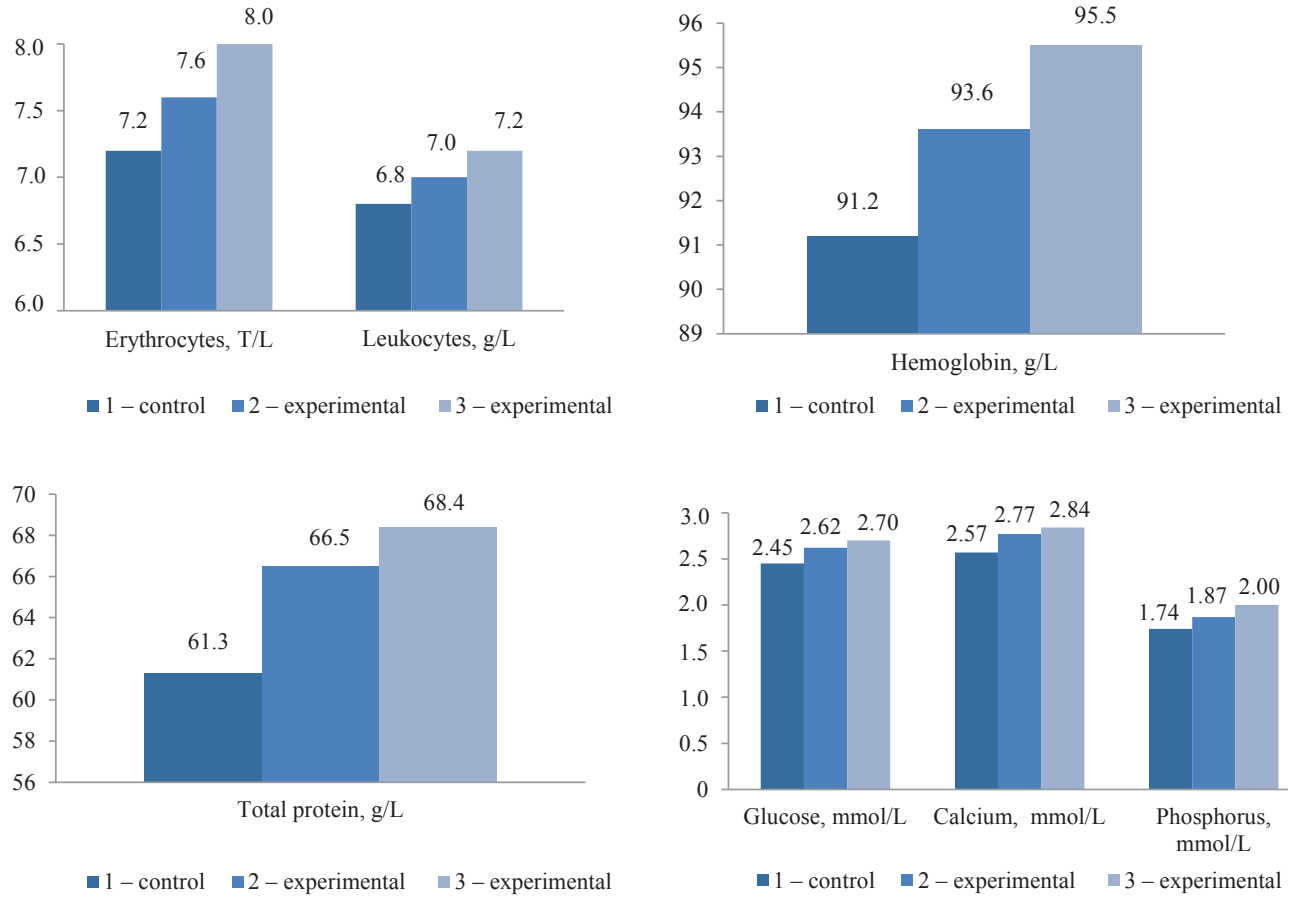


Figure 2 Hematological and biochemical parameters of young sheep that received: 1 – standard feed (control); 2 – starter feed with the whole milk replacer, 3 – starter feed with the ORGANIC supplement

well as protein synthesis in the liver. This finding was confirmed by the live weight gain indicators. In particular, total protein in the experimental groups that received the MR and the OS was 8.5 and 11.6% ($P < 0.05$) higher in comparison with the control group. Glucose contents were higher by 6.9 ($P < 0.05$) and 10.2% ($P < 0.05$), respectively, indicating a better supply of body cells with energy. Similar results were reported by Khompodoeva and Pashtetskaya *et al.* [15, 16].

