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Differential thermal analysis of moisture binding in zephyr with different contents of glucose syrup

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Abstract:
When formulating a product, it is just as important to measure changes in free and bound moisture as it is to analyze quality indicators. Zephyr, a Russian whipped dessert, made with sugar dries quickly during storage, gradually losing its moisture. Its crystalline sugar crust thickens and its entire mass saccharifies, resulting in higher firmness and poor appearance. In this study, we aimed to determine the effect of high-conversion glucose syrup on the amount of moisture and its binding forms in zephyr after storage.

We studied four samples of pectin-based zephyr with different carbohydrate profiles after three months of storage. Differential scanning calorimetry, thermogravimetry, and non-isothermal kinetics were applied to assess moisture contents and forms of binding in zephyr.

Thermograms with thermoanalytical curves were used to analyze the thermolysis of zephyr samples with different contents of glucose syrup in the temperature range from 20 to 300°C. We also studied the endothermic effects at various stages of thermolysis and measured free and bound moisture in the samples. Four stages of their dehydration were identified on the basis of graphical dependences between weight changes and heating temperatures.

The control zephyr sample contained more capillary and polymolecular bound moisture, while the experimental samples in which sugar and confectioner's syrup were partially or completely replaced with high-conversion glucose syrup had more polymolecular and monomolecular bound moisture. The use of high-conversion glucose syrup instead of sugar and confectioner's syrup reduced the amount of free moisture and therefore increased the amount of bound moisture, keeping zephyr fresh throughout its shelf life.

Keywords: Zephyr, glucose syrup, differential thermal analysis, moisture content, forms of moisture binding

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INTRODUCTION

Modern quality control methods in Russia are used to evaluate food products with various technological, structural, mechanical, thermophysical, and electrophysical properties throughout their shelf life [1]. They include chromatographic, spectral, optical, and thermovimetric instruments. It is particularly important to control changes in moisture binding, namely the ratio of free and bound moisture in the product based on the content and properties of moisture as it interacts with dry substances. This also requires a comprehensive determination of the product's thermodynamic, thermophysical, structural, and mechanical characteristics, as well as its mass-exchange properties [2].

Whipped pastille mass has a colloidal capillary-porous coagulation structure. It is a two-phase gas-liquid system, where the dispersed phase is air (or gas) bubbles (up to 25 microns in size) separated by protein films and the dispersion medium is a solution of sugar, glucose syrup, acids, pectin, and other components. The dispersion medium, which is formed due to the

pectin frame of colloidal particles, is a stabilized foamy jelly with certain mechanical strength, plasticity, and elasticity [3].

Zephyr mass has a foamy structure and an intermediate moisture content of 18 to 25% that contributes to its increased plasticity and elasticity. In zephyr mass, water mainly moves along the skeleton of the jelly but it can evaporate and condensate inside the pores (cells).

Zephyr is produced with various types of gelling agents, including pectin, agar-agar, agaroid, furcellaran, gelatin, etc. These agents are high-molecular compounds that are similar to polysaccharides with a chain-like structure of molecules [4, 5]. In a soluble form, they contribute to the stability of foams. In particular, they are adsorbed in the liquid films covering air bubbles and increase their strength. Egg or milk-protein foaming agents are used to stabilize the foam structure.

Strong pectin jelly is produced from pectins, sugar (or its substitutes), sugary starch hydrolysates (glucose syrups of various types), organic acids, salts of alkali metals and weak organic acids, as well as water. Pectin forms a dense, elastic network of the frame which firmly holds the liquid phase of the jelly. To cause pectin substances to coagulate, water polarity is changed and the surface tension is increased by adding sugar or sugar-containing products. The amount of added sugar depends on the amount of pectin.

Greater esterification of molecules correlates with their lower polarity, which requires a lower sugar content in the solution, and vice versa [6]. Higher concentrations of sugar lead to higher surface tension, causing sucrose molecules to bind and hold more water molecules and resulting in lower water polarity. If the concentration of pectin with a medium gelling capacity is 0.8–1.0% of the jelly weight, the amount of sugar should be at least 65%, corresponding to the concentration of a saturated solution at 70°C.

Molded zephyr halves gradually cool down as they mature, with pectin jelly forming in the dispersion medium. As they dry, a thin crystalline crust forms on their surface, preventing them from sticking. As a result, zephyr halves can be easily removed from the trays, fastened together, and sprinkled with powdered sugar.

During storage, zephyr dries out quite quickly, gradually losing its moisture. Its crystalline crust thickens and its entire mass gets saccharified, resulting in better strength but poorer appearance (reduced volume).

To keep zephyr fresh, its moisture should be evenly distributed and retained throughout its entire shelf life [7]. Free moisture is actively involved in various biochemical and microbiological processes. Bound moisture is associated with proteins and carbohydrates through physical and chemical interactions and least contributes to product spoilage. Bound moisture can be increased by using polysaccharide hydrocolloids (dietary fibers, gums, pectin-containing materials, starch, polysaccharides, carrageenans, etc.) [8]. Chemically, they are linear or branched high-molecular compounds with hydrophilic groups. They easily enter into physical interaction with moisture in the product and ensure its reliable retention.

Glucose syrups, depending on their type, contain from 15 to 60% of polysaccharides (dextrins, tetra- and trisaccharides), which are branched high-molecular compounds [9]. In addition, they contain hygroscopic reducing substances (glucose and maltose), which are good at retaining moisture for a long time.

The traditional zephyr formulation contains 40–50% of confectioner's syrup (by weight of sugar) with 36–44% of reducing substances on a dry basis (dextrose equivalent, DE), 42–34% of dextrins, and 65–75% of sugar (of the total weight). In addition, the syrup contains 0.1–0.4% of minerals, such as potassium, phosphorus, sodium, calcium, magnesium, and iron, as well as organic acids [10]. Its pH varies from 3.5 to 6.0.

In our previous studies, we tried to prolong zephyr's freshness during storage by slowing down its drying and gradual saccharization. For this, we partially or completely replaced sugar and confectioner's syrup in the control sample with various types of syrups [11]: high-conversion glucose syrup (DE = 62.6%) with 15.4% of dextrins, confectioner's syrup (DE = 40.4%) with 37.6% of dextrins, and low-conversion glucose syrup (DE = 32.3%) with 45.7% of dextrins. According to our results, confectioner's or low-conversion syrups used instead of sugar significantly increased the zephyr's viscosity and density, but decreased its shape-holding ability, causing the mass to spread after molding due to a high content of polysaccharides in these syrups. The use of high-conversion glucose syrup provided the zephyr mass with good viscosity and plasticity, as well as a density of 460 ± 10 kg/m^3, which allowed the mass to be shaped by using a depositor [12].

Foamy zephyr mass can be obtained by two methods: 1) by prolonged mechanical whipping of an apple-sugar-pectin mixture at atmospheric pressure in the presence of a foaming agent, followed by adding a glucose syrup and 2) by saturating the mass from an apple-sugar-pectin mixture and a glucose syrup with air at excess pressure. In this study, the zephyr mass was obtained in laboratory conditions using the first method using a Kitfort KT-1338 planetary mixer at a rotation speed of 4.3 s^{-1}.

We aimed to study how the content of high-conversion glucose syrup changed the amount of water and its binding forms in zephyr during storage by using differential thermal analysis.

STUDY OBJECTS AND METHODS

We studied zephyr samples based on pectin with different carbohydrate profiles resulting from partial or complete replacement of sugar and confectioner's syrup with high-conversion glucose syrup. After manufacturing, the samples were packed in food-grade plastic transparent containers made of polyethylene terephthalate and stored for three months under controlled external conditions (21 ± 1.5°C, relative humidity of 82 ± 2%). The control sample was prepared according to the traditional method by using sugar and acid confectioner's syrup at a ratio of 1:0.2. Samples No. 1 and No. 2

Table 1 Zephyr formulations with various carbohydrate profiles

Ingredient	Dry matter, %	Zephyr samples							
		Control		No. 1		No. 2		No. 3	
		Content of ingredients, g							
		Natural	Dry matter	Natural	Dry matter	Natural	Dry matter	Natural	Dry matter
		Syrup							
White sugar	99.85	34.65	34.60	34.65	34.60	34.65	34.60	–	–
Confectioner's syrup (DE = 40.4%)	83.40	13.98	11.65	13.98	11.65	13.98	11.65	–	–
High-conversion syrup (DE = 62.6%)	84.20	–	–	–	–	–	–	48.63	40.95
Total	–	54.41	46.25	53.78	46.25	53.16	46.25	46.53	40.95
Dry matter in syrup, %		85.0		86.0		87.0		88.0	
		Whipped mixture							
White sugar	99.85	30.87	30.82	15.42	15.40	–	–	–	–
High-conversion syrup (DE = 62.6%)	84.20	–	–	15.45	13.01	30.87	25.99	30.78	25.92
Pectin citrus	92.00	1.98	1.82	1.98	1.82	1.98	1.82	1.98	1.82
Apple puree	10.00	29.30	2.93	26.20	2.62	22.9	2.29	21.90	2.19
Egg white	12.00	6.33	0.76	6.33	0.76	6.33	0.76	6.33	0.76
Sodium lactate	40.00	0.65	0.26	0.65	0.26	0.65	0.26	0.65	0.26
Lactic acid	40.00	1.17	0.47	1.17	0.47	1.17	0.47	1.17	0.47
Flavoring	–	0.13	–	0.13	–	0.13	–	0.13	–
Total	–	70.43	37.06	67.33	34.34	64.03	31.49	62.94	31.42
Dry matter in mixture, %		52.6		51.0		49.2		50.0	
Total	–	124.84	83.31	121.11	80.59	117.19	77.74	109.47	72.37
Dry matter in zephyr mass, %		66.7		66.5		66.3		66.1	

Control contained sugar and acid confectioner's syrup at a ratio of 1:0.2 (traditional formulation)

Samples No. 1 and No. 2 included 50 and 100% of high-conversion glucose syrup instead of sugar

Sample No. 3 had sugar and acid confectioner's syrup completely replaced with high-conversion glucose syrup

had sugar partially (50%) and completely (100%) replaced with high-conversion glucose syrup in the whipped formulation mixture, respectively. Sample No. 3 had sugar and acid confectioner's syrup completely replaced with high-conversion glucose syrup in the whipped mixture and syrup.

The control sample was prepared as formulated in Table 1 by whipping an apple-sugar-pectin mixture with egg white and then adding, with constant stirring, confectioner's syrup with a moisture content of 85.0 ± 0.5% at 85–90°C until the zephyr mass reached a moisture of 33.0 ± 0.5% and a density of 440 ± 10 kg/m^3.

The partial or complete replacement of sugar with high-conversion glucose syrup gradually decreased the sweetness of the zephyr mass, intensified the aroma and taste of the fruit puree and flavoring additives (acids, flavors), and increased the mass's viscosity and strength. The content of reducing substances increased from 16.6% (control) to 38.7% (sample No. 3). Sucrose crystallization slowed down or stopped completely. Larger amounts of high-conversion glucose syrup in the zephyr samples increased the contents of mono- and disaccharides (glucose and maltose), whose molecules are highly hydrophilic, compared to those of sucrose. In particular, they bind moisture better and retain it for a long time, keeping zephyr fresh [13].

Using high-conversion glucose syrup instead of sugar increased the moisture content in the zephyr mass. To obtain the required moisture of 33.5 ± 0.3%, the syrup was boiled down to a higher dry matter content of 86–88%. The content of apple puree was reduced from 23.5% (control) to 20% (sample No. 3) of the total mass in line with State Standard R 702.1.015-2021. After three months of storage, the control and experimental samples No. 1, 2, and 3 had moisture contents of 10.1, 11.5, 12.1, and 14.3%, respectively. Moisture was determined by the refractometric method in accordance with State Standard 5900-2014.

Changes in moisture and its binding forms in the zephyr samples after storage were determined by the differential thermal method involving differential scanning calorimetry (DSC), thermogravimetry (TG), and non-isothermal kinetics. Thermoanalytical curves (thermogravimetry and differential scanning calorimetry), which were used for quantitative processing by the method of non-isometric kinetics, showed changes in temperature, weight, and enthalpy [14]. Particularly, the thermogravimetry curve indicated weight losses caused by increased temperatures; the derivative thermogravimetry (DTG) curve showed the rate of weight losses; and the differential scanning calorimetry curve characterized the thermal effects of reactions at linearly increasing temperatures.

The differential scanning calorimetry method determines the direction and magnitude of enthalpy changes associated with changes in the product's moisture caused by heating. It registers thermal effects in the form of absorbed energy during physical, chemical, and structural changes in the product. The experimental curves show the dependence of the heat flux of absorbed energy on the exposure temperature [15]. The differential scanning calorimetry method measures differences in heat fluxes and temperatures between the experimental samples and the control.

The thermogravimetry method measures changes (losses) in the weight of a sample during its heating in a wide temperature range (from 20 to 300°C), corresponding to various phase transformations of moisture [16].

Thermal curves, or thermograms, resulting from thermal analysis depend on the chemical composition and structure of the sample. The differential scanning calorimetry and thermogravimetry curves are processed using the MS Excel and NETZSCH Proteus software [17]. The resulting DSC and DTG curves show the rate of absorbed energy and the rate of weight loss due to moisture removal by heating, respectively. The dDSC curve determines the rate of absorbed energy at different stages of sample heating, the initial and final temperatures of thermal reactions, and the temperature peaks with maximum rates of moisture evaporation or volatilization of other substances. The region of the derivative thermogravimetry curve with a constant drying rate corresponds to the removal of free moisture, while the region with a decreasing drying rate characterizes the removal of bound moisture [18].

In this study, we used an STA 449 F3 Jupiter synchronous thermal analysis (TG-DSC) apparatus (NETZSCH, Germany) for various gas atmosphere values. This apparatus has the advantages of a highly sensitive thermobalance and a differential scanning calorimeter.

The thermal analysis of the zephyr samples was carried out in the analytical center of Voronezh State University of Engineering Technologies at atmospheric pressure, maximum temperature of 573 K (300°C), and a temperature change rate of 5°C/min. Zephyr samples were placed in 20-μL oxidized aluminum crucibles with a pierced lid. Since zephyr mass foams when heated, the sample weights were lower than the allowable volume of a crucible (12 mg), namely 6.3930, 7.8803, 5.1045, and 8.0919 mg for the control and samples No. 1, 2, and 3, respectively. The crucibles were placed in a nitrogen gas atmosphere of class 5.0, with a flow rate of 60 and 20 mL/min for the active sweeping gas and the protective gas, respectively.

RESULTS AND DISCUSSION

When heated, zephyr mass gradually loses its free moisture and then bound moisture. This is accompanied by wide endothermic effects characterizing moisture loss and decomposition of nutrients. Moreover, these processes depend on the chemical composition, quantity, and ratio of carbohydrates introduced with sugar and glucose syrup; pectins and dietary fibers introduced with apple puree; protein substances of the foaming agent; and sugar decomposition products forming during the boiling of syrups [19].

High temperature causes significant physical and chemical changes in organic compounds, resulting in moisture release which transforms nutrients inside the product [20]. The weight of zephyr samples decreases due to moisture evaporation, protein denaturation, and carbohydrate decomposition [21].

The thermolysis of the zephyr samples in the temperature range from 20 to 300°C is shown in the thermoanalytical curves (Figs. 1–4). As can be seen, the weight of the control sample decreased by 55.83%, with the residual weight of 2.8238 mg or 44.17%; the weight of sample No. 1 decreased by 54.80%, with the residual weight of 3.5619 mg or 45.20%; that of sample No. 2, by 55.03%, with the residual weight of 2.2955 mg or 44.97%; and that of sample No. 3, by 53.31%, the residual weight of 3.7781 mg or 46.69%.

The initial heating of the zephyr samples was in the temperature range of 20–25°C. Further heating intensified moisture removal and, accordingly, increased the energy of breaking the bond between water and the material. The differential scanning calorimetry curves showed a number of endothermic effects characterized by changes in the enthalpy index and accompanied by heat absorption [22]. The thermogravimetry curves revealed changes in the weights of the zephyr samples and peaks of the endothermic effects (differential scanning calorimetry curves). The endothermic effects identified in various temperature ranges (Table 2) indicated a gradual removal of moisture from the samples according to the forms and energy of moisture binding with the biopolymers of the product [23].

During heat exposure, the zephyr samples passed from a structured to a highly elastic state due to moisture removal and many physicochemical processes [24].

The differential scanning calorimetry curve showed three endothermic effects for the control sample (Fig. 1), namely:

1. In the temperature range from 25.45 to 138.37°C, with a large amount of internal energy (328.9 J/g) absorbed at a medium rate (1.04%/min) at the peak of the derivative thermogravimetry curve (t = 87.82°C) and a weight loss of 0.8596 mg (13.44%) (thermogravimetry curve) due to dehydration with a removal of capillary, poly- and monomolecular bound moisture (0.6460 mg) at the beginning of melting, thermal decomposition of monosaccharides (fructose and maltose), denaturation and subsequent decomposition of protein substances, and the formation of various chemical compounds with volatile substances released in the form of gases (0.2136 mg).

2. In the temperature range from 155.49 to 167.95°C, with a small amount of internal energy (3.833 J/g) absorbed at a low rate (0.12%/min) at the peak of the derivative thermogravimetry curve (t = 165.99°C) and a weight loss of 0.0774 mg (1.21%) (thermogravimetry curve) due

to the melting of glucose and sucrose, the decomposition and caramelization of fructose and maltose, as well as the formation of anhydrides, reversion (condensation) products, hydroxymethylfurfural, organic (formic and levulinic) acids, and colored compounds (carameline, caramelene), with volatile substances released in the form of gases (0.0774 mg).

3. In the temperature range from 188.46 to 231.89°C, with a small amount of internal energy (92.6 J/g) absorbed at a high rate (4.0%/min) at the peak of the derivative thermogravimetry curve (t = 218.13°C) and a weight loss of 1.5044 mg (23.52%) (thermogravimetry curve) due to the thermal decomposition and caramelization of sucrose and glucose, the formation of anhydrides, reversion products, hydroxymethylfurfural, organic acids, and colored compounds, as well as the decomposition of dextrins and protein substances, with volatile substances released in the form of gases (1.5044 mg).