Calcium was significantly higher in the blood serum of experimental lambs, namely by 7.8 ($P < 0.05$) and 10.5% ($P < 0.05$) in the groups that received the MR and the OS, respectively. A similar trend was observed in the amount of phosphorus: it was 7.5 ($P < 0.05$) and 14.9% ($P < 0.01$) higher in the experimental groups, compared to the control. This was indicative of better saturation of the bone tissue and stronger bones.

The control slaughter (Fig. 3) proved a possibility of obtaining high-quality dietetic meat from lambs aged 4 months.

We established (Fig. 4) that starter feeds enriched with the milk replacer and the ORGANIC supplement, increased the slaughter weight by 36.5 and 42.05% ($P \leq 0.001$) and slaughter yield by 2.50 and 2.96 abs.%, respectively, compared to the control.

Next, we studied the morphological composition of animal carcasses to assess meat productivity. For this, we deboned the carcasses and analyzed various meat characteristics, especially the meat coefficient (the ratio of the weight of meat to the weight of bones and tendons) (Fig. 5).

Figure 3 Carcasses of young sheep

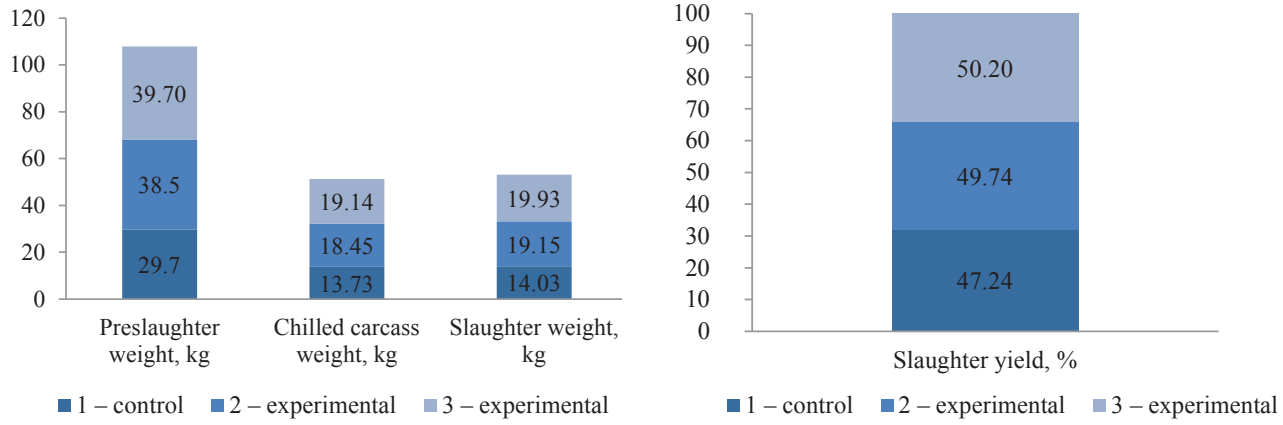


Figure 4 Slaughter characteristics of ram lambs that received: 1 – standard feed (control); 2 – starter feed with the whole milk replacer, 3 – starter feed with the ORGANIC supplement; n = 3