The differential scanning calorimetry curve revealed two endothermic effects for sample No. 1 (Fig. 2), namely:

1. In the temperature range from 33.72 to 140.85°C, with a large amount of internal energy (336.6 J/g) absorbed at a medium rate (1.05%/min) at the peak of the derivative thermogravimetry curve (t = 84.26°C) and a weight loss of 1.0181 mg (12.92%) (thermogravimetry curve) due to dehydration processes with a removal of capillary, poly- and monomolecular bound moisture (0.9062 mg) and the beginning of melting and thermal decomposition of monosaccharides (fructose, maltose),

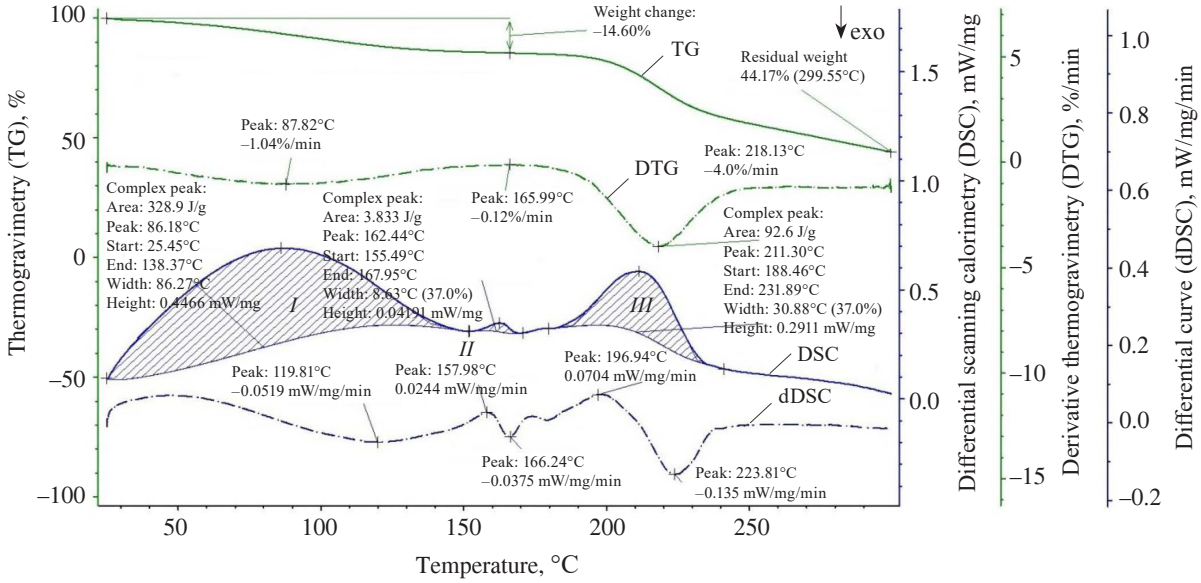

Figure 1 Thermogram of the control zephyr sample made from sugar and confectioner's syrup (DE = 40.4%) at a ratio of 1:0.2

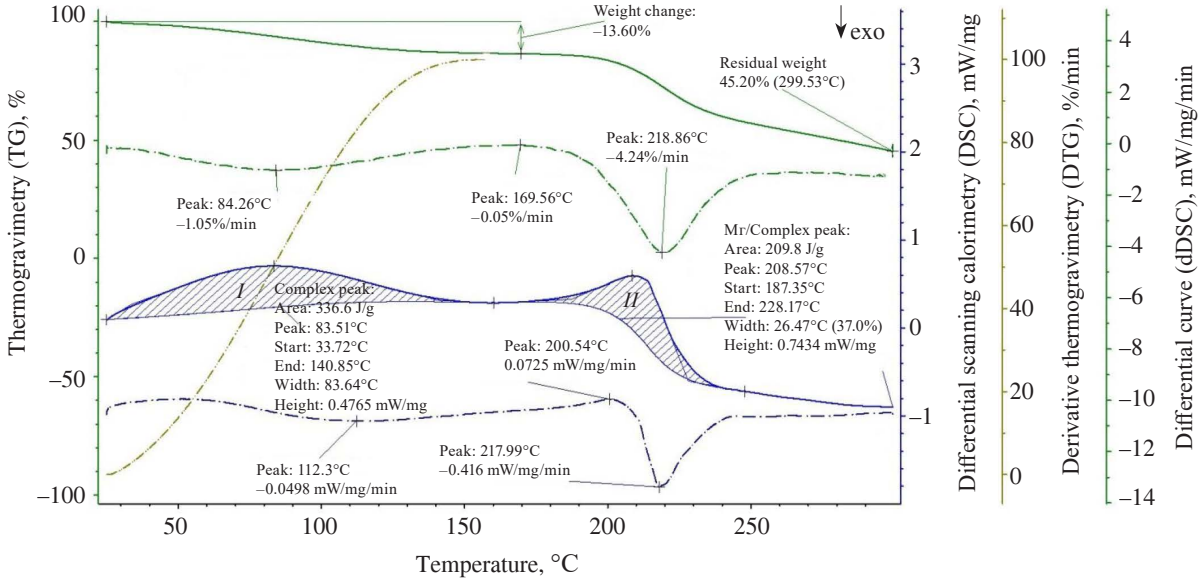

Figure 2 Thermogram of zephyr sample No. 1 with 50% sugar in the whipped mixture replaced with high-conversion glucose syrup (DE = 62.6%)

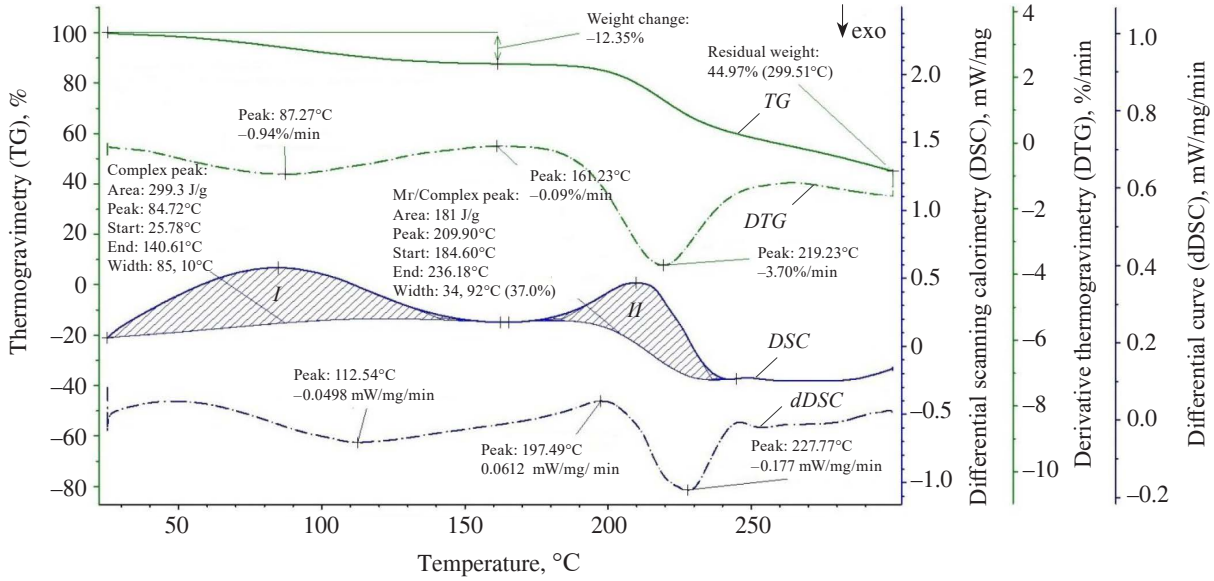

Figure 3 Thermogram of zephyr sample No. 2 with 100% sugar in the whipped mixture replaced with high-conversion glucose syrup (DE = 62.6%)

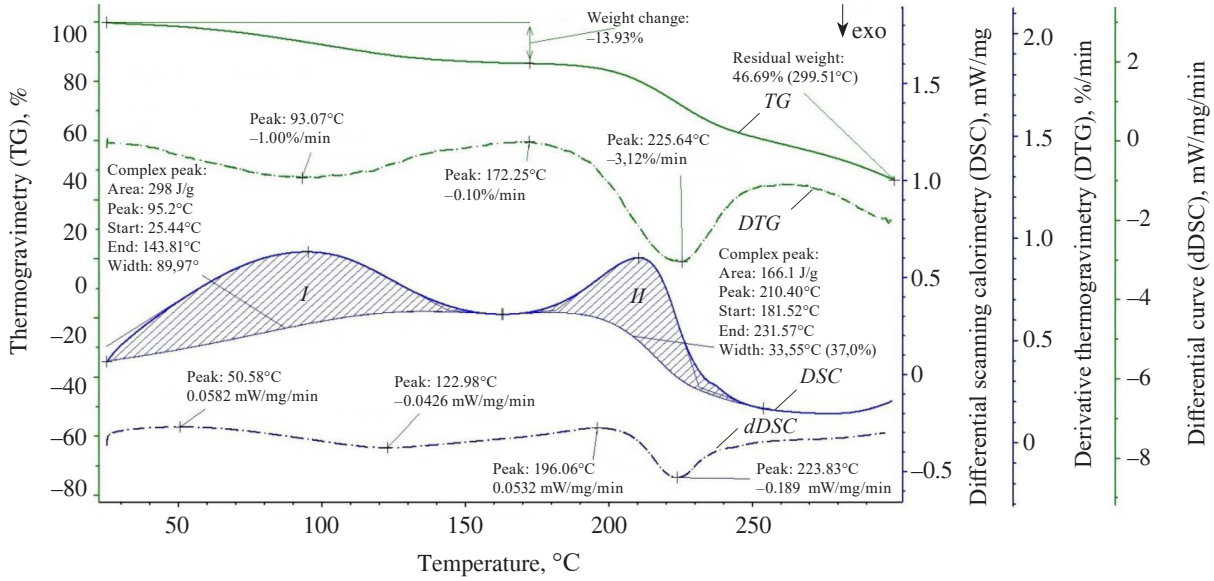

Figure 4 Thermogram of zephyr sample No. 3 with 100% sugar and confectioner's syrup (DE = 40.4 %) replaced with high-conversion glucose syrup (DE = 62.6%) in the whipped mixture and syrup

denaturation and subsequent decomposition of protein substances, and the formation of various chemical compounds with a release of volatile substances in the form of gases (0.1119 mg).

2. In the temperature range from 187.35 to 228.17°C, with a smaller amount of internal energy (209.8 J/g) absorbed at a high rate (4.24%/min) at the peak of the derivative thermogravimetry curve (t = 218.86°C) and a weight loss of 1.7321 mg (21.98%) (thermogravimetry curve) due to the thermal decomposition and caramelization of mono- and disaccharides (sucrose, glucose, fructose, and maltose), the formation of anhydrides, reversion products, hydroxymethylfurfural, organic acids, and colored compounds, as well as the decomposition of dextrins and protein substances into by-products with ta release of volatile substances in the form of gases (1.7321 mg).

The thermolysis of sample No. 2 had two endothermic effects, as shown on the differential scanning calorimetry curve (Fig. 3), namely:

1. In the temperature range from 25.78 to 140.61°C, with a large amount of internal energy (299.3 J/g) absorbed at a medium rate (0.94%/min) at the peak of the derivative thermogravimetry curve (t = 87.27°C) and a loss weight of 0.5972 mg (11.70%) (thermogravimetry curve) due to dehydration processes with a removal of capillary, poly- and monomolecular bound moisture (0.5972 mg) during the denaturation of protein

Table 2 The kinetic characteristics of endothermic effects and weight changes during the thermolysis of zephyr samples with different carbohydrate profiles

Zephyr sample	Endothermic effect	The beginning and end of the endothermic effect, ΔT, °C (DSC curve)	Enthalpy ΔH, J/g (DSC curve)	The peak of the endothermic effect, °C (DSC curve)	Weight loss during thermolysis (TG curve)		
					Weight loss range, %	Weight difference in the range, %	Total weight loss after thermolysis, %
Control	I	25.45–138.37	328.9	86.18	0–13.44	13.44	38.17
	II	155.49–167.95	3.833	162.44	13.44–14.65	1.21	
	III	188.46–231.89	92.6	211.3	14.65–38.17	23.52	
No. 1	I	33.72–140.85	336.6	83.51	0–12.92	12.92	34.90
	II	187.35–228.17	209.8	208.57	12.92–34.90	21.98	
No. 2	I	25.78–140.61	299.3	84.72	0–11.70	11.70	37.01
	II	184.6–236.18	181.0	209.9	11.70–37.01	25.31	
No. 3	I	25.44–143.81	298.0	95.2	0–12.90	12.90	31.46
	II	181.52–231.57	166.1	210.4	12.90–31.46	18.56	

Control contained sugar and acid confectioner's syrup at a ratio of 1:0.2 (traditional formulation)

Samples No. 1 and 2 included 50 and 100% of high-conversion glucose syrup instead of sugar

Sample No. 3 had sugar and acid confectioner's syrup completely replaced with high-conversion glucose syrup

substances and the beginning of melting of monosaccharides (fructose and maltose).

2. In the temperature range from 184.6 to 236.18°C, with a smaller amount of internal energy (181.0 J/g) absorbed at a high rate (3.70%/min) at the peak of the derivative thermogravimetry curve (t = 219.23°C) and a weight loss of 1.8892 mg (25.31%) (thermogravimetry curve) due to dehydration with a removal of monomolecular bound moisture (0.0205 mg) during the thermal decomposition and caramelization of mono- and disaccharides (sucrose, glucose, fructose, and maltose), the formation of anhydrides, reversion products, hydroxymethylfurfural, organic acids, and colored compounds, as well as the decomposition of dextrins and protein substances into by-products with a release of volatile substances in the form of gases (1.292 mg).

The differential scanning calorimetry curve showed two endothermic effects for sample No. 3 (Fig. 4), just as for samples No. 2 and 3, namely:

1. In the temperature range from 25.44 to 143.81°C, with a large amount of internal energy (298.0 J/g) absorbed at a medium rate (1.0%/min) at the peak of the derivative thermogravimetry curve (t = 93.07°C) and a weight loss of 1.0439 mg (12.9%) (thermogravimetry curve) due to dehydration processes with a removal of capillary, poly- and monomolecular bound moisture (1.0439 mg) during the denaturation of protein substances and the beginning of melting of monosaccharides (fructose and maltose).

2. In the temperature range from 181.52 to 231.57°C, with a smaller amount of internal energy (166.1 J/g) absorbed at a high rate (3.12%/min) at the peak of the derivative thermogravimetry curve (t = 225.64°C) and a weight loss of 1.5018 mg (18.56%) (thermogravimetry curve) due to dehydration processes with a removal of monomolecular bound moisture (0.1132 mg) during the thermal decomposition and caramelization of mono- and disaccharides (sucrose, glucose, fructose, and maltose), the formation of anhydrides, reversion products, hydroxymethylfurfural, organic acids, and colored compounds, as well as the decomposition of dextrins and protein substances into by-products with a release of volatile substances in the form of gases (1.3886 mg).

According to our data, the temperature ranges at which the first endothermic effect occurred were wider for the experimental samples than the control, indicative of a higher degree of moisture binding in them. At the beginning of thermolysis, after the first endothermic effect, when the largest amount of free moisture was removed, the enthalpy values were higher for the control and sample No. 1 (328.9 and 336.6 kJ/g, respectively) than for samples No. 2 and 3 (299.3 and 298.0 kJ/g, respectively). This explains the greater decrease in the weight of the control and sample No. 1 (13.44 and 12.92%, respectively) compared to samples No. 2 and 3 (11.7 and 12.9%, respectively). At this stage of dehydration, the control and sample No. 1 not only lost capillary, poly- and monomolecular bound moisture, but also began to undergo the processes of melting and thermal decomposition of monosaccharides and protein substances into by-products. With further heating, the weight of the control and sample No. 1 decreased by 24.73 and 21.98%, respectively, while the weight of samples No. 2 and 3 reduced by 25.31 and 18.56%, respectively. The total weight loss after thermolysis amounted to 38.17, 34.90, 37.01, and 31.46% for the control and experimental samples No. 1, 2, and 3, respectively. The total amount of by-products was 1.7954, 1.844, 1.292, and 1.3886 mg for the control and samples No. 1, 2, and 3, respectively.

To quantify the changes in moisture and the forms of its binding with the sample components, the dependence between weight change on the thermogravimetry curves and dehydration in the specified temperature ranges is converted into the dependence between the degree of weight change, or the degree of substance transformation (α, mg/mg), and the heating temperature (T, K) [25]. For this, weight changes (Δm_i, %) were registered on the thermogravimetry curves at certain temperatures after

every rise of 5°C, which corresponded to the amount of water released at temperature T_i. The indicator α was calculated as a ratio of the current weight change (Δm_i) at a certain time (τ_i) to the total weight change at the end of dehydration (Δm_{max}, %) according to the formula [26]:

$$\alpha = \frac{\Delta m_i}{\Delta m_{max}} \quad (1)$$

Based on the thermogravimetry curves (Figs. 1–4), we presented the initial weights of the zephyr samples (m_i), their changes during thermolysis (Δm_i), and the α indicator in the temperature range from 24 to 183°C (Table 3). After three months of storage, the moisture contents, as determined by the arbitration method, were 10.1, 11.5, 12.1, and 14.3% in the control and samples No. 1, 2, and 3, respectively.

Figure 5 shows graphical dependences between moisture changes in the zephyr samples and thermolysis temperatures in the range from 24 to 183°C.

The dependence curves $\alpha = f(T)$ (Fig. 6) have an S-shaped form [27] which shows a complex nature of interaction between water and dry substances in the zephyr samples and the process of dehydration with different rates of release of moisture and binding energy at different stages of thermal analysis [28]. These curves

Table 3 Changes in the weight of zephyr samples during thermolysis according to thermogravimetry curves

Dehydration temperature, *T*, K (°C)	Zephyr samples											
	Control			No. 1			No. 2			No. 3		
	m_i, %	Δm_i, %	α, mg/mg	m_i, %	Δm_i, %	α, mg/mg	m_i, %	Δm_i, %	α, mg/mg	m_i, %	Δm_i, %	α, mg/mg
297 (24)	100	0	0	100	0	0	100	0	0	100	0	0
305 (32)	99.244	0.76	0.075	99.441	0.56	0.049	99.529	0.47	0.039	99.469	0.53	0.037
310 (37)	98.960	1.04	0.103	99.162	0.84	0.073	99.293	0.71	0.059	99.254	0.75	0.052
315 (42)	98.673	1.33	0.132	98.882	1.12	0.097	99.057	0.94	0.078	99.024	0.98	0.069
320 (47)	98.306	1.69	0.167	98.542	1.46	0.127	98.759	1.24	0.102	98.741	1.26	0.088
325 (52)	97.881	2.12	0.210	98.202	1.80	0.157	98.461	1.54	0.127	98.394	1.61	0.113
330 (57)	97.391	2.61	0.258	97.710	2.30	0.200	98.040	1.96	0.162	97.978	2.02	0.141
335 (62)	96.851	3.15	0.312	97.111	2.89	0.251	97.487	2.51	0.207	97.518	2.48	0.173
340 (67)	96.254	3.75	0.371	96.551	3.45	0.231	96.915	3.09	0.255	97.012	2.99	0.209
345 (72)	95.595	4.41	0.437	95.941	4.06	0.353	96.319	3.68	0.304	96.449	3.55	0.248
350 (77)	94.889	5.11	0.506	95.351	4.65	0.404	95.729	4.27	0.353	95.816	4.18	0.292
355 (82)	94.131	5.87	0.581	94.584	5.42	0.471	95.184	4.82	0.398	95.131	4.87	0.341
360 (87)	93.351	6.65	0.658	93.871	6.13	0.533	94.555	5.45	0.450	94.407	5.59	0.391
365 (92)	92.560	7.44	0.737	93.187	6.81	0.592	93.913	6.09	0.503	93.657	6.34	0.443
370 (97)	91.762	8.24	0.816	92.502	7.49	0.652	93.272	6.73	0.556	92.883	7.12	0.498
375 (102)	90.965	9.04	0.895	91.743	8.26	0.718	92.619	7.38	0.610	92.097	7.90	0.552
380 (107)	90.188	9.81	0.971	90.943	9.06	0.788	91.843	8.16	0.674	91.315	8.69	0.608
382 (109)	**89.900**	**10.10**	1.000	90.664	9.34	0.812	91.585	8.42	0.696	91.001	8.90	0.622
385 (112)	89.432	10.57	–	90.225	9.78	0.850	91.094	8.91	0.736	90.544	9.46	0.662
390 (117)	88.722	11.28	–	89.450	10.55	0.917	90.344	9.66	0.798	89.785	10.22	0.715
395 (122)	88.069	11.93	–	88.868	11.13	0.968	89.655	10.35	0.855	89.078	10.92	0.764
400 (127)	87.501	12.50	–	**88.515**	**11.50**	1.000	89.195	10.81	0.893	88.471	11.53	0.806
405 (132)	87.011	12.99	–	87.970	12.03	–	88.964	11.04	0.912	87.957	12.04	0.842
410 (137)	86.624	13.38	–	87.697	12.30	–	88.706	11.29	0.933	87.550	12.45	0.871
415 (142)	86.313	13.69	–	87.424	12.58	–	88.324	11.68	0.965	87.223	12.78	0.894
420 (147)	86.059	13.94	–	87.077	12.92	–	88.115	11.89	0.983	86.925	13.08	0.915
425 (152)	85.853	14.15	–	86.904	13.10	–	87.949	11.05	0.913	86.668	13.33	0.932
428 (155)	85.745	14.26	–	86.817	13.18	–	**87.866**	**12.10**	1.000	86.538	13.46	0.941
430 (157)	85.682	14.32	–	86.730	13.27	–	87.782	12.22	–	86.464	13.54	0.947
435 (162)	85.519	14.48	–	86.545	13.46	–	87.606	12.39	–	86.308	13.69	0.957
440 (167)	85.381	14.62	–	86.453	13.55	–	87.518	12.48	–	86.185	13.82	0.966
445 (172)	85.244	14.76	–	86.360	13.64	–	87.430	12.57	–	86.000	13.99	0.979
450 (177)	85.073	14.93	–	86.274	13.73	–	87.321	12.68	–	85.886	14.11	0.987
455 (182)	84.832	15.17	–	86.188	13.81	–	87.219	12.78	–	85.755	14.25	0.997
456 (183)	84.769	15.23	–	86.012	13.99	–	87.119	12.88	–	**85.700**	**14.30**	1.000

Note: m_i is the initial weight of a sample; Δm_i is a weight change during thermolysis; α is the degree of weight change

Control contained sugar and acid confectioner's syrup at a ratio of 1:0.2 (traditional formulation)

Samples No. 1 and 2 included 50 and 100% of high-conversion glucose syrup instead of sugar

Sample No. 3 had sugar and acid confectioner's syrup completely replaced with high-conversion glucose syrup

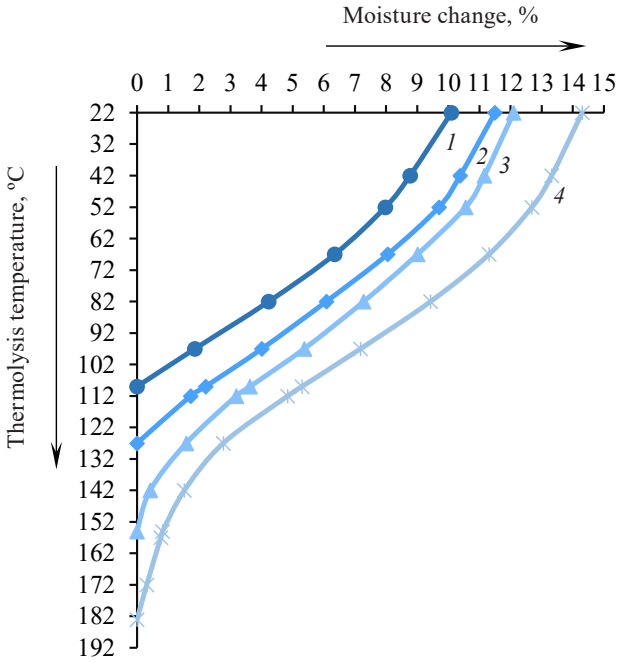

Figure 5 Moisture changes in the zephyr samples with different carbohydrate profiles during thermolysis: 1 – control, 2 – sample No. 1, 3 – sample No. 2, and 4 – sample No. 3

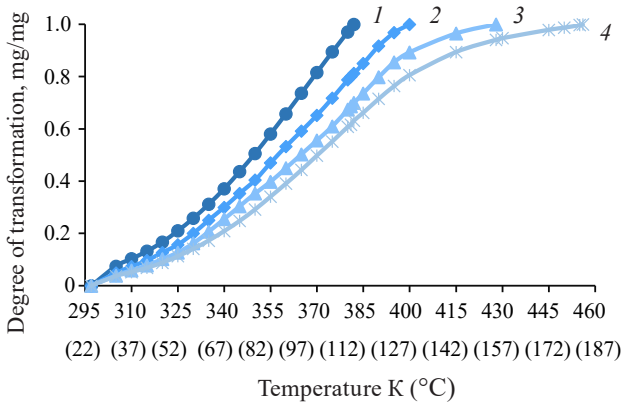

Figure 6 Correlations between the degree of transformation (α) and absolute heating temperatures (T) in the zephyr samples with different carbohydrate profiles: 1 – control, 2 – sample No. 1, 3 – sample No. 2, and 4 – sample No. 3

reveal various kinetically unequal forms of moisture binding in the samples at different rates of dehydration.

To establish the forms of moisture binding and the mechanism of moisture removal in the zephyr samples, we used the curves in the α-T coordinates, as well as the data on temperature ranges and the amounts of bound moisture, and plotted the same curves in the coordinates $(-lg\alpha)$-$(10^3/T)$. The dependences $-lg\alpha = f(10^3/T)$ were made for the temperature range of 297–456 K (Fig. 7) [29–31].

Based on the curves showing graphic dependences between the changes in α converted into $(-lg\alpha)$ and the changes in temperature $(10^3/T)$, we identified four stages of dehydration of the zephyr samples with a removal of moisture in various forms and with different binding energy. The curves that indicate weight changes at certain temperatures had characteristic inflection points B_i, C_i, and D_i, showing changes in degradation. The graphic dependences (Fig. 7) were approximated in the form of four-line splines in order to establish the rates of moisture removal in relation to the energy levels of moisture binding in the samples [32, 33]. Approximating curves were constructed for each temperature range, which were linear in nature ($R^2 \approx 1$).

Four linear sections marked with a dash-dotted line (Fig. 7) confirm a gradual (stage by stage) removal of moisture depending on the forms of its binding with biopolymers [34, 35] in the zephyr samples with different carbohydrate profiles, namely:

– stage 1 (section A_iB_i) characterizes the removal of free, physically and mechanically bound (capillary-bound) moisture from zephyr micro- and macrocapillaries (pores) that has a low energy of binding with the material (the desorption of capillary moisture has a lower activation energy compared to the moisture released in the second stage) [36];

– stage 2 (section B_iC_i) characterizes the removal of physically and chemically bound (capillary- and polymolecule-bound) moisture that is more strongly bound with zephyr nutrients than capillary moisture and is mainly contained in the form of hydrated moisture in the closed cells of nutrient micelles and around carbohydrate molecules [27];

– stage 3 (section C_iD_i) characterizes the removal of physically and chemically bound (poly- and monomolecular bound) moisture during the partial decomposition of nutrients that is strongly bound with nutrients and is involved in the swelling of proteins, pectins, and dietary fibers [37, 38]; and

– stage 4 (section D_iK_i) characterizes the removal of physically and chemically bound (poly- and monomolecular bound) moisture and gaseous substances formed during the decomposition of nutrients.

Table 4 shows the degrees of transformation ($\Delta\alpha$) corresponding to the four-stage dehydration kinetics of the zephyr samples with different carbohydrate profiles.

As can be seen, the first section (A_1B_1–A_4B_4), which refers to the temperature range of 297–312 K (24–39°C), is where the "water-water" bonds are destroyed and the "free", physically and mechanically bound (capillary-bound) moisture is released from the surface of the zephyr samples in insignificant amounts: 10.30, 8.26, 6.78, and 5.94% for the control and samples No. 1, 2, and 3, respectively.

The second section (B_1C_1–B_4C_4) refers to a more intensive removal of physically and chemically bound (capillary- and polymolecular bound) moisture in the temperature range of 310–361 K (37–88°C) in the following amounts: 40.20, 40.43, 35.37, and 36.02% for the control and samples No. 1, 2, and 3, respectively.

The third section (C_1D_1–C_4D_4) is where physically and chemically bound (poly- and monomolecular bound) moisture is removed in the temperature range of 349–403 K (76–130°C) in the following amounts: 31.09,

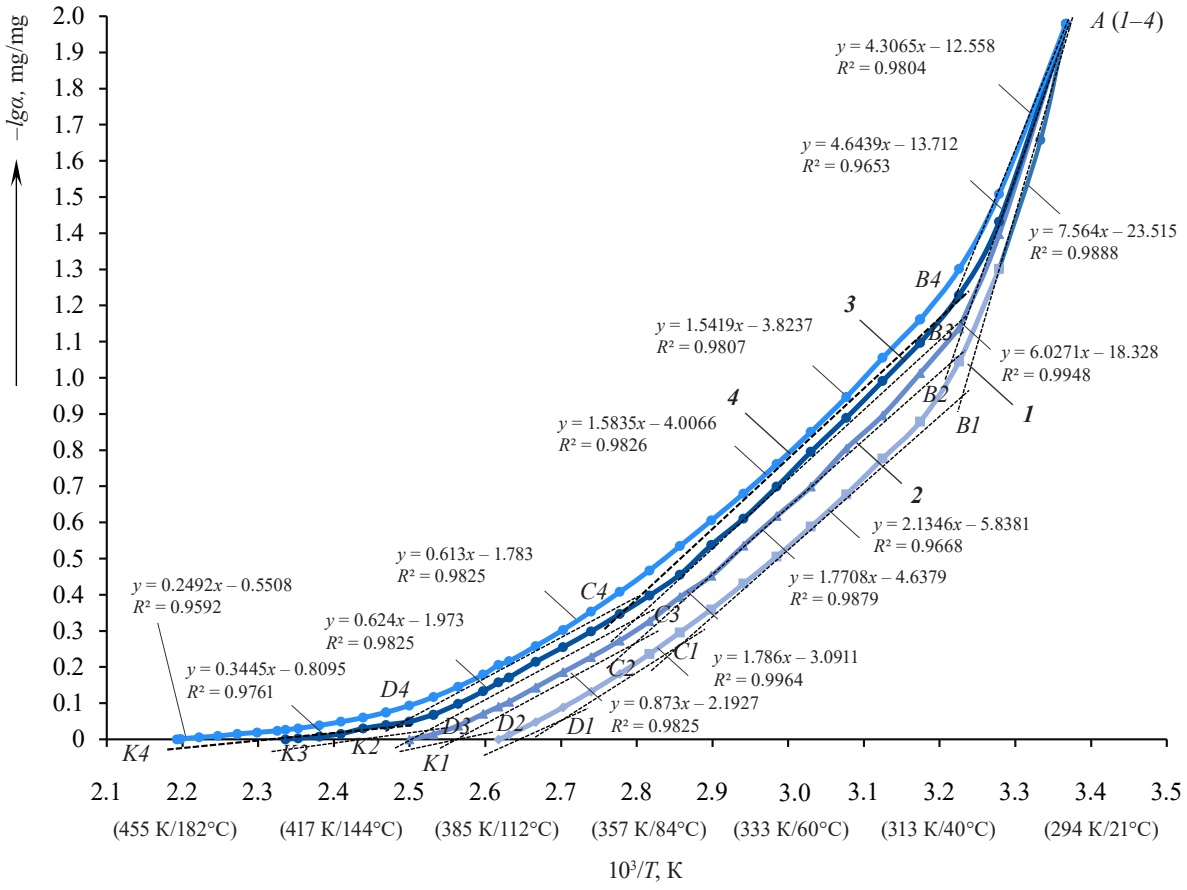

Figure 7 Dependence (*-lgα*) on temperature ($10^3/T$) at an air heating rate of 5°C/min for the zephyr samples with different carbohydrate profiles: 1 – control; 2 – sample No. 1; 3 – sample No. 2; and 4 – sample No. 3

44.35, 43.80, and 39.86% for the control and samples No. 1, 2, and 3, respectively.

The last section (D_1K_1–D_4K_4) refers to the removal of physically and chemically bound (poly- and monomolecular bound) moisture and gaseous substances in the temperature range of 370–456 K (97–183°C) in the following amounts: 18.41, 10.44, 14.05, and 18.18% for the control and samples No. 1, 2, and 3, respectively.

CONCLUSION

In this study, we used thermal analysis to determine the content of moisture and forms of its binding in zephyr during storage. The samples in which sugar and confectioner's syrup were partially or completely repced with high-conversion glucose syrup gradually lost some of their sweetness and had a more pronounced taste and smell of fruit puree, as well as flavoring agents, a higher degree of moisture binding due to an increased content of reducing sugars (glucose and maltose), and slower, or completely eliminated, crystallization of sucrose during storage.

In addition, the samples had a lower content of fructose (or no fructose at all), a highly hygroscopic sugar formed during the boiling of glucose syrup that contributes to a rapid wetting of zephyr. Although glucose and maltose are less hygroscopic, they are highly hydrophilic – they bind moisture firmly and retain it for a long time, keeping zephyr fresh.

The thermal analysis of the zephyr samples with different carbohydrate profiles showed that at the initial stage of dehydration, the largest amount of "free", physically and mechanically bound moisture was removed from the control sample, which had a lower content of reducing sugars and dextrins. The wider temperature ranges in all sections of heating of the experimental samples indicated a higher content of physically and chemically bound moisture in them, compared to the control. Therefore, replacing sugar and confectioner's syrup with high-conversion glucose syrup results in less "free", capillary-bound moisture and more poly- and monomolecular bound moisture in zephyr. This is due to a higher content of reducing sugars which have high hydrophilic properties allowing them to firmly bind and retain moisture for a long time, prolonging the freshness of zephyr and slowing down its drying during storage [39].

RECOMMENDATION

The differential thermal analysis used to assess moisture and forms of its binding in zephyr samples with different carbohydrate profiles allowed us to determine the effect of high-conversion glucose syrup on the

Table 4 Dehydration kinetics of the zephyr samples with different carbohydrate profiles

Zephyr sample	Stage of dehydration	Δ*T*, K (°C)	1000/*T*	Δ*α*, mg/mg	–*lgα*, mg/mg	Amount of moisture removed during thermolysis (TG curve)	
						Δ*m*, %	% of the total amount
Control	I	297–310 (24–37)	3.37–3.23	0–0.11	1.98–0.95	0–1.04	10.30
	II	310–349 (37–76)	3.23–2.86	0.11–0.51	0.95–0.29	1.04–5.10	40.20
	III	349–370 (76–97)	2.86–2.70	0.51–0.82	0.29–0.08	5.10–8.24	31.09
	IV	370–382 (97–109)	2.70–2.62	0.82–1.00	0.08–0	8.24–**10.10**	18.41
No. 1	I	297–312 (24–39)	3.37–3.22	0–0.09	1.98–1.04	0–0.95	8.26
	II	312–357 (39–84)	3.22–2.80	0.09–0.51	1.04–0.29	0.95–5.60	40.43
	III	357–391 (84–118)	2.80–2.55	0.51–0.95	0.29–0.02	5.60–10.30	40.87
	IV	391–400 (118–127)	2.55–2.50	0.95–1.00	0.02–0	10.30–**11.50**	10.44
No. 2	I	297–311 (24–38)	3.37–3.10	0–0.07	1.98–1.14	0–0.82	6.78
	II	311–357 (38–84)	3.10–2.80	0.07–0.44	1.14–0.35	0.82–5.10	35.37
	III	357–395 (84–122)	2.80–2.53	0.44–0.91	0.35–0.04	5.10–10.40	43.80
	IV	395–428 (122–155)	2.53–2.34	0.91–1.00	0.04–0	10.40–**12.10**	14.05
No. 3	I	297–312 (24–39)	3.37–3.22	0–0.06	1.98–1.23	0–0.85	5.94
	II	312–361 (39–88)	3.22–2.77	0.06–0.41	1.23–0.38	0.85–6.00	36.02
	III	361–403 (88–130)	2.77–2.48	0.41–0.87	0.38–0.06	6.00–11.70	39.86
	IV	403–456 (130–183)	2.48–2.19	0.87–1.00	0.06–0	11.70–**14.30**	18.18

Control contained sugar and acid confectioner's syrup at a ratio of 1:0.2 (traditional formulation)

Samples No. 1 and 2 included 50 and 100% of high-conversion glucose syrup instead of sugar

Sample No. 3 had sugar and acid confectioner's syrup completely replaced with high-conversion glucose syrup

content of free and bound moisture in zephyr and to predict a possibility of extending its freshness during storage. In the future, our results may be used to create a database of ingredients that contribute to the product's quality and extended shelf life.

CONTRIBUTION

I.V. Plotnikova developed the research concept, prosed a methodology for the experiment, and edited the manuscript for submission. G.O. Magomedov supervised and monitored the experiment. D.A. Kazartsev processed the experimental data, performed calculations, and edited the manuscript for submission. M.G. Magomedov processed the experimental data and advised on the experiment. K.K. Polansky organized the production tests and advised on the experiment. V.E. Plotnikov reviewed literary sources on the research problem and conducted the experiment. All 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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Effects of drying methods on the biochemical and antioxidant properties of *Volvariella volvacea* from Côte d'Ivoire

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Abstract:
The wild mushroom *Volvariella volvacea* is widely picked and consumed in Côte d'Ivoire. However, it is highly perishable due to its high moisture content. This study aimed to determine the effects of three drying methods on the biochemical and mineral composition, as well as antioxidant properties, of *V. volvacea* powders.
Three *V. volvacea* powders were obtained by sun drying, oven drying, and freeze-drying. Each powder was analyzed for its biochemical and mineral composition according to standard analytical methods. The powder methanolic extracts were analyzed for their antioxidant components by colorimetric methods or titration, while their antioxidant capacities were determined by using DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) scavenging and the ferric reducing antioxidant power.
The freeze-dried powder of *V. volvacea* had a lower moisture content than the oven-dried and sun-dried powders. The highest protein, ash, and fiber contents were also recorded in the freeze-dried powder. In addition, freeze-drying provided the highest contents of iron, magnesium, sodium, and potassium. Regarding the antioxidant components, the freeze-dried powder showed the highest levels of total phenolic compounds, flavonoids, and vitamin C. Similarly, freeze-drying provided the best antioxidant capacities in terms of DPPH scavenging and the ferric reducing antioxidant power.
Our study showed that freeze-drying ensured a better retention of essential nutrients and antioxidant components in the mushroom *V. volvacea*, while sun-drying led to greater losses of these compounds.