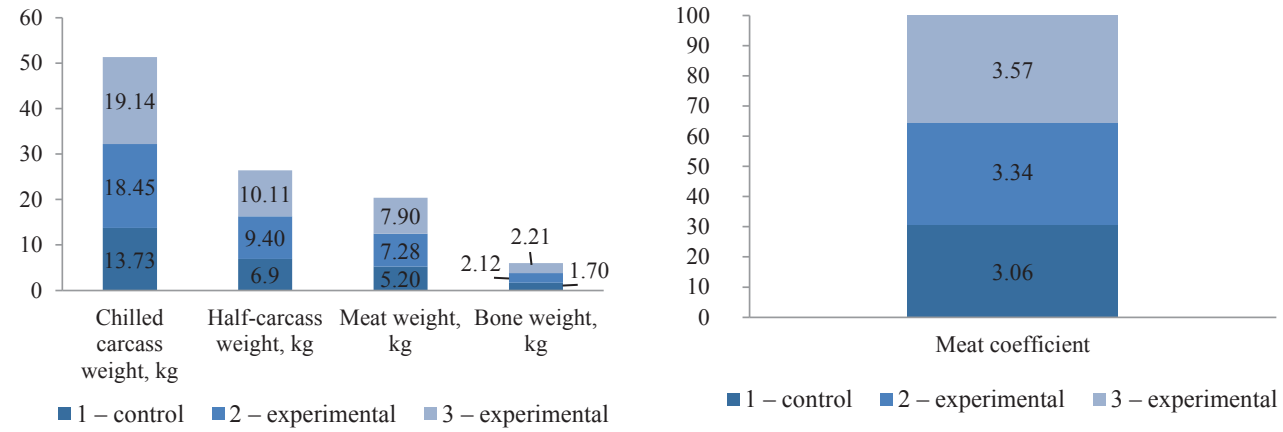


Figure 5 Morphological composition of lamb carcasses aged 4 months that received: 1 – standard feed (control); 2 – starter feed with the whole milk replacer, 3 – starter feed with the ORGANIC supplement; n = 3

The ratio of muscle, bone, and connective tissues in the body of sheep affects the nutritional and biological value of carcasses. However, if sheep had different live weight, the nutritional value of their carcasses can vary greatly [17].

Apparently, higher feed intake and better assimilation of nutrients in the experimental groups contributed to a more uniform deposition of subcutaneous fat and the formation of muscle mass.

We found maximum meat weight in the carcasses of lambs that received feed with the milk replacer and the ORGANIC supplement, respectively. Particularly, their meat weight was 40.0 and 51.9% ($P \leq 0.001$) higher than in the control group, with a meat coefficient of 3.34 and 3.57, respectively. Our data were consistent with those reported by Khayitov and Dzhuraeva [18].

Histological studies of the *Longissimus dorsi* muscle showed better microstructural characteristics in the meat of experimental lambs (Table 5).

The meat of young sheep from the experimental groups that received the MR and the OS had smaller muscle bundles, but it contained more fibers than the meat of the control animals, namely by 8.45 pcs./1 mm^2 (2.1%) and 13.34 pcs./1 mm^2 (3.3%), respectively. The marbling coefficient of meat in the group that received the OS exceeded that of meat in the groups fed on standard feed and the OS feed by 11.7 and 7.9% ($P \leq 0.05$), respectively.

The microstructural analysis of muscle tissue samples (Fig. 6) showed that the *Longissimus dorsi* muscle of lambs had a greater number of muscle fibers of a smaller diameter with fatty layers, indicative of high nutritional and commercial qualities.

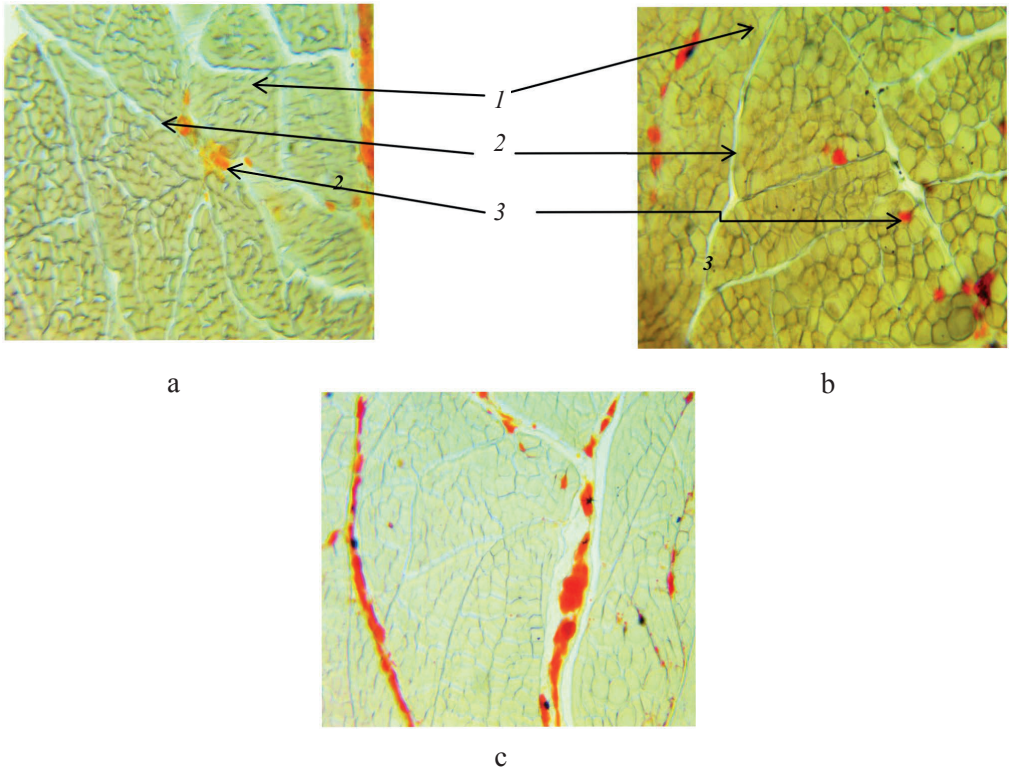
The biological value is the main indicator of the product's nutritional value.