Keywords: Mushroom, *Volvariella volvacea*, drying methods, mushroom powders, biochemical and nutritional properties, antioxidant capacity

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INTRODUCTION

In Africa, as in several developing countries around the world, wild edible mushrooms have always been an important component of foodstuffs intended for human consumption [1–4]. Several studies have reported that edible mushrooms are valuable healthy and nutritious foods, low in calories and rich in vegetable proteins, vitamins, and minerals [5–9]. In addition to their food value, they play a role in traditional medicine. Indeed, certain species of mushrooms have, among other things, antioxidant, antimicrobial, and anticancerous properties [10–13]. During rainy seasons in Côte d'Ivoire, wild edible mushrooms are collected and sold by women and even men in rural and peri-urban areas [14].

According to a recent study, the species *Psathyrella tuberculata* ranks first in terms of importance, followed by *Volvariella volvacea* and *Termitomyces letestui* [15]. *V. volvacea* has been the subject of ecological, as well as biochemical and nutritional studies in Côte d'Ivoire [4, 8, 16]. This saprophytic species, which generally grows on the trunks of dead oil palms, is rich in proteins, carbohydrates, fibers, minerals, and natural antioxidants. However, it is highly perishable in the fresh state, like other species of mushrooms, due to a high water content (over 80%) which promotes bacterial proliferation. Moreover, the oxidation of phenolic compounds under the action of enzymes, such as polyphenol oxidases, causes browning that affects the quality [17].

In tropical Africa, mushrooms are traditionally sun-dried, which is a cheap and therefore accessible method [18]. Although this method has an undeniable advantage resulting from the conversion of ergosterol into vitamin D, its impact on the dried mushroom does not guarantee its quality in terms of health safety and nutrient retention [19]. Therefore, other methods such as oven-drying and freeze-drying seem appropriate to improve the quality of the dried mushrooms. Previous works have indicated satisfactory results for the drying of several species of mushrooms [20–22].

Thus, we aimed to analyze and compare three methods of drying the wild edible mushroom *V. volvacea* harvested in Côte d'Ivoire in terms of the chemical composition and antioxidant properties of the resulting products.

STUDY OBJECTS AND METHODS

***Volvariella volvacea* collection.** Fresh fruiting bodies of *V. volvacea* mushrooms were harvested from dead trunks of oil palms in the plantations of Dimbokro, the N'zi region (Côte d'Ivoire). Once harvested, the mushrooms were packaged in ventilated baskets and carefully transported on the same day to the laboratory for subsequent analyses.

Sampling. In the laboratory, the mushrooms were sorted and cleared of debris. Then, they were washed three times with tap water and rinsed with distilled water. Finally, the mushrooms were divided into three 200-g samples. Each of the samples was subsequently dried and ground into a powder.

Drying and obtaining *V. volvacea* powders. The first sample was placed on a table covered with aluminum foil and subjected to direct sunlight for 5 successive days. As soon as the sun set, the table was removed to the laboratory to avoid rehydration due to the humidity of the air. The second sample was dried in a ventilated oven (BOBASE, China) at 45°C for 48 h. The third sample was first frozen at –80°C, quickly transferred to a pre-cooled freeze-dryer (BIOBASE, China), and dried for 48 h. The freeze-dryer had a cold trap temperature of –80°C and a vacuum of 1 bar. After the application of these drying methods, the mushrooms of each sample were crushed with a flat-hammer grinding mill and sifted through a 60-mesh screen. The three powdered samples were packaged in labeled bottles, which were previously dried and hermetically sealed. These bottles were stored in a desiccator at 25°C until further use.

Biochemical and mineral composition. The moisture, ash, fat, and protein contents of the mushroom powders were determined according to the AOAC [23]. The moisture content was determined by drying a sample in an oven at 105°C to a constant weight. Fat contents were determined by continuous extraction in a Soxhlet apparatus for 4 h using hexane as a solvent. After evaporation of the solvent, the fat content was obtained by the gravimetric method. Ash was measured from the residual mass obtained after incinerating the samples at 550°C for 2 h in a muffle furnace. The protein content was calculated by nitrogen ×6.25. The contents of fibers and total carbohydrates were respectively determined gravimetrically and by difference. The energy value of each sample was estimated by multiplying protein, fat, and available carbohydrates (total carbohydrates minus fibers) by 4, 9, and 4, respectively. Total and reducing sugars were quantified using the methods of Chow & Landhäusser and Garriga *et al.*, respectively [24, 25]. To determine the contents of different mineral elements, the ash residue of each sample (1 g) was digested with a mixture of concentrated nitric acid (14.44 mol/L), sulfuric acid (18.01 mol/L), and perchloric acid (11.80 mol/L). After cooling, the samples were filtered through Whatman filter paper No. 4. Then, each sample solution was made up to a final volume of 25 mL with distilled water. An aliquot of each solution was used to determine the contents of Zn, Cu, Fe, Ca, Mg, Mn, and Na by measuring atomic absorption [26]. *P* was determined colorimetrically using the method of Taussky & Shorr [27].

Antinutritional factors. Oxalate contents in the *V. volvacea* powder were determined according to the method described by Day & Underwood using a potassium permanganate solution (0.05 M $KMnO_4$) [28]. Phytate contents were determined according to the method of Latta & Eskin using a Wade reagent [29].

The phenolic compounds of each *V. volvacea* powder were extracted with 80% (v/v) methanol. For this, 10 g of *V. volvacea* powder was extracted by stirring with 50 mL of 80 % (v/v) methanol at 25°C for 24 h and filtered through Whatman paper No. 4. The residue was then extracted with two additional 50 mL portions of methanol. The combined methanolic extracts were evaporated at 35°C in a Heildolph Laborota 4003 Control rotary evaporator (Schwabach, Germany) until the volume of 25 mL. The extracts obtained were used to estimate the contents of phenolic compounds.

The content of total phenolic compounds in each *V. volvacea* powder was determined using the Folin-Ciocalteu reagent as described by Singleton *et al.* [30]. The results were expressed as mg gallic acid equivalent (GAE)/100 g DW. The content of flavonoids was estimated by the method of Meda *et al.* using aluminum chloride [31]. The results were expressed as mg of quercetin equivalent (QE)/100 g DW. The tannin content was determined according to the method described by Bainbridge *et al.* using a vanillin reagent [32]. The results were expressed as mg of tannic acid equivalent (TAE)/100 g DW.

The vitamin C content in each powder was estimated by titration with 2,6-dichloroindophenol as reported by Pongracz *et al.* [33].

Antioxidant capacities. The capacity of the *V. volvacea* powders to scavenge DPPH radicals was monitored according to the method described by Hatano *et al.* [34]. For this, *V. volvacea* powder solutions (2.5 m) at various concentrations ranging from 0 to 100 mg/mL and a Trolox solution (standard reference of antioxidant) were added to 1 mL of a methanolic solution of DPPH (3 mM).

The mixture was shaken vigorously and left to stand for 30 min in the dark. The DPPH inhibition was determined by measuring absorbance at 517 nm. A check was made by also measuring the absorbance of the DPPH solution. The antioxidant activity expressed as the DPPH radical scavenging activity of each powder and Trolox was marked by the discoloration of the DPPH solution. It was calculated as a percentage of DPPH inhibition, % using the following equation:

$$\text{DPPH inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (1)$$

where A_{control} is the absorbance of the DPPH solution, A_{sample} is the absorbance of the solution with the sample extract.

The concentration of each powder solution causing 50% inhibition (EC_{50}) was estimated from the graph of the DPPH inhibition percentage against the powder solution concentration.

The ferric reducing antioxidant power of the methanolic solution of each of the *V. volvacea* powders or the Trolox methanolic solution was determined according to the method described by Barros *et al.* with slight modifications [35]. For this, 0.1 mL of the *V. volvacea* powder solution or the Trolox solution prepared at various concentrations (0 to 100 mg/mL) was mixed with 2.0 mL of phosphate buffer (0.2 M, pH 6.6) and then with 2 mL of 1% potassium hexacyanoferrate [$K_3Fe(CN)_6$] (w/v). The mixture was incubated at 50°C in a water bath for 20 min and then cooled. A volume of 2 mL of 10% (w/v) trichloroacetic acid was then added and the mixture was centrifuged at 3000 rpm for 10 min. Finally, 2 mL of the supernatant was mixed with 2 mL of distilled water and 0.4 mL of ferric chloride ($FeCl_3$). A blank without a powder was prepared under the same conditions. Absorbance was measured at 700 nm against the blank. Increased absorbance indicated higher reducing power. The concentration of each powder solution causing half maximal effective concentration (EC_{50}) was estimated from the graph of absorbance at 700 nm against the powder solution concentration.

Statistical analysis. All chemical analyses and assays were carried out in triplicate. The results were expressed as mean values ± standard deviation (SD). Analysis of variance (ANOVA) followed by Duncan's test was performed to test for differences between the means by employing Kyplot (version 2.0 beta 15, ©1997–2001, Koichi Yoshioka) statistical software. Significance of differences was defined at the 5% level ($p < 0.05$).

RESULTS AND DISCUSSION

The biochemical composition and energy values of the *Volvariella volvacea* powders obtained by sun drying, oven drying, and freeze-drying are presented in Table 1.

In terms of moisture, freeze-drying allowed better dehydration of the mushroom *V. volvacea* with a powder water content of 4.21%, compared to oven drying and sun drying with the water contents of 5.32 and 8.11%, respectively. It is well-known that dehydration by conventional drying methods is an important process in the food industry because it considerably reduces the water activity which affects the microbiological stability and the physicochemical deterioration reactions [21, 36].

Various studies dedicated to the drying of edible mushrooms have indicated that freeze-drying is the best form of dehydration since it transforms water into ice and then directly into water vapor, skipping the liquid phase [21, 22, 37]. On the other hand, oven drying, which uses a uniform temperature, resulted in a higher removal of moisture compared to sun drying [22, 38]. With regard to the retention of different nutrients, freeze-drying also gave better results than oven drying. This could be due to the fluctuation of temperature for efficient moisture removal with a good retention of proteins, total and reducing sugars, ash, and fibers.

The sun-dried *V. volvacea* powder presented the worst results for nutrient retention. Several reports have indicated a similar trend regarding the impact of the drying method on the protein content of mushrooms [21, 22, 39, 40]. The capacity of freeze-drying to ensure a better retention of proteins could be explained by the fact that drying with hot air (sun drying and oven drying) can cause denaturation of some proteins due to relatively high temperatures, resulting in a substantial protein

Table 1 Biochemical composition, % DW and energy values of *Volvariella volvacea* powders by different drying methods

Parameters	Volvariella volvacea powder obtained by		
	Sun-drying	Oven drying	Freeze-drying
Moisture, %	8.11 ± 0.01[a]	5.32 ± 0.04[b]	4.21 ± 0.01[c]
Proteins, %	24.43 ± 0.03[a]	27.25 ± 0.03[b]	30.10 ± 0.00[c]
Fat, %	1.40 ± 0.04[a]	1.47 ± 0.07[b]	1.50 ± 0.08[c]
Total carbohydrates, %	53.84 ± 0.02[a]	51.93 ± 0.01[b]	48.65 ± 0.02[c]
Total sugars, %	17.33 ± 0.14[a]	18.14 ± 0.11[b]	19.66 ± 0.03[c]
Reducing sugars, %	0.42 ± 0.09[a]	0.49 ± 0.02[b]	0.51 ± 0.07[c]
Ash, %	12.22 ± 0.11[a]	14.03 ± 0.05[b]	15.54 ± 0.04[c]
Fibers, %	13.67 ± 0.13[a]	14.87 ± 0.09[b]	15.50 ± 0.01[c]
Energy value, Kcal/100 g DW	271.20 ± 013[a]	270.47 ± 0.12[b]	266.50 ± 0.01[c]

n = 3; the means ± standard deviations with different letters on the same line are significantly different at $p < 0.05$ according to Duncan's test

loss [22, 41]. In addition, the low protein content of the sun-dried and oven-dried *V. volvacea* powders, compared to that of the freeze-dried powder, could be partly due to the leaching of soluble proteins by the washing water and losses during browning reactions (Maillard reactions) [41, 42].

The low total and reducing sugar contents of the sun-dried and oven-dried powders could be attributed to the fact that some sugars are consumed by heat-induced Maillard reactions [43]. The low content of fibers in the sun-dried *V. volvacea* powder could be explained by the fact that this method required a long duration, i.e., a long exposure to the sun rays, which resulted in a rupture of the cell walls, thus causing a decrease in fibers as a beta-glucan content [44, 45]. However, it is generally recognized that the method of drying does not affect the fiber content in mushrooms [20, 22].

The fat contents were quite low for the *V. volvacea* powders from the three drying methods, as previously reported [4, 8]. The few slight differences between the powders obtained by hot air drying and freeze-drying could be due to oxidation. The mushroom fat is mainly made up of unsaturated fatty acids which are very prone to oxidation when exposed to heat and ambient air [22, 38]. The good ash retention observed during the freeze-drying of the *V. volvacea* mushroom is in agreement with the results reported by Bashir *et al.* for the mushroom *Pleurotus florida* [21]. The considerable decrease in ash during the sun drying could be due to the diffusion of some minerals into the water which migrates out of the mushroom during the process [21, 45]. With respect to energy, the *V. volvacea* powder from sun drying recorded the highest value (271.20 ± 0.13 Kcal/100 g DW), followed by the oven-dried powder (270.47 ± 0.12 DW) and the freeze-dried powder (266.50 ± 0.01 Kcal/100 g DW). This resulted from the fact that the powder from freeze-drying has the lowest levels of available carbohydrates estimated by difference, as reported by Dunkwal *et al.* [46].

Oxalate and phytate are antinutritional factors, also called antinutrients, which interfere with the absorption of certain minerals (iron, calcium, zinc, etc.). Table 2 shows the contents of these two antinutritional factors in the *V. volvacea* powders.

The highest levels of oxalate and phytate were observed in the freeze-dried powder of *V. volvacea* with the respective values of 11.91 ± 0.12 and 8.50 ± 0.03 mg/100 g DW. The lowest contents of oxalate and phytate were recorded in the powder obtained by sun drying. It is well known that oven drying significantly reduces the antinutrient levels of vegetables, which could justify the fact that the oven-dried powder showed lower oxalate and phytate contents than the freeze-dried powder [47]. The report of Bello *et al.* indicated that drying oyster mushroom *Pleurotus sajur-caju* in the oven at 60°C considerably reduced the levels of antinutritional factors, including phytate and oxalate [48]. The low contents of these compounds in the sun-dried *V. volvacea* powder could be attributed to the fact that these two antinutrients are more or less soluble in water and an important amount of them could have migrated with the water that came out of the mushroom during drying. We found that the freeze-drying showed better retention of phytate and oxalate. However, the levels obtained were well below the limits reported by the WHO, which are 22.10 and 105.00 mg/100 g for phytate and oxalate, respectively [49].

The mineral composition of each *V. volvacea* powder was evaluated in terms of microelements and macroelements. Table 3 presents the microelement (zinc, copper, iron, and manganese) contents of the *V. volvacea* powders.

Overall, only the iron content varied significantly from one drying method to another. The powder from freeze-drying had the highest iron content of 4.850 ± 0.014 mg/100 g against 4.15 ± 0.10 and 3.41 ± 0.20 mg/100 g for the oven-dried and sun-dried powders, respectively. This result was comparable to that reported by Bashir *et al.* for the oyster mushroom *P. florida* [21]. In their study, the freeze-dried powder of this mushroom showed the best iron content of 5.10 mg/100 g compared to other methods. On the other hand, Maray *et al.* reported that the sun drying of *Pleurotus ostreatus* resulted in the highest iron content of 11.6 mg/100 g [45]. The other microelements did not show any significant differences depending on the drying method. The values were around 10.30, 1.32, and 2.45 mg/100 g for zinc, copper, and manganese, respectively.

The contents of the macroelements (magnesium, sodium, potassium, phosphorus, and calcium) are presented in Table 4. These contents showed significant differences. The highest contents of most of the macroelements

Table 2 Contents of antinutritional factors (oxalate and phytate) in *Volvariella volvacea* powders dried by different methods

Parameter	*Volvariella volvacea* powder obtained by		
	Sun-drying	Oven drying	Freeze-drying
Oxalate, mg/100 g DW	6.51 ± 0.02[a]	10.10 ± 0.21[b]	11.91 ± 0.12[c]
Phytate, mg/100 g DW	4.37 ± 0.05[a]	6.09 ± 0.09[b]	8.50 ± 0.03[c]

n = 3; the means ± standard deviations with different letters on the same line are significantly different at $p < 0.05$ according to Duncan's test

Table 3 Contents of microelements in *Volvariella volvacea* powders obtained by different drying

Microelements, mg/100g	*Volvariella volvacea* powder obtained by		
	Sun-drying	Oven drying	Freeze-drying
Zn	10.69 ± 0.04[a]	10.21 ± 0.05[a]	10.30 ± 0.05[a]
Cu	1.30 ± 0.15[a]	1.32 ± 0.02[a]	1.38 ± 0.10[a]
Fe	3.41 ± 0.20[a]	4.15 ± 0.10[b]	4.85 ± 0.01[c]
Mn	2.33 ± 0.11[a]	2.45 ± 0.03[a]	2.51 ± 0.09[a]

n = 3; the means ± standard deviations with different letters on the same line are significantly different at $p < 0.05$ according to Duncan's test

were obtained in the freeze-dried *V. volvacea* powder. Thus, freeze-drying allowed for a good retention of these minerals. This same trend was observed by Bashir *et al.* for the contents of magnesium, potassium, phosphorus, and calcium during the drying of the oyster mushroom *P. florida* [21].

In addition, potassium showed the highest content in each of the *V. volvacea* powders, while sodium showed the lowest content. Due to a high K/Na ratio, the *V. volvacea* powders may have an advantage for patients with hypertension and other cardiovascular diseases. Such a result was reported very recently for powders from the oyster mushrooms *P. sajor-caju* and *P. djamor* [50].

Total phenolic compounds, flavonoids, tannins and vitamin C. Table 5 shows the contents of total phenolic compounds, flavonoids, tannins, and vitamin C in the *V. volvacea* powders obtained by different drying methods.

The *V. volvacea* powder obtained by freeze-drying generated the highest contents of total phenolic compounds, flavonoids, tannins, and vitamin C compared to those obtained by oven drying and freeze-drying. These compounds are widely described as major antioxidant components of edible mushrooms [4, 51, 52]. Thus, we found that freeze-drying ensured a better retention of the antioxidant components in the mushroom *V. volvacea*. The findings reported by Tarafdar *et al.* indicated a good retention of vitamin C during the freeze-drying of the button mushroom *Agaricus bisporus* [53]. The decreased retention of these antioxidants in the sun-dried and oven-dried *V. volvacea* powders could be attributed to the fact that high temperatures are likely to degrade heat-sensitive components such as vitamin C and antioxidants [54, 55].

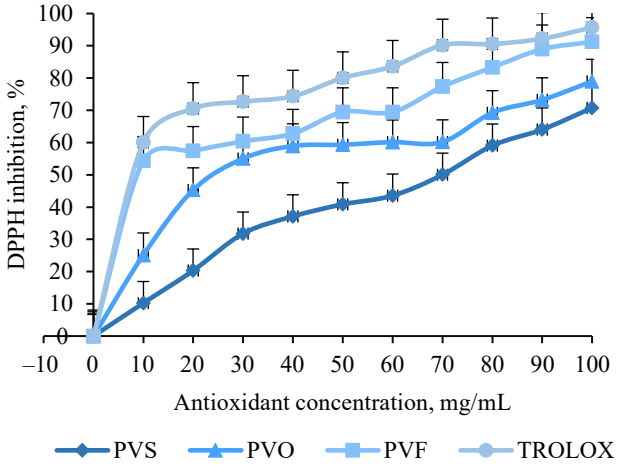
Antioxidant capacities. Two methods of *in vitro* assay of antioxidant activities were used to evaluate the *V. volvacea* powders, namely the DPPH scavenging and the ferric reducing antioxidant power. Figure 1 represents the percentage of DPPH inhibition as a function of the *V. volvacea* powder solution. We observed higher inhibition percentages with increased concentrations of *V. volvacea* powders in the following order: freeze-drying > oven drying > sun-drying (Fig. 1). Trolox used as a reference antioxidant showed the highest percentage of DPPH inhibition. At 100 mg/mL, the Trolox, freeze-dried, oven-dried, and sundried powders had DPPH inhibition percentages of 95.6, 91.2, 78.9, and 70.7%, respectively.

The graphically determined effective concentrations (EC_{50}) of the *V. volvacea* powders are presented in Table 6. As can be seen, the powders showed effective concentrations in the following increasing order: Trolox < freeze-drying < oven drying < sun-drying. The values of effective concentrations were 10.2 ± 0.1, 24.3 ± 0.2, and 69.8 ± 0.5 mg/mL for the *V. volvacea* powders obtained by freeze-drying, oven drying, and sun drying, respectively.

Table 4 Contents of macroelements in *Volvariella volvacea* powders from different drying methods

Macroelements, mg/100g	*Volvariella volvacea* powder obtained by		
	Sun-drying	Oven drying	Freeze-drying
Mg	79.43 ± 0.04[a]	82.64 ± 0.20[a]	86.50 ± 0.04[b]
Na	3.78 ± 0.02[a]	4.11 ± 0.12[b]	4.50 ± 0.01[c]
K	500.04 ± 0.13[a]	678.00 ± 0.16[b]	831.04 ± 0.03[c]
P	183.10 ± 0.17[b]	180.02 ± 0.10[b]	138.13 ± 0.05[a]
Ca	124.01 ± 0.14[b]	132.76 ± 0.03[c]	110.41 ± 0.01[a]

n = 3; the means ± standard deviations with different letters on the same line are significantly different at $p < 0.05$ according to Duncan's test



PVS, PVO, and PVF are *V. volvacea* powders obtained by sun-drying, oven drying, and freeze-drying, respectively

Figure 1 Antioxidant activities via DPPH radical scavenging as a function of *Volvariella volvacea* powders. Each value is expressed as mean ± standard deviation (n = 3)

Table 5 Contents of total phenolic compounds, flavonoids, tannins, and vitamin C in *Volvariella volvacea* powders obtained by different drying methods

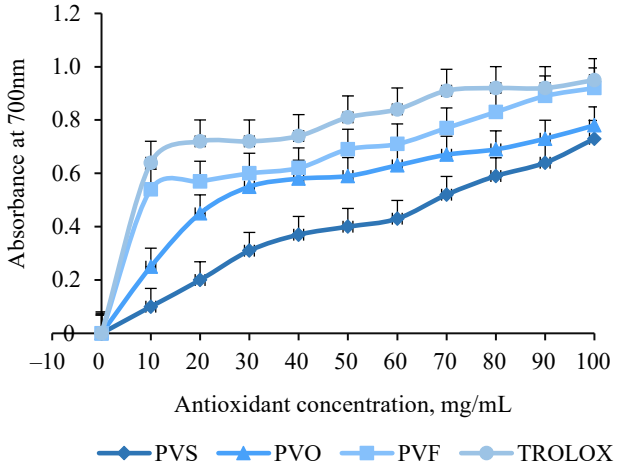
Parameter	*Volvariella volvacea* powder obtained by		
	Sun-drying	Oven drying	Freeze-drying
Total phenolic compounds, mg EG/A100 g	251.30 ± 0.02[a]	269.34 ± 0.03[b]	340.26 ± 0.17[c]
Flavonoids, mg EQ/100 g	50.22 ± 0.05[a]	79.30 ± 0.03[b]	92.66 ± 9.15[c]
Tannins, mg ETA/100 g	39.38 ± 0.07[a]	52.57 ± 0.17[b]	74.59 ± 0.11[c]
Vitamin C, mg/100 g	1.60 ± 0.01[a]	2.44 ± 0.03[b]	5.21 ± 0.02[c]

n = 3; the means ± standard deviations with different letters on the same line are significantly different at $p < 0.05$ according to Duncan's test

Table 6 Effective concentrations (EC_{50}) of *Volvariella volvacea* powders from freeze-drying, oven drying, and sun-drying for DPPH scavenging

Antioxidant	Effective concentrations EC_{50}, mg/mL
Trolox	9.4 ± 0.2^{d}
Freeze-dried powder	10.2 ± 0.1^{c}
Oven dried powder	22.3 ± 0.2^{b}
Sun-dried powder	698.0 ± 0.5^{a}

Each value is expressed as mean ± standard deviation (n = 3). The values with different letters within the same column are significantly different ($p < 0.05$)



PVS, PVO, and PVF are *V. volvacea* powders obtained by sun-drying, oven drying, and freeze-drying, respectively

Figure 2 Antioxidant activities of *Volvariella volvacea* powders via ferric ion reducing antioxidant power. Each value is expressed as mean ± standard deviation (n = 3)

According to the results, the freeze-dried powder of *V. volvacea* showed the best antioxidant capacity in terms of DPPH scavenging due to its lower effective concentration EC_{50}. The decrease in DPPH radical scavenging activity in the sun-dried and oven-dried powders could be the consequence of thermal degradation of vitamin C and some phenolic compounds, as reported by Bashir *et al.* [55]. Similar results were reported for the mushroom *Lentinus edodes*, with the lowest effective concentration obtained by its powder from freeze-drying [56].

With regard to the ferric reducing antioxidant power, Fig. 2 represents the absorbance at 700 nm as a function of the concentration of the *V. volvacea* powder solution. We found that the reducing power expressed by increased absorbance at 700 nm was higher with increased concentrations of the *V. volvacea* powders. Trolox showed the highest absorbances at all concentrations, followed respectively by the powders from freeze-drying, oven drying, and sun drying. At 100 mg/mL, the absorbances were 0.95, 0.92, 0.78, and 0.72 for Trolox, freeze-dried, oven-dried, and sun-dried powders, respectively.

Table 7 Effective concentrations (EC_{50}) of *Volvariella volvacea* powders for ferric reducing antioxidant power

Antioxidant	Effective concentration EC_{50}, mg/mL
Trolox	8.5 ± 0.2^{d}
Freeze-dried powder	10.1 ± 0.1^{c}
Oven dried powder	25.6 ± 0.4^{b}
Sun-dried powder	68.5 ± 0.3^{a}

Each value is expressed as mean ± standard deviation (n = 3). Values with different letters within the same column are significantly different ($p < 0.05$)

The effective concentrations (EC_{50}) for the ferric reducing antioxidant power (Table 7) followed the same trend as those for the DPPH trapping activities: Trolox < freeze-drying < oven drying < sun-drying. The values of effective concentrations were 10.1 ± 0.1, 25.6 ± 0.4, and 68.5 ± 0.3 mg/mL for the *V. volvacea* powders obtained by freeze-drying, oven drying, and sun drying, respectively.

Similar patterns were reported by some authors about the reducing power of mushroom powders from different drying methods [55]. Better antioxidant activity of the freeze-dried samples can be attributed to low temperature and vacuum used in the freeze-drying process which cause less thermal degradation and oxidation of phenolic compounds [56].

CONCLUSION

Our results clearly indicate that freeze-drying is the best method of drying the mushroom *Volvariella volvacea* from Côte d'Ivoire because it results in a better retention of nutrients and bioactive compounds responsible for antioxidant activities. This could promote the use of the freeze-dried powder of *V. volvacea* to formulate functional foods and fortify certain conventional foods.

CONTRIBUTION

E.J.P. Kouadio designed the research concept, provided the analysis tools, wrote the manuscript, and submitted it. B.B. Koffi, O.J. Gbotognon, and S. Soro were responsible for data collection and analysis.

CONFLICT OF INTEREST

The authors declare no conflict of interest regarding this publication.

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Storage stability and anti-caking agents in spray-dried fruit powders: A review

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Abstract:
Fruit powders possess numerous benefits compared to fresh raw materials, i.e., extended shelf-life, convenient transportation and storage, a wide range of applications, etc. Nonetheless, the storage time of fruit powders depends on such factors as storage conditions, packaging, etc.

This review suggests a comprehensive analysis of articles, reviews, reports, and books indexed in Scopus, Web of Science, and eLIBRARY.RU, as well as reported at conference proceedings and other scholarly resources in 2005–2022.

Due to their high hygroscopicity, powders tend to absorb moisture from the environment and become prone to caking. Anti-caking agents can prevent powders from this process. Different packaging materials also affect the compounds and properties of fruit powders. Accelerated degradation and temperature models can predict shelfp-life. This review featured the effectiveness of different anti-caking agents, as well as the impact of various packaging methods on the storage of powders. Calcium phosphate demonstrated excellent anti-caking properties, reduced hygroscopicity, and enhanced flowability. Aluminum laminated packaging proved effective in protecting powders during storage. As the storage time increased, powders demonstrated only a slight increase in moisture content. Their L^* value (light to dark) and b^* value (yellow to blue) decreased while the a^* value (green to red) and the total color change increased. Caking increased as the flowability, pigment content, and antioxidant content went down.

The review has practical implications for developing new technologies aimed at prolonging the storage time of spray-dried fruit powders.

Keywords: Fruit powder, physico-chemical properties, anti-caking agents, kinetics, packaging, shelf-life, spray-dried products

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INTRODUCTION

Stickiness and caking of food powders are one of the most important problems that the modern food industry has to solve [1]. Spray-dried powder has many water-soluble amorphous substances that are prone to caking [2, 3]. Caking can be caused by such factors as inter-particle forces that develop under moisture absorption, increased temperature, or pressure during processing, transportation, and storage [4]. Humidity caking is the most common caking phenomenon that damagees food powder. It usually occurs as bridging, agglomeration, compaction, or liquefaction [5].

Caking happens when amorphous food powders turn into an undesirable sticky material [2]. Anti-caking agents are substances that can prevent caking, clumping, and aggregation of hygroscopic powders by improving their flowability [2, 6]. An anti-caking agent competes with the host powder for moisture and acts as a moisture-protective barrier [5]. Anti-caking agents improve the powder flowability. They inhibit caking by acting as a physical surface barrier between particles. As a result, they increase the glass transition temperature (Tg) of the amorphous phase, thus or creating a moisture-protective barrier on the surface of hygroscopic particles [2]. In addition, anti-caking agents also decrease inter-particle forces and reduce stickiness [6].

Anti-caking agents are extremely important components of food production because they make it possible to obtain non-sticky and free-flowing powders [5]. Calcium phosphate, silicon dioxide, silicates, phosphates,

stearic acid salts, and modified carbohydrates resolve the stickiness problem and increase the spray-dried powder yield [7]. Anti-caking agents must be effective at low concentrations, e.g., 3%. As a rule, their allowable concentration is restricted to a very low level [8]. In practice, the percentage of anti-caking agents does not exceed 1% [6, 9].

Packaging means that a product is placed in a protective container or wrapped for storage and transport [10]. Package has a three-fold function: it protects the product against heat, light, moisture, and oxygen, inhibits evaporation, and prevents microbial contamination [11]. The right selection of packaging materials is essential to maintain product quality and freshness during distribution [12]. Packaging materials affect the physicochemical profile and quality of the final product, which the consumer acceptability depends on [13].

Dehydrated fruits serve either as food or ingredients for bakery products, soups, and instant fruit powders. Spray-drying converts fruits into powder, which is a more stable product [14, 15]. Spray-drying can be applied to such exotic fruits as pineapple, papaya, *cempedak* (*Artocarpus integer* Thunb), and *terung asam* (*Solanum ferox*) [16–20]. Powder requires protection against moisture and oxygen, as well as against the loss of volatile flavorings and color [21]. High humidity and temperature conditions are not favorable for powder storage: powder starts to melt and solidify, thus decreasing in quality [4]. As a consequence, food products lose consumer attraction.

Shelf-life prediction usually relies on the data generated under accelerated storage conditions. The test measures the stability of the product under abusive storage conditions, such as high temperature and humidity [22]. The obtained data then help estimate the shelf-life value [23]. This method is fast and cheap, which makes it advantageous for food product development.

Food powders possess numerous advantages over fresh products: they have a longer shelf-life, they are easy to store and transport, and they have a wide range of application as food ingredients. However, the storage time of food powders depends on storage conditions and packaging. Thus, scientific community needs to improve storage technologies and gather data on storage conditions for food powders.

This research features different types of powders, their storage conditions, and packaging methods, with an emphasis on preventing caking and assessing their impact on the properties of spray-dried powders. The findings may aid in developing long-term storage technologies for spray-dried food powders.

STUDY OBJECTS AND METHODS

The research consisted of an exhaustive analysis that defined the anti-caking capabilities of spray-dried powders. It covered articles, reviews, reports, books, etc. published in Scopus, Web of Science, eLIBRARY.RU, conference proceedings, and other scholarly resources in 2005–2022. Such a protracted period provided a thorough comprehension of the subject matter represented in various studies, discoveries, and improvements in the field of spray-dried powder and its anti-caking properties. As a result, the review included findings and conclusions that were well-informed, reliable, and representative of the current body of knowledge on the subject.

RESULTS AND DISCUSSION

Types of anti-caking agents and their effect on powder properties. Table 1 shows different types of anti-caking agents that are incorporated into fruit powders. Calcium phosphate and calcium silicate proved to be the main anti-caking agents [9, 21]. The percentage range of anti-caking agents in food formulations was 0.05–0.25% [24, 25]. However, Lipasek *et al.* described some extreme cases when the share of an anti-caking agent was as high as 50% [26].

Fruit powder producers use different anti-caking agents. Pui *et al.* studied calcium silicate, silicon dioxide, and calcium phosphate incorporated into *cempedak* powder: 0.66% of calcium phosphate demonstrated the best results in hygroscopicity reduction [31]. On the other hand, Addo *et al.* reported calcium stearate to be the most effective anti-caking agent in improving the flowability of *jujube* powder, as compared to magnesium stearate and silicon dioxide [32].

Calcium phosphate. Calcium phosphate (E341) is a calcium salt of phosphoric acid. Its chalky texture makes it a useful free-flowing agent as it can take up to 10% of its weight in moisture [33]. Calcium phosphate inhibits

Table 1 Application of anti-caking agent in fruit and vegetable powders

Powder	Anti-caking agent	Concentration, %	References
Kokum (*Garcinia indica* L.)	Calcium phosphate	0.25	[25]
Guava and pineapple powder	Calcium phosphate, calcium silicate, calcium oxide	0.05, 0.1, and 0.15	[24]
Lemon	Powdered sugar	50	[27]
Lime	Silicon dioxide	10	[28]
Mango	Glycerol monostearate, calcium phosphate	1.5	[21]
Pineapple	Calcium phosphate, calcium silicate, calcium oxide	0.25	[9]
Powdered drink mix	Silicon dioxide	0.2	[29]
Strawberry	Calcium carbonate	–	[30]
Vitamin C	Calcium phosphate, calcium silicate, calcium stearate, corn starch	2.0; 50.0	[26]

caking in cases when a lot of multiwall bags are stacked on top of each other [34].

Jaya & Das reported the optimal requirement of 0.015 kg calcium phosphate and glycerol monostearate per 1 kg mango solid [7]. The degree of caking decreased when the concentration of the anti-caking agents increased. Calcium phosphate also proved effective in hygroscopicity reduction [7]. Nayak & Rastogi added 0.25% of calcium phosphate to *Garcinia indica* L. powder, while Phanindrakumar *et al.* incorporated the same concentration into pineapple powder [9, 25]. Tricalcium phosphate also reduced hygroscopicity in crystalline coconut sugar [35].

Calcium silicate. Calcium silicate (E552) is made from chalk, limestone, or diatomaceous earth. It serves as an anti-caking agent in dry products [8]. Due to its extensive surface area, calcium silicate can draw up quite a lot of moisture [34]. Phanindrakumar *et al.* incorporated 0.25% of calcium silicate into pineapple powder, while Lipasek *et al.* used as many as 2% in vitamin C powder [9, 26]. However, other studies reported a much lower concentration of calcium silicate in guava and pineapple powder, namely 0.05–0.15% [24].

Silicon dioxide. Silicon dioxide (E551), also known as silica, is the oxide of silicon. It absorbs water and improves the flowability of dry products [8]. Silicon dioxide is known to attract and soak up moisture in seasoning blends. However, after silicon dioxide reached its moisture limit, it stopped inhibiting moisture caking [34].

Castro *et al.* applied 0.2% of silicon dioxide to a powder drink mix [29]. Nortuy *et al.* calculated the optimal percentage of 0.73% silicon dioxide for instant date powder [36]. On the other hand, Rostapour *et al.* came up with a much higher optimal concentration of 10% for lime powder [28]. The maximal limit of silicon dioxide was 2% because higher doses gave powder a sandy texture [8].

Packaging materials. Table 2 describes different storage packaging materials for different powders. Quite a few popular packaging materials can be applied to food powders, e.g., polyethylene and polypropylene. Pouches with a metalized barrier are another type of powder packaging, e.g., metalized co-extruded bi-axially-oriented polypropylene, metalized polyester polyethylene, and aluminum foil laminated polyethylene [37–40].

New powder packaging materials appear in scientific publications every day. For instance, Kardile *et al.* used low density polyethylene and coextruded laminated pouches to store instant *puran* powder [45]. Ding *et al.* studied black garlic powder stored in polyethylene tetraphtalate bottles, kraft paper bags, and aluminum laminated polyethylene bags [46]. Kuchi *et al.* determined the quality of *Burfi* banana packaged in aluminium foil, butter paper, and polyethylene film [47]. Varastegani *et al.* used low density polyethylene to store *Nigella sativa* instant beverage powder [48].

The thickness of these packaging is usually 90–100 μm [40, 41, 43]. However, metalized films of high barrier can be as thin as 50 μm; they are used as packaging for apple peel powder [41]. Rao *et al.* studied polyethylene and metalized polyester polyethylene with a thickness of 25 and 20 μm, respectively [39].