The chemical composition of muscle tissue largely depends on fatness and productivity type (wool-and-meat or other). In our study (Fig. 7), the muscle tissue of the experimental lambs in the groups fed on the MR and OS feeds contained less moisture (by 4.52–6.47 abs.% ($P \leq 0.05$)), but more dry matter (by 1.47–4.52 abs.%), fat (by 0.58–0.80 abs.%), and protein (by 0.87–3.67 abs.% ($P \leq 0.05$)), compared to the control. Our data were consistent with those of Bogatirevet *et al.* [19].

Table 5 Microstructural analysis of the *Longissimus dorsi* muscle in sheep aged 4 months that received various diets (n = 3)

Indicator	Group		
	Standard feed (control)	Starter feed with whole milk replacer (experiment)	Starter feed with ORGANIC supplement (experiment)
Number of muscle fibers, pcs.	401.33 ± 7.22	409.78 ± 7.38	414.67 ± 7.47
Muscle fiber diameter, μm	28.45 ± 0.51	25.84 ± 0.46	25.34 ± 0.35*
"Marbling" assessment, score	28.95 ± 0.52	29.99 ± 0.54	32.35 ± 0.58*
Connective tissue, %	8.00 ± 0.14	7.60 ± 0.13	7.20 ± 0.11*

* – significant difference from the control group ($P \leq 0.05$)



1 – muscle fibers; *2* – connective tissue; *3* – fatty interfiber and interfascicular inclusions ("marbling")

Figure 6 Histosection of the longissimus dorsi muscle tissue of young sheep (colored with hematoxylin-eosin, magnification ×500): a) group fed on standard feed (control); b) group fed on starter feed with whole milk replacer (experiment); c) group fed on starter feed with ORGANIC supplement (experiment)