Aluminum is a good barrier to oxygen, water vapor, and light. Barrier properties can be measured by the oxygen and water vapor permeation [49]. However, the

Table 2 Different packaging material in storing various fruit powders

Sample	Packaging	Size, cm	Thickness, μm	Water vapor transmission rate, kg/m^{2}day Pa	Oxygen transmission rate, L/m^2 day atm	References
Apple peel powder	High-density polyethylene (HDPE)	10×8	100	1900 cm^3/m^2 day	5700 cm^3/m^2 day	[41]
	Metalized films of high barrier (MFHB)	10×8	50	0.3 g/m^2 day	< 50 cm^3/m^2 day bar	
Jackfruit powder	Aluminum laminated polyethylene (ALP)	10×15	89	1.2×10^{-6} kg/m^2 day	0.0197	[42]
	Metalized co-extruded bi-axially oriented polypropylene (BOPP/MCPP)	10×15	75	3.56×10^{-5} kg/m^2 day	0.0233	
Mango powder	Polyester polypropylene (PP)	18×13	40.2	–	–	[38]
	Metalized polyester polyethylene (MPP)	18×13	62.2	–	–	
Papaya	Aluminum laminated polyethylene (ALP)	15×18	117	6.44×10^{-8}	0.0213	[43]
	Polyamide/polyethylene (PA/PE)	15×18	90	2.25×10^{-7}	0.1200	
Pink quamachil aril powder	Polyethylene (PE)	14×12	25	–	–	[39]
	Metalized polyester polyethylene (MPE)	14×12	20	–	–	
Sour cherry powder	High barrier metalized polypropylene (Pet/PPmet/PE)	12×12	130	–	–	[44]
	Aluminum packaging (Pet/Al/PE)	12×12	106	–	–	

permeability of plastic films to gases and water vapor varies according to the type and thickness of the plastic used [50].

Pua *et al.* described the high-water vapor transmission rate and oxygen transmission rate of 3.56×10^{-5} kg/m^2 day Pa and 0.0233 L/m^2 day atm, respectively [42]. On the other hand, aluminum foil laminated polyethylene had a lower water vapor transmission rate of 1.58×10^{-8} kg/m^2 day Pa [51]. Udomkum *et al.* reported oxygen transmission rate value of 0.12 L/m^2 day atm for polyamide/polyethylene [43].

Aluminum laminated polyethylene. The barrier properties are the main requirement in choosing a high-performance packaging material. A good packaging material prevents oxygen, water, light, flavor, and grease from entering or leaving the package [49]. Aluminum foil laminates have a wide application in food packaging. Table 2 demonstrates quite clearly that aluminum laminated polyethylene is a better packaging material than aluminum foil laminated polyethylene in terms of water vapor permeability [37, 43, 51].

Powder packed in aluminum laminated polyethylene retains more nutrients and catches less moisture. Yu *et al.* reported that powder packaged in polyethylene terephthalate pouches gained more moisture than that in aluminum laminated polyethylene pouches [51]. Aluminum laminated polyethylene had a lower water vapor transmission rate than metalized co-extruded biaxially oriented polypropylene.

Pua *et al.* reported that jackfruit powder packaged in aluminum laminated polyethylene exhibited a lower moisture uptake and had a higher kinetic constant for the total color [42]. In addition, spray-dried bovine colostrum powder packaged in aluminum laminated polyethylene had a longer shelf-life than that packaged in polyethylene terephthalate pouches [51]. Zorić *et al.* studied *marasca* powder preserved with laminated packaging [52].

Loo & Pui reported that aluminum laminated polyethylene pouches were more effective than polyethylene terephthalate pouches in retaining moisture content, water solubility, carotenoid content, flowability, and hygroscopicity [53]. Dried carrots packaged in aluminum laminated pouches and stored under refrigerated conditions showed a minimal increase in moisture content, water activity, pH, and sugar. The carrots also retained the highest amounts of carotenoids, total phenolics, and antioxidants [54].

Phahom *et al.* also reported that aluminum laminated polyethylene was more effective that polyester poly in storing *Thunbergia laurifolia* L. leaves: it had a smaller decrease in hue angle and a smaller increase in total color difference [55]. Suhag & Nanda studied honey powder stored in aluminum laminated polyethylene [56]. The sample had better antioxidant properties and minimal hygroscopicity as compared to those stored in high-density polyethylene. According to Barooah *et al.*, spray-dried ripe banana powder stored in aluminum laminated polyethylene was sensory acceptable even after one year of storage, while metalized polyester pouches were able to preserve its qualities for three months only [57].

Accelerated storage of powder. Table 3 summarizes the storage conditions for different powders. Accelerated storage tests usually include high relative humidity and temperature. Accelerated storage at 90% relative humidity and 38 ± 1°C can be applied to model moisture adsorption and storage time relationships [58].

Some publications report models that predict variations in food quality and shelf-life, e.g., for aloe vera gel powder and apple peel powder [37, 41, 62]. Kinetic modeling based on the Arrhenius principle relates temperature to shelf-life. For dried products, their shelf-life can be calculated from their critical moisture content [63, 64].

Table 4 illustrates the effects of storage stability on the properties and shelf-life of powders. Generally, the moisture content in packaged powder increases together with storage time. Sornsomboonsuk *et al.* reported that extended storage under elevated temperature increased the water activity, moisture content, bulk density, and tapped density of *bael* fruit powder [65]. Apart from storage time, relative humidity, and temperature, the packaging material also affected the moisture gain in jackfruit powder because water vapor migrated from the storage environment into the packaging material [42].

An increased oisture content deteriorates the physical, chemical, and technological properties of the product [67]. Jaya & Das studied mango powder and reported that accelerated storage time decreased the flowability and increased caking [21].

Color is an important attribute as it is the first property noticed by the consumer [68]. Hence, color retention is a predictor of food deterioration rate [69]. Non-enzymatic browning during storage depends on temperature, moisture, water activity, oxygen, and chemical composition [70].

Table 3 shows that total color change increases together with storage period temperature and relative humidity, as well as the type of packaging. Kumar & Mishra studied the total color change in yogurt powder fortified with mango soy and stored under accelerated storage conditions [61]. Packaged powders lost their pigment content and total phenolic content (Table 4) under the effect of temperature, acidity, light, and oxygen exposure caused by the porosity of the packaging.

In addition, a higher moisture uptake eventually leads to degradation of phenolic compounds [71]. Li *et al.*, who studied plum powder, reported that phenolic components were stable for 40 days at room temperature and decreased slightly to 85% after 60 days of storage [72]. Pereira *et al.* managed to preserve the bioactive compounds in *juçara* powder for 103 days [73]. Zhang *et al.* reported that cranberry powder retained its phenolic content after 12 weeks of storage at 25°C [74]. Food quality requires a minimal retention of 50% initial phenolics. Loss of phenolics may result from the excessive gas permeability of the packing material [75].

Table 3 Storage condition for various powders

Powder	Packaging	Temperature, °C	Relative humidity, %	Time	References
Aloe vera gel powder	Aluminum laminated polyethylene (AF) Metalized co-extruded bi-axially oriented polypropylene (BOPP) Polypropylene (PP)	38	90	49 days (intervals of 7 days)	[37]
Aonla (Indian gooseberry)	High density polyethylene bag (HDPE) Polyethylene terephthalate (PET)	15	–	6 months (2, 4, and 6 months)	[59]
Apple peel powder	High-density polyethylene (HDPE)	4 10	– –	120 days (30, 60, and 120 days)	[41]
	Metalized films of high barrier (MFHB)	25 38	– 90		
Bovine colostrum powder	Aluminum laminated polyethylene (ALPE) Polyethylene terephthalate (PET)	4 25 50	40–70 50 20–50	90 days	[51]
Coconut milk powder	Aluminum foil laminated polyethylene (ALP)	38	90	49 days (intervals of 7 days)	[22]
Guava	High-density polyethylene (HDPE) Aluminum laminated polyethylene (ALPE) Coextruded pouches (COEX)	7 26	–	6 months	[60]
Jackfruit powder	Aluminum laminated polyethylene (ALP) Metalized co-extruded bi-axially oriented polypropylene (BOPP/MCPP)	28 38	50 90	12 weeks	[42]
Mango powder	Polyester poly (PP) Metalized polyester polyethylene (MPP)	27–32	–	6 months (0, 2, 4, and 6 months)	[38]
Mango soy fortified yogurt powder	High-density polypropylene (HDPE) Aluminum laminated polyethylene (ALP)	38	90	49 days (intervals of 7 days)	[61]
Papaya	Aluminum laminated polyethylene (ALP) Polyamide/polyethylene (PA/PE)	30	40–45	9 months	[43]
Pink quamachil aril powder	Polyethylene (PE) Metalized polyester polyethylene (MPE)	26	–	6 months (2, 4, and 6 months)	[39]
Pomegranate arils	Aluminum laminated polyethylene (ALP) High-density polypropylene (HDPP)	38	90	3 months	[40]
Sour cherry powder	High barrier metalized polypropylene (Pet/PPmet/PE) Aluminum packaging (Pet/Al /PE)	4 20 37	–	12 months	[44]

Kinetic modeling is essential to predict food changes during storage [62]. Most studies in Table 4 reported losses in food quality by zero or first-order degradation reaction kinetics [61, 76, 77].

According to Singh, the zero-order rate was useful in describing such reactions as enzymatic degradation, non-enzymic browning, and lipid oxidation, which cause rancidity [77]. On the other hand, food deterioration reactions showing first-order losses indicated vitamin and protein losses, as well as microbial growth.

Syamila *et al.* reported reaction kinetics and half-life based on carotenoid content for spray-dried spinach powder [78]. Muzzafar & Kumar assessed the storage stability of spray-dried tamarind powder [79]. They found that the color change followed the zero-order reaction kinetics. The zero-order kinetics was also observed for L^* and a^* parameters, moisture content, ascorbic acid, and total sugar in Khodifad *et al.*, who studied custard apple powder [80]. Similarly, Chang *et al.* reported that total color difference in *soursop* powder was also caused by a zero-order kinetic reaction [81].

Different specific models can predict product shelf-life [82]. The shelf-life of a food product ends when the product is no longer sensory stable or safe, or when its nutrients have degraded [83]. According to Entrup *et al.*, the actual shelf-life depends on the formulation, processing, packaging, and storage conditions [80].

Table 4 shows that powders packaged in aluminum laminated polyethylene had a longer shelf-life, which ranged from 30.28 to 425.5 days. This shelf-life determination is commonly based on the free-flow properties of the powder. In Ramachandra & Rao, aloe vera

Table 4 Effects of storage stability on properties and shelf-life of various powders

Sample	Moisture/water activity	Hygroscopicity	Color L^*	Color a^*	Color b^*	Color difference	Caking	Flowability	Pigment content	Antioxidant/phenolic	Total soluble solids/sugar	Kinetic order	Shelf-life, days	References
Aloe vera powder	+ve	–	–ve	+ve	–ve	+ve	–	–	–	–	–	1 (color change)	33.87 (bi-axially-oriented polypropylene) 42.58 (polypropylene) 51.05 (aluminum laminated polyethylene) (free-flowing)	[37]
Apple peel powder	–	–	–	–	–	–	–	–	–	–	–	0 (phenolic content)	96 (high-density polyethylene) 120 (metalized films of high barrier)	[41]
Bovine colostrum powder	+ve	–	–	–	–	+ve	–	–	–	–	–	1 (Ig concentration)	425.5 (aluminum laminated polyethylene) 86.5 (polyethylene terephthalate)	[51]
Coconut milk powder	+ve	–	–	–	–	–	–	–	–	–	–	–	30.28 (aluminum foil laminated polyethylene)	[22]
Jackfruit powder	+ve	–	–ve	+ve	–ve	+ve	–	–	–	–	–	0 (color change)	–	[42]
Mango milk powder	–	–	+ve	+ve	–ve	–	–	–	–	–	–	–	10 months (free-flowing)	[66]
Mango powder	–	–ve	–	–	–	–	+ve	–ve	–	–	+ve	1 (color change)	114.68	[21]
Mango powder	+ve	–	–	–	–	–	–	–	–ve	–ve	+ve	–	–	[38]
Mango soy yoghurt	–	–	–	–	–	–	–	–	–	–	–	0	45 (high-density polypropylene) 54 (aluminum laminated polyethylene)	[61]
Papaya powder	+ve	–	–	–	–	–	–	–	–	–ve	–	1 (ascorbic acid)	8 months (aluminum laminated polyethylene) 6 months (polyamide/ polyethylene)	[43]
Quamachil aril	+ve	–	–	–	–	+ve	–	–	+ve		–	–	–	[39]
Pomegranate arils	+ve	–	–	–	–	+ve	–	–	–ve	–ve	+ve	0 (color change)	96 (high-density polypropylene) 187 (aluminum polyethylene)	[40]
Sour cherry	–	–	+ve	–ve	–ve	+ve	–	–	–ve	–ve	–	1 (anthocyanin)	12 months (polyphenol)	[44]

+ve = positive effect; –ve = negative effect; – = not reported

powder packaged in aluminum laminated polyethylene had a shelf-life of 51.05 days [37]. However, Kumar & Mishra reported a shelf-life of 54 days for mango soy yogurt powder packaged in aluminum laminated polyethylene [61].

Some powders have a shelf-life of 6–12 months when subjected to lower storage temperature, which is another basis for shelf-life determination, e.g., polyphenol content [44]. Instant *puran* powder had a predicted shelf-life of 13.41 months while the predicted shelf-life for *soursop* powder was 242 days [45, 85].

CONCLUSION

Prolonged storage time makes powders prone to caking. This review featured various anti-caking agents, e.g., calcium phosphate, calcium stearate, silicon dio-

xide, etc., in fruit powders, as well as different packaging materials used to preserve spray-dried powders. Aluminum laminated polyethylene, polypropylene, polyethylene, polyethylene terephthalate, and metalized films proved to be the most common packaging materials adopted for spray-dried powders. The review also included storage conditions for different fruit powders, as well as the effects of storage stability on their roperties and shelf-life. Most powders stored in aluminum laminated polyethylene followed zero- or first-order kinetics with predicted powder shelf-life ranging from 51 to 425 days, deding on the storage temperature.

CONTRIBUTION

All the authors were equally involved in the research analysis and manuscript writing.

CONFLICT OF INTEREST

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

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Plant proteases and anti-bacterial substances in *Allium sativum* L. varieties

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Abstract:
Allium sativum L. protease still remains largely understudied although new varieties of garlic appear quite often, e.g., *lanang* garlic. This study tested the antibacterial effect of garlic and the effectiveness of various *A. sativum* proteases as meat tenderizers. The research involved powder extracts of four varieties of *A. sativum*: *kating*, *lanang*, black garlic, and *sin-chung*. The degradation kinetics was defined based on the Lineweaver-Burk equation. The degradation zones were measured using sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE). Scan electron microscopy served to test the changes in meat connective tissue.
Lanang demonstrated the largest inhibition zones against *Escherichia coli* (9.75 ± 0.15 mm) and *Staphylococcus aureus* (1.04 mm). *Sin-chung* protease degraded beef protein with the highest V_{max} of 0.1818 μg/μL/min at 10–22 KDa (small peptide, troponin C, and troponin I), 25–40 KDa (myosin light chain, troponin T, *α*- and *β*-tropomyosin, actin), and 100–140 KDa (protein C). The same garlic variety degraded mutton meat protein at 10–17 KDa (small peptide) and 25–40 KDa (myosin light chain, troponin T, *α*- and *β*-tropomyosin, actin) with V_{max} of 0.1135 μg/μL/min.
All four *A. sativum* proteases proved to be quite effective meat tenderizers.

Keywords: *Allium sativum* protease, *lanang* garlic, *kating* garlic, black garlic, *sin-chung* garlic

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INTRODUCTION

The benefits of garlic (*Allium sativum* L.) remain a relevant scientific issue, even though some of them have become a long-established concept. Almost all researchers agree on the role of *A. sativum* as a source of prose inhibitors and antibiotics [1–11]. Garlic is known for its antifungal, antibacterial, hypolipidemic, anti-atherosclerosis, and anticarcinogenic properties, not to mention that garlic is a world-famous culinary spice [8, 12–19].

This research aimed at identifying the connection between the *A. sativum* protease inhibitor and various scientifically-established concepts regarding the role of protease in *A. sativum* plants. Proteases are important for the metabolic processes of plant cell growth [20–22]. Researchers have reached no consensus about the relationship between *A. sativum* protease and its inhibitors. The role of *A. sativum* protease remains quite vague because its effectiveness is often disguised by the functions of other spices [23, 24]. For instance, the effect of *A. sativum* protease as a meat tenderizer cannot be separated from other ingredients in the marinating process [23, 25]. Figure 1 proves that very few studies report the role of *A. sativum* protease as a meat tenderizer. We collected around 750 papers published in 2010–2023 that featured *A. sativum* and searched for *A. sativum* protease only to find some 82 results (0.1%). Thus, the poor scientific coverage of *A. sativum* protease and its properties became the background for this research.

In Indonesia, *A. sativum* is represented by such varieties as black garlic, *kating*, and *sin-chung*. *Lanang* garlic, or single garlic, is an accidental new variety that appeared as a result of unsuitable planting environment in

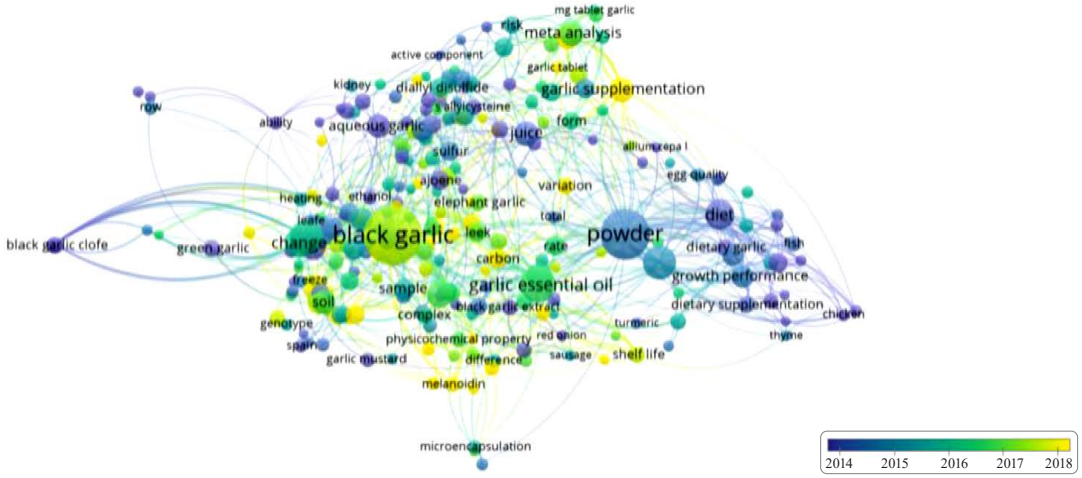


Figure 1 *Allium sativum* L. in scientific publications

the Sarangan area, Magetan, East Java. *Lanang* is more of a medicinal plant than a culinary spice. Poor shoot growth in the canopy inhibits the clove budding, which results in a single big garlic clove. Such growth is suspected to be due to the effect of plant protease on the formation of plant cells [22]. In this regard, the new variety represents a prospective material for protease studies. We compared *lanang* garlic and its properties with other three varieties of *A. sativum* by testing their effect on the inhibition of Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) bacteria.

The research objectives included the following tasks:
– to test the inhibitory power of Gram-positive and Gram-negative bacteria of the four varieties of *A. sativum*; and
– to compare the effectiveness of *A. sativum* protease in four garlic varieties.

We measured the effectiveness based on the following parameters:

1. Degradation kinetics. This parameter included such measurements as the maximal speed (V_{max}) of protein degradation in beef and mutton substrates and the Michaelis Menten constant (K_M);

2. Meat protein degradation zone. It involved sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE); and

3. Effect of *A. sativum* protease on muscle connective tissue, perimysium, endomysium, and collagen in mutton and beef using scan electron microscopy.

STUDY OBJECTS AND METHODS

We purchased *lanang*, *kating*, black garlic, *sin-chung*, beef, and mutton at a local supermarket. The pure bacterial cultures of *Eschericia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were obtained from Merck KGaA, Darmstadt, Germany. The control samples were beef and mutton without *Allium sativum* L., while experimental samples included beef and mutton meat with different varieties of *A. sativum*, namely *lanang*, *kating*, black garlic, *sin-chung*.

Sample treatment. To powder the garlic samples, we peeled the garlic skin and cut each clove into three pieces. After that, we baked it in an oven at 70°C for 72 h. After cooling it at room temperature (25°C) for 15 min, we pulverized the mass with a blender and sieved it. Finally, we re-ovened the powder at 70°C for 24 h.

We extracted 100 g of dried garlic powder using water (100 mL) for 72 h, then filtered and evaporated it in an evaporator for 1 h to obtain a thick extract. The final extract concentration used for analysis was 20% (20 mg/mL).

The beef and mutton were sliced into thin slices (4 cm×4 cm×2 mm). The meat was smeared with 20% garlic extract as the weight of the garlic extract to the weight of the meat. After letting it rest for 30 min, we stored the meat for 60 min at 30–35°C to prevent thermal changes. Untreated meat served as control. The protein degradation kinetics analysis (SDS PAGE) took place at 30 and 60 min, but the scan electron microscopy test was performed at 60 min.

Bacterial inhibition test. After extracting 100 g of the dry garlic powder for 72 h, we filtered and evaporated it in an evaporator for 1 h. The procedure was followed by the water bath. The resulting thick extract had a concentration of 20% (20 mg/mL) each.

The strains of *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) were taken at 200 µL and spread out with a sterile spreader glass in a petri dish containing Muller Hinton Agar and Mannitol Salt Agar. Disk blanks were impregnated with 15 µL of stock extract. One disk served as a negative control whereas the other disc was filled with sample extracts. After incubating them at 37°C for 24 h, we observed the diameter of the inhibition zone in line with the procedure we described in [26].

Protein content. We used the biuret method with a UV-Vis spectrophotometer at λ = 595 nm to determine the protein content in the meat samples [27].

Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE). We performed the analysis of protein degradation using SDS-PAGE in line with the

procedure specified by the Association of Official Analytical Chemists [28]. The analysis involved acrylamide gel electrophoresis: top (5% stacking gel) and bottom (12% separating gel).

Scanning electron microscopy. This procedure relied on Běhalová *et al.* [29]. The meat structure was analyzed using a scanning electron microscope (ZEISS, type EVOMA 10). The image was displayed using a secondary electron (SE) detector.

Maximal speed (V_{max}) and Michaelis Menten constant (K_M). The kinetics of protein degradation involved the Lineweaver-Burk equation, which is the inverse of the Michaelis Menten equation [30]. The relationship between reaction rate V and substrate concentration S changed to $1/V$ and $1/S$:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{S} + \frac{1}{V_{max}} \quad (1)$$

where $1/V$ is the y-axis and $1/S$ is the x-axis; $y = bx + a$; $V_{max} = 1/a$; $K_m = V_{max} \times b$.

RESULTS AND DISCUSSION

Bacterial inhibition. *Lanang* garlic had the greatest bacterial inhibition among all *Allium sativum* L. varieties, both in relation to Gram-negative and Gram-positive bacteria. The inhibition power for Gram-negative *Eschericia coli* was 9.75 ± 0.15 mm. For Gram-positive *Staphylococcus aureus*, the inhibition zone was 1.04 mm.

Kating garlic was able to inhibit *E. coli* with an inhibition zone of 7.54 ± 0.25 mm. For *S. aureus*, it did not exceed 1 mm. Black garlic and *sin-chung* garlic extracts yielded no inhibition results.

Table 1 sums up the results for all the *A. sativum* varieties in this research.

Lanang demonstrated the best inhibition results for Gram-negative and Gram-positive bacteria. It might owe its effectiveness to homogenate allicin (S-(2-propenyl)2-propene-1-sulfinothionate), which is known for its antibacterial activity [31]. *Kating* also inhibited *E. coli* and *S. aureus*, but to a much lesser extent. Black garlic and *sin-chung* produced no inhibition effect at a concentration of 20 mg/mL. Probably, the process of forming active bacterial substances in the cell enlargement process and the growth area were far from optimal [32].

Kinetics of meat protein degradation. The control samples of beef and mutton, which were untreated with garlic extracts, showed no significant difference ($p > 0.05$). The mutton samples treated with extracts of *kating*, *lanang*, black garlic, and *sin-chung*, on the contrary, demonstrated significant differences ($p < 0.05$) after 30 and 60 min of processing. The same was true for the experimental beef ($p < 0.05$).

The *sin-chung* garlic extract was able to reduce the protein content, followed by black garlic and *kating*. *Lanang*, however, showed little effect on the degradation of meat protein. Table 2 illustrates the changes in protein levels for the mutton and beef samples.

Protein degradation kinetics was analyzed using the Lineweaver-Burk equation. Figure 2 shows the relationship of velocity ($1/V$) to substrate degradation ($1/S$). Figure 3 illustrates the changes in the concentration of protein substrate (S).

Table 1 *Allium sativum* L. varieties: bacterial inhibition effect

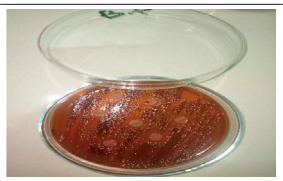
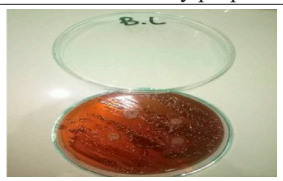
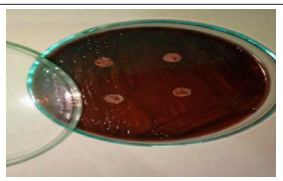
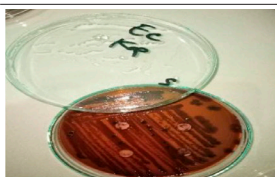
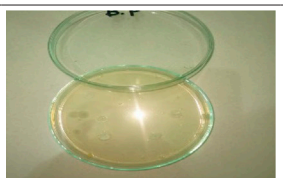
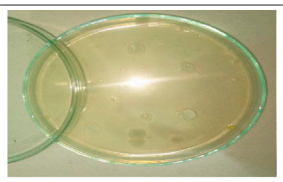
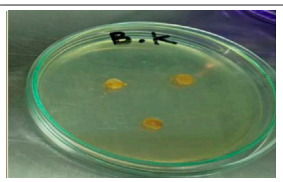
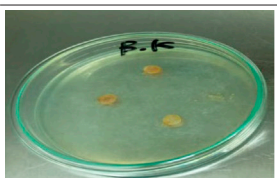
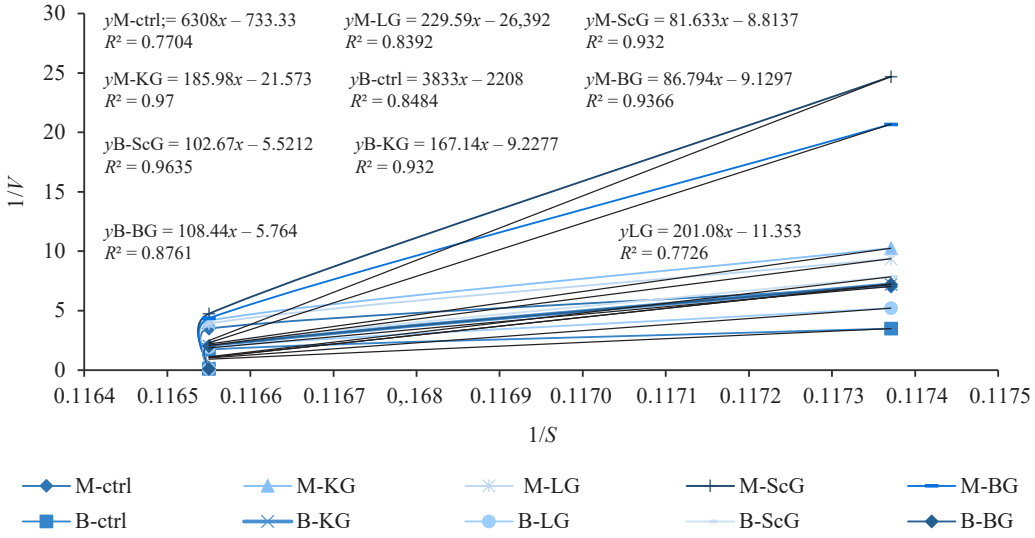
	Kating	*Lanang*	Black garlic	*Sin-chung*
Appearance				
Characteristics	Small wrinkled cloves clustered together	Single cloves; the smallest size among other varieties	Clustered black cloves	Large clustered cloves
		Inhibitory properties		
Escherichia coli				
	Diameter of inhibition zone: 7.54 ± 0.25 mm	Diameter of inhibition zone: 9.75 ± 0.15 mm	No zone of inhibition detected	No zone of inhibition detected
Staphylococcus aureus				
	Diameter of inhibition zone: < 1 mm	Diameter of inhibition zone: 1.04 mm	No zone of inhibition detected	No zone of inhibition detected

Table 2 Protein contents, μg/μL in meat samples

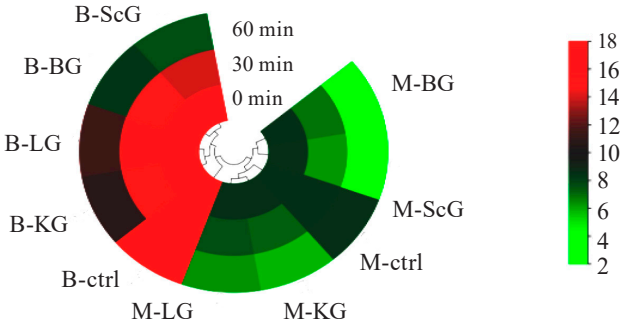
Sample	0 min	30 min	60 min
Mutton, control	9.39 ± 0.15[a]	9.41 ± 0.05[a]	9.52 ± 0.12[a]
Mutton + *kating*	8.38 ± 0.13[c]	7.32 ± 0.11[b]	5.78 ± 0.06[a]
Mutton + *lanang*	8.52 ± 0.15[c]	7.60 ± 0.05[b]	6.30 ± 0.08[a]
Mutton + black garlic	8.50 ± 0.18[c]	6.90 ± 0.04[b]	2.90 ± 0.05[a]
Mutton + *sin-chung*	8.47 ± 0.11[c]	6.29 ± 0.15[b]	2.34 ± 0.18[a]
Beef, control	18.35 ± 0.25[a]	18.41 ± 0.15[a]	18.31 ± 0.35[a]
Beef + *kating*	17.35 ± 0.25[c]	15.17 ± 0.35[b]	10.77 ± 0.13[a]
Beef + *lanang*	17.35 ± 0.15[c]	15.47 ± 0.15[b]	11.40 ± 0.15[a]
Beef + black garlic	17.35 ± 0.15[a]	14.70 ± 0.35[b]	8.23 ± 0.18[a]
Beef + *sin-chung*	17.32 ± 0.14[c]	14.10 ± 0.05[b]	7.60 ± 0.17[a]

Anova Tukey HSD post-hoc test was performed at standard $p < 0.05$. The same superscripts indicate no significant difference between the samples. Mutton and beef samples smeared with extracts of *kating*, *lanang*, black garlic, and *sin-chung* were compared with the untreated control



M – mutton; B – beef; ctrl. – control; KG – *kating* garlic; ScG – *sin-chung* garlic; BG – black garlic; LG – *lanang* garlic

Figure 2 Kinetics of protein degradation per 1 min in beef and mutton samples



M – mutton; B – beef; ctrl. – control; KG – *kating* garlic; ScG – *sin-chung* garlic; BG – black garlic; LG – *lanang* garlic

Figure 3 Changes in protein content per 1 min in beef and mutton samples

In the mutton samples, *sin-chung* protease had the highest V_{max} in the protein degradation process of 0.1135 μg/μL/min using a substrate of 9.19 μg/μL. The *lanang* protease showed the lowest V_{max}, namely 0.0378 μg/μL/min. The results for black garlic and *kating* were 0.1096 and 0.0464 μg/μL/min, respectively. The *sin-chung* protease had the best degrading results for beef protein with V_{max} of 0.1818 μg/μL/min and substrates ranging from 18.54 μg/μL. The black garlic protease produced V_{max} of 0.1735 μg/μL/min while *kating* protease was 0.1078 μg/μL/min. The *lanang* protease had the lowest effect: 0.088 μg/μL/min (Table 3).

The process of forming antibacterial active substances does not always coincide with the formation of plant cells during cell formation/growth process, which involves plant proteases. This research succeeded in proving that black garlic and *sin-chung* were in that phase. In both cases, protease tenderized the meat. The *sin-chung* variety had the highest protein degradation V_{max} in mutton (0.1135 μg/μL/min) and beef (0.1818 μg/μL/min). Black garlic protease had the second-best result, followed by *kating* and *lanang*. The obtained results confirm those reported by Bar *et al.* regarding the formation of anti-bacterial substances and Sharma & Gayen regarding the growth process of *A. sativum* [22, 32].

Meat protein degradation zone. To measure the protein degradation zone, we smeared the mutton and beef

Table 3 Kinetics of beef and mutton protein degradation under the effect of *Allium sativum* L. protease enzyme

Sample	V_{max}, µg/µL/min	K_M, µg/µL
Mutton, control	0.001363698	8.602209
Mutton + *sin-chung*	0.113459727	9.190238
Mutton + *lanang*	0.037878788	8.674242
Mutton + black garlic	0.109649123	9.429825
Mutton + *kating*	0.046360686	8.576727
Beef, control	0.004545455	17.42273
Beef + *sin-chung*	0.181818182	18.54545
Beef + *kating*	0.107793468	18.01660
Beef + black garlic	0.173490632	18.81332
Beef + *lanang*	0.088082445	17.70457

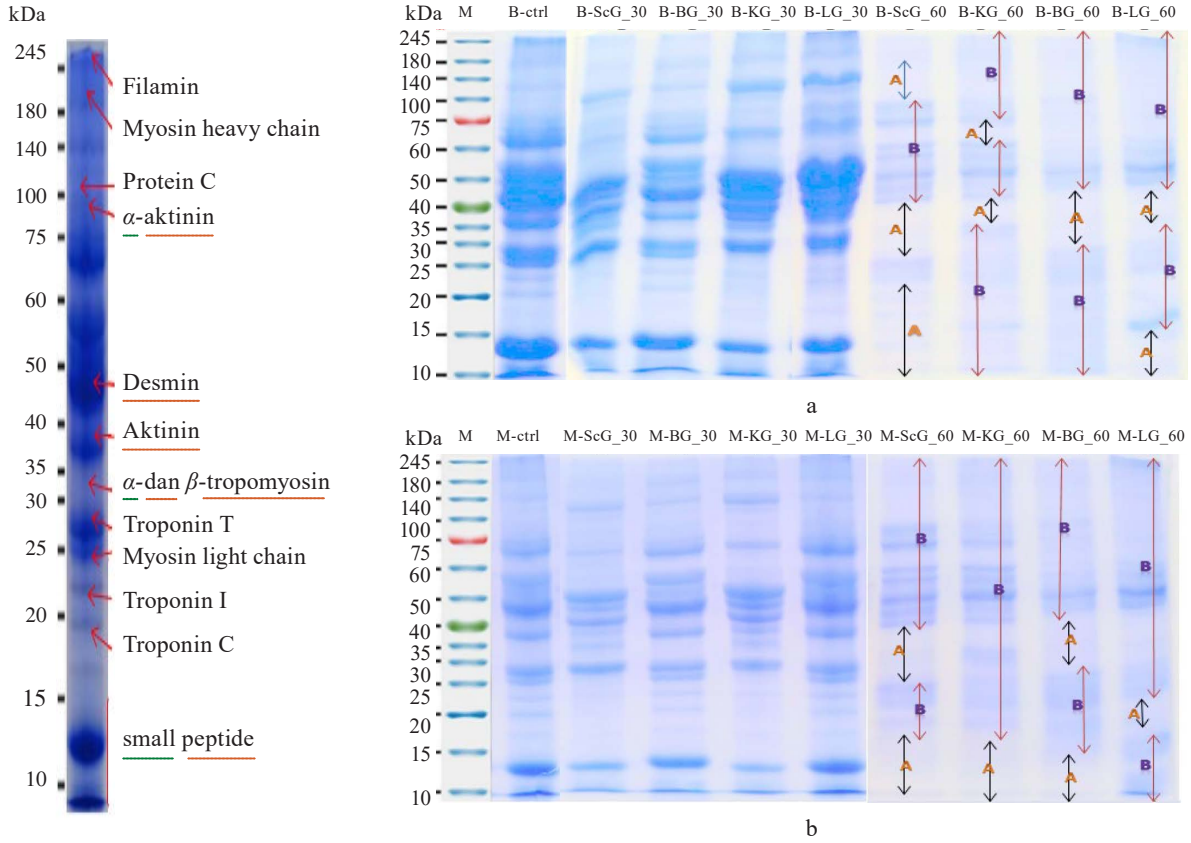
samples with extracts of *kating*, *lanang*, black garlic, and *sin-chung* at 30 and 60 min and compared the obtained results with those for the untreated control samples.

In beef (Fig. 4a), the garlic extracts produced no mild degradation within the first 30 min. Mild degradation is marked by a change in color from blue to purple while complete degradation means a loss of color. Both were clearly visible after 60 min. The *sin-chung* protease demonstrated complete degradation in the area of 10–22 KDa (small peptide, troponin C, and troponin I), 25–40 KDa (myosin light chain, troponin T, *α*- and *β*-tropomyosin, actin), and 100–140 KDa (Protein C). Other areas showed mild degradation only.

The *kating* protease was able to degrade proteins in the 35–40 KDa (actin) and 60–62 KDa zones. Other areas were only slightly degraded. The black garlic protease produced a degradation effect in the 30–42 KDa zone (*α*- and *β*-tropomyosin, actin). Meanwhile, *lanang* had the smallest degradation area of 10–17 KDa (small peptide) and 40–50 KDa (desmin).

In mutton (Fig. 4b), the proteases produced no degradation effect within the first 30 min. For all garlic varieties, degradation started at 60 min. *Lanang* was able to degrade only 20–23 KDa (troponin I). S*in-chung* had a degradation zone at 10–17 KDa (small peptide) and 25–40 KDa (myosin light chain, troponin T, *α*- and *β*-tropomyosin, actin). The back garlic protease was in the 10–15 KDa and 35–40 KDa ranges. *Kating* was effective in the 10–17 KDa range.

Therefore, all four *A. sativum* proteases proved to be effective meat tenderizers. For beef, the *sin-chung* extract succeeded in degrading proteins in a fairly wide area of 10–22 KDa (small peptide, troponin C, troponin I), 25–40 KDa (myosin light chain, troponin T, *α*- and



M – mutton; B – beef; ctrl. – control; KG – *kating* garlic; ScG – *sin-chung* garlic; BG – black garlic; LG – *lanang* garlic; A – complete degradation; B – mild degradation; KDa – protein molecular weight (Kilo Dalton); M – markers.

Example of reading code: B-BG_30 stands for beef + black garlic for 30 min; M-BG_60 stands for mutton + black garlic for 60 min. Other codes follow the same pattern.

Figure 4 Complete and mild degradation zones for beef (a) and mutton (b): SDS-PAGE

β-tropomyosin, actin), and 100–140 KDa (protein C). As for the mutton samples, the *sin-chung* extract managed to degrade only 10–17 KDa (small peptide) and 25–40 KDa (myosin light chain, troponin T, *α*- and *β*-tropomyosin, actin). Meanwhile, *lanang* had the smallest degradation zone: 10–17 KDa (small peptide) and 40–50 KDa (desmin) for beef and 20–23 KDa (troponin I) for mutton.

Effect of *A. sativum* protease on meat connective tissue. Collagen is the most abundant component in muscle tissue, where it forms perimysium and endomysium. Perimysium separates muscle fibers while endomysium coats them. Perimysium and endomysium release muscle tissue in the form of tears or cracks.

Figure 5 illustrates the effect of *A. sativum* protease on beef connective tissue. In the control meat (Fig. 5a), muscle tissue remained tight, and collagen dominated. In the sample treated with black garlic (Fig. 5b), endomysium connective tissue looked elongated and wide, with perimysium predominating. Some strong muscle tissue remained, and collagen was no longer predominant. The *lanang* protease (Fig. 5c) affected endomysium and perimysium at several points. Strong-bound muscle tissue predominated, but collagen was reduced. *Kating* (Fig. 5d) resulted in dominant perimysium. Small endomysium tissue was visible in some areas, and strong muscle tissue was detected in all directions. *Sin-chung* (Fig. 5e) was able to change the dominance of collagen and muscle tissue, and they became less dominant. Large endomysium predominated at several points, although perimysium was visible only on one side.

Figure 6 illustrates the effect of *A. sativum* protease on connective mutton tissue. The control sample (Fig. 6a) was predominated by strong muscle tissue and collagen. No visible loss of muscle tissue was detected:

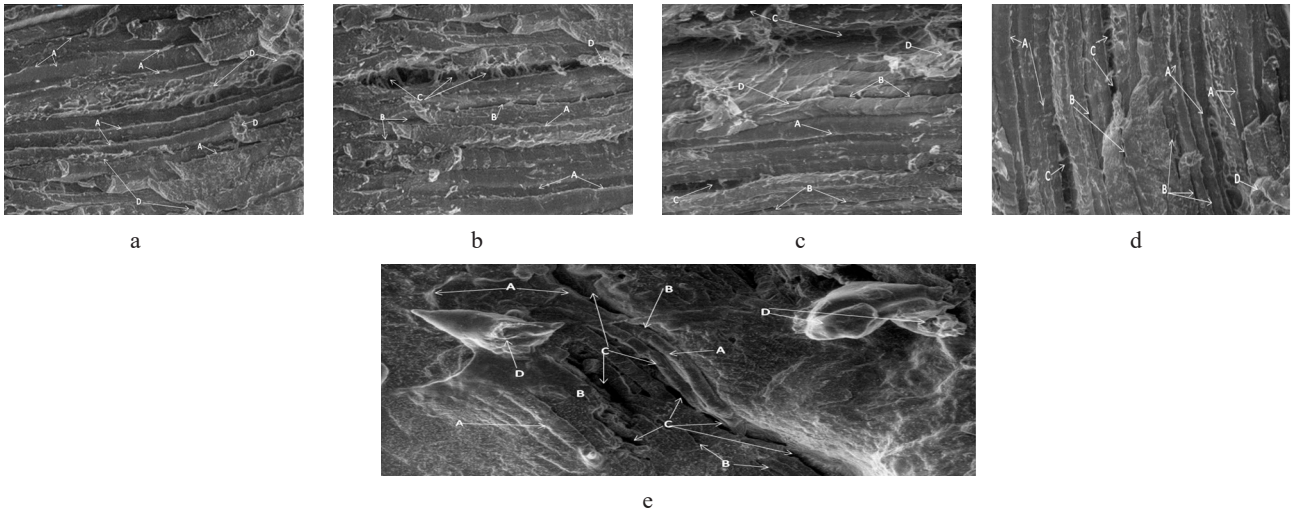


Figure 5 Effect of *Allium sativum* L. proteases on beef connective tissue. Connective tissue in the control sample (a) was degraded by black garlic (b), *lanang* (c), *kating* (d), and *sin-chung* (e), which affected muscle tissue (A), perimysium (B), endomysium (C), and collagen (D)

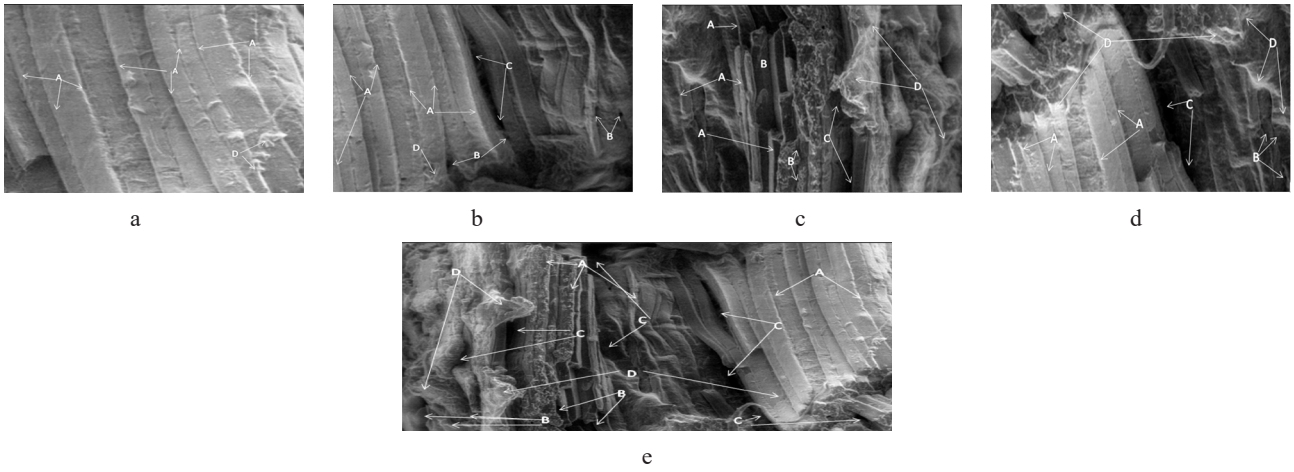


Figure 6 Effect of *Allium sativum* L. proteases on mutton connective tissue. Connective tissue in the control sample (a) was degraded by black garlic (b), *lanang* (c), *kating* (d), and *sin-chung* (e), which affected muscle tissue (A), perimysium (B), endomysium (C), and collagen (D)

perimysium and endomysium did not appear under these conditions. Black garlic (Fig. 6b) resulted in a long and wide endomysium network, though at one point only. Perimysium appeared at several points. Muscle tissue predominated but collagen was no longer visible. *Lanang* (Fig. 6c) had very little effect on perimysium and endomysium formation. Tightly bound muscle tissue and collagen still predominated on all sides of the meat sample. The *kating* protease (Fig. 6d) produced large endomysium and small clustered perimysium visible on a few sides. Muscle tissue and collagen still predominated on all sides of the meat sample. *Sin-chung* (Fig. 6e) had a very different effect. Large and long endomysium dominated on all sides of the meat sample. Perimysium demonstrated a similar picture but was scattered at several points. In this sample, collagen and muscle tissue were only seen in one order and were clustered together.

All four *A. sativum* proteases were able to separate myofibers from the perimysium, which is the most vulnerable tissue. This experiment was able to catalyze the effect of perimysium as it separated muscle fibers in muscle connective tissue. Perimysium is a fascicle that can be classified into primary, secondary, and tertiary fascicles, based on the diameter [33].

Sin-chung and black garlic had a prominent effect on the formation of dominant endomysium, which is the first step in meat tenderizing. Probably, when endomysium detached from sarcomere, it surrounded the muscle fibers of basal lamina, proteoglycans, collagen, and lamina. As a result, the endomysium formation left tears or cracks on the meat surface. The obtained results were consistent with those reported by Swasdison & Mayne regarding endomysium formation [34]. The control samples (Figs. 5a and 6a) revealed no endomysium and perimysium tissue because the cross-linked tissue was still strong in muscle tissue and collagen.

CONCLUSION

In this research, the *lanang* garlic variety demonstrated the greatest antibacterial properties: its inhibition zone was 9.75 ± 0.15 mm against *Escherichia coli* and 1.04 mm against *Staphylococcus aureus*. Black garlic and *sin-chung* demonstrated no inhibitory power, probably, because the process of forming anti-bacterial substances does not always coincide with the process of plant growth, which involves plant proteases.

All four *Allium sativum* L. proteases proved to be effective meat tenderizers. The *sin-chung* extract possessed the most effective plant protease in this process. Its protease was able to degrade beef protein with the highest V_{max} of 0.1818 μg/μL/min in the 10–22 KDa range (small peptide, troponin C, and troponin I), 25–40 KDa (myosin light chain, troponin T, α- and β-tropomyosin, actin), and 100–140 KDa (protein C). In mutton, it was effective only in the 10–17 KDa (small peptide) and 25–40 KDa (myosin light chain, troponin T, α- and β-tropomyosin, actin) ranges with V_{max} of 0.1135 μg/μL/min. The *lanang* protease showed the weakest protease enzyme activity: a small degradation zone in the area of 10–17 KDa (small peptide) and 40–50 KDa (desmin) with V_{max} of 0.0881 μg/μL/min for beef. For the mutton samples, its result was 20–23 KDa.

CONTRIBUTION

All the authors were equally involved in the research analysis and manuscript writing.

CONFLICT OF INTEREST

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

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Anti-lipidemic and protein restoration potential of *Monodora myristica* (Gaertn.) in rats fed with cassava containing crude oil

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Abstract:
Consumption of cassava meal affected by crude oil has significant effects on lipid and protein metabolism. The hepatoprotective action of spices is mostly attributed to the suppression of lipid oxidation and protein breakdown. This study examined the protein restoration and anti-lipidemic potential of *Monodora myristica* (Gaertn.) in rats fed with cassava contaminated with crude oil.
The research involved 36 albino rats separated into six groups (n = 6). Group 1 (control) consumed cassava without crude oil. Group 2 received cassava with crude oil. Groups 3, 4, and 5 fed on cassava with crude oil and various extracts of *M. myristica*, i.e., aqueous, ethanol, and diethyl ether, respectively. Group 6 received non-ionic synthetic surfactant Tween 80. The experiment relied on standard methods.
Blood serum and liver obtained from the rats of Group 2 showed a significant ($p < 0.05$) increase in total cholesterol, low density lipoprotein cholesterol, triacylglycerol, and malondialdehyde, as well as a decrease in total protein, albumin, and high-density lipoprotein cholesterol. The groups that received *M. myristica* extracts showed a significant increase ($p < 0.05$) in total protein, albumin, and high-density lipoprotein cholesterol. They also had lower total cholesterol, low density lipoprotein cholesterol, triacylglycerol, and malondialdehyde as compared to Group 2, which dieted on cassava contaminated with crude oil without additives.
In this research, crude oil-contaminated cassava affected proteins and lipids in rats. Diethyl ether extract of *M. myristica* demonstrated the best anti-lipidemic and protein restoration.

Keywords: Cassava meal, crude petroleum oil, lipids, *Monodora myristica*, protein

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INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a primary food crop growing in South America. This plant is believed to have been introduced to Nigeria by Portuguese explorers and colonists at the height of the slave trade in the 16th century [1]. Due to cassava drought-resistance, its now grows in all parts of Africa. It is a perennial woody shrub that can withstand water shortages and has a starch content of up to 32% (fresh). These days, cassava is one of the most important staple food crops in sub-Saharan Africa. In some regions, its average consumption exceeds 300 kg per person annually [2].

Cassava is a remarkably adaptable crop with a wide range of applications and by-products. Its leaves can be fed to cattle as a protein feed additive. People can eat them dry or in soups. Stems are used for plant propagation and grafting. Roots are typically processed for human and industrial consumption as a good source of carbohydrates [2]. Cassava yields a variety of goods, including cassava starch, fried cassava granules, *lafun* (cassava flour), and *garri* (fried cassava).

Cassava is a source of both animal feed and biofuel. Oil spillages affect the economy by destroying vegetation, food crops, and soil fertility, which reduces food productivity [3]. However, poor crop yield and productivity decline are not the only problems related to

oil spillage. Cassava grown in crude oil-impacted soils accumulates toxic hydrocarbons and heavy metals with a high consequence of transfer within the food web [4].

The chemical composition of crude oil depends on the earth crust development. In fact, it is a complex of up to 6000 potentially unique hydrocarbons and metals. A rising crude oil exposure may be dangerous for people and animals [3]. Toxicants generate free radicals, and free radicals may lead to oxidative stress [5]. If followed by oxidative alteration, free radicals cause lipid peroxidation, thus harming such vital cellular components such as proteins, lipids, and DNA [6–8]. The oxidation and digestion of lipids and carbohydrates obtained with food provides most of the energy required by the human body [4].

The liver has a higher capacity for the metabolism of xenobiotics, as well as other hydrocarbons and elements found in petroleum and petrochemical goods. Akinbule *et al.* studied the changes in lipid profiles and health lipid quality of some Nigerian composite meals [9]. Spices such as turmeric, bay leaf, cinnamon, and cloves demonstrated a positive effect on human health. Herbs and spices are essential components of daily diet [10]. In addition, they also enhance the flavor and taste of food [11, 12]. Even in very small quantities, spices can make an average meal appealing and fragrant. For instance, the calabash nutmeg (*Monodora myristica* (Gaertn.)) may participate in lipid metabolism. *M. myristica* is a tropical tree that belongs to the *Annonaceae* family of flowering plants. Valuable yet neglected, *M. myristica* has a lot of names in African languages: *ehirior ehuru* (Ibo); *gujiyadanmiya* (Hausa), *ariwo*, *arigbo*, *abolakoshe*, or *eyinaghose* (Yoruba), and *ehinawosin* (Ikale). Its fruit contains seeds that are typically 1.5 cm long and covered in a white, sweet-smelling mush. Olatoye *et al.* reported that *M. myristica* seeds serve mostly as a flavoring in various foods, including soups and salads [13].

Our study is part of a research project that features the changes in lipid and protein parameters in rats fed with cassava contaminated with crude oil. However, the effect of *M. myristica* on the toxicity of crude oil in cassava has not been acknowledged scientifically. In future, this project may prove the efficacy of *M. myristica* in treating crude oil-induced lipidemia in Wistar rats. As *M. myristica* is a popular component of African diet, the research results may eventually find dietary application in treating people exposed to cassava foods contaminated with crude oil.

STUDY OBJECTS AND METHODS

***Monodora myristica* (Gaertn.) and crude oil.** The *M. myristica* and cassava were obtained from a market in Amai, Delta State, Nigeria, in October 2021. *M. myristica* (voucher number FHI107259) was identified at Forest Research Institute of Nigeria, Ibadan. The crude oil was obtained from a refinery in Warri that belongs to the Nigerian National Petroleum Cooperation (NNPC), Delta State, Nigeria.

Preparing cassava diet. The fresh cassava tubers that had just reached maturity were purchased in October 2021 from local farmers in Amai community, Delta State. They were washed in clean water to remove dirt and peeled. After being manually cut into smaller bits, they were rinsed in clean water, dried in an oven at 40°C, and milled. The samples included cassava meal with crude oil and cassava meal without crude oil. The rats diet consisted of dried cassava (54.64%), casein (11.26%), corn cob cellulose (5%), bone meal (2%), oyster shell (1%), Vitmin premix (1%), glucose monohydrate (5%), and salt (5%). The difference between the two groups was that the control mix without crude oil contained 10% of sucrose, which was substituted with 10% of crude oil in the experimental mix. The ingredients were mixed together manually before being fed to albino rats.

Preparing spice extracts. The dried *M. myristica* was crushed into fine particles in a high-speed blender. After that, we dissolved 100 g of the powder in 500 mL of the following solvents: ethanol (95% by volume), hot water (60°C), and diethyl ether (95% by volume). This stage lasted for 48 h. After being filtered through a cotton cloth, the solutions underwent a water bath at 45°C and were concentrated to dryness.

Experimental procedures. The experiment featured 36 rats that were 7–8 weeks old. The average weight of the rats were 130–140 g. The rats were allowed to acclimatize to laboratory condition for one week. After that, they were divided into six groups with six rats in each. Group 1 was normal control and included rats fed on cassava without crude oil. Group 2 was the experimental control and included rats consumed nothing but cassava contaminated with crude oil. In Group 3, the rats had a diet of cassava with crude oil plus aqueous *M. myristica* extract. Group 4 combined cassava with crude oil and ethanol *M. myristica* extract. In Group 5, the rats ate cassava with crude oil plus a diethyl ether extract of *M. myristica.* In Group 6, there were rats fed on cassava with crude oil plus 1 mL/kg of 5% Tween 80. The cassava meal and extracts were administered orally for 28 days using cannulas.

Ethical approval. Approval for the current study was granted by the Ethics Committee of Novena University (case No. NUO/PGD/21/890). The research followed the Ethics of Animal Research [14].

Blood collection and tissue homogenate. The rats were sacrificed on day 29 of the experiment after an overnight fast. We collected blood samples from the heart using a syringe and a needle to transfer the samples to an anticoagulant-free test tube. The clotted blood was centrifuged at 2500 rpm for 15 min to isolate serum for further examination. The serum was stored in a freezer at –4°C. One gram of liver and kidney were homogenized in 10 mL of normal saline and centrifuged at 2500 g for 15 min. The obtained supernatant was kept in a freezer at –4°C for biochemical examination.

Biochemical examination. *Total cholesterol.* We used the method described by Allan *et al.* to determine the total cholesterol (T-Chol) [15]. According to the pro-

cedure, we added 1 mL of cholesterol reagent to a labelled test tube. After that, 10 µL of each sample was added to a respective tube and mixed. The tubes with the reagent and sample mix were left to stand for 5 min. When the solution turned pinkish-red, the absorbance test took place at 530 nm after blanking.

Triacylglycerol. The triacylglycerol assay followed the method described by Young in [16]. We placed 1 mL triacylglycerol regent in labelled tubes. After that, we transferred 10 µL sample, blank (distilled water), and standard reagent into the respective tubes and mixed the contents. The tubes with reagents and samples were stored at room temperature for 5 min. The absorbance was determined at 540 nm after blanking.

High-density lipoprotein cholesterol. We applied the method specified by Badimon *et al.* to determine high-density lipoprotein cholesterol (HDL-C) [17]. In line with the procedure, we added precisely 500 µL diluted precipitant to 200 µL sample. The resulting mix was allowed to settle at room temperature for 10 min. After that, we centrifuged them at 4000 rpm for 10 min or at 12 000 rpm for 2 min. Subsequently, 1 mL cholesterol reagent was placed in a labelled test tube, upon which we transferred 25 µL supernatant, blank (distilled water), and standard reagent to the respective tubes. The content of each tube was thoroughly mixed for 5 min, and the values were recorded at 500 nm.

Low-density lipoprotein cholesterol. The low-density lipoprotein cholesterol (LDL