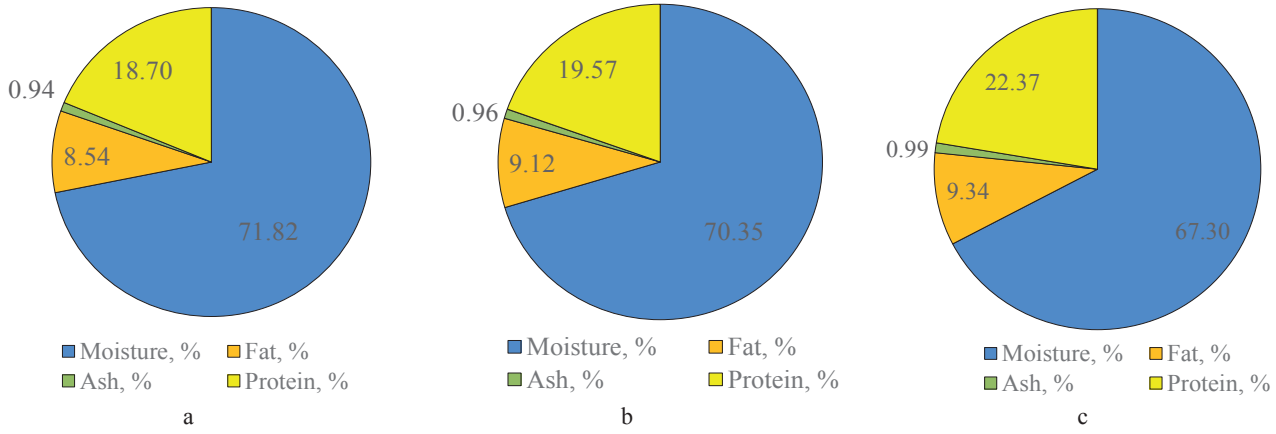


Figure 7 Chemical composition of muscle tissue of young sheep that received a) – standard feed (control); b) – starter feed with the whole milk replacer, c) – starter feed with the ORGANIC supplement; n = 3

Table 6 Economic efficiency of lamb production (per head, in 2021 prices)

Indicator	Group		
	Standard feed (control)	Starter feed with whole milk replacer (experiment)	Starter feed with ORGANIC supplement (experiment)
Weight gain during experimental period, kg	25.62	34.40	35.61
Additional gain, kg	–	8.78	9.99
Average daily gain, g	214	287	297
Costs per 1 kg of gain: energy feed units	3.02	2.31	2.22
digestible protein, g	336	259	282
Cost of 1 kg of live weight gain, rubles	189.0	145.5	138.7
Total costs, rubles	4842.2	5005.2	4939.1
including feed supplements, rubles	–	161.3	96.3
Selling price of 1 kg of live weight gain, rubles		219.0	
Sales proceeds, rubles	5610.8	7533.6	7798.6
Profit, rubles	768.6	2528.4	2859.5
Additional profit, v	–	1761.5	2091.5
Level of profitability, %	15.8	50.5	57.9
Return on 1 rbl. of feed supplement costs, rubles	–	10.9	21.7

Thus, the morphological and microstructural analyses of the longissimus dorsi muscle tissue revealed higher quality indicators in the lamb fed on the OS feed, compared to the groups that received standard feed and the MR feed. Particularly, it had the largest number of muscle fibers of a smaller diameter, a higher marbling score, and a lower content of connective tissue. Therefore, the meat in the group fed on the OS feed was characterized as tender and juicy. The most nutritious and valuable were those animal carcasses which had a greater content of muscle tissue, since connective tissue contains fatty tissue that make meat a high-calorie product and give it a characteristic taste, color, and aroma.

The economic analysis (Table 6) proved the efficiency of using starter feeds with the high-protein milk replacer of the ORGANIC supplement when growing wool-and-meat lambs up to 4 months of age.

Particularly, the absolute increase in live weight of lambs in the experimental groups that received the MR and the OS feeds amounted to 8.8 and 10.0 kg or 34.27 and 38.99% ($P \leq 0.001$), respectively.

The additional costs of the milk replacer and the ORGANIC feed supplement for the experimental groups were 161.3 and 96.3 rubles, respectively. The same selling price of 219.0 rubles per 1 kg of live weight allowed for an extra profit of 1761.5 and 2091.5 rubles respectively. The level of profitability of lamb production increased by 34.7 and 42.1%, respectively. The return on one ruble of feed supplement costs amounted to 10.9 and 21.7 rubles, respectively.

CONCLUSION

Including starter feeds enriched with 5% of skimmed powered milk as a whole milk replacer or 3% of the ORGANIC feed supplement in the diet of sheep aged up to four months enhanced their nutritional value, namely metabolic energy (by 4.7 and 6.6%), raw protein (by 19.8 and 27.3%), lysine (by 23.7 and 18.6%), methionine + cystine (by 32.7%), and threonine (by 23.1 and 21.2%). By the age of four months, the lambs had an increase in live weight (8.8 and 10.0 kg) and average daily weight gain (73.0 and 83.0 g). The supplements also improved the quality of meat, as well as feed efficiency (by 0.71 and 0.98 energy feed units) and the profitability of lamb production.

CONTRIBUTION

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

CONFLICT OF INTEREST

The authors declare no conflict of interest regarding the publication of this article.

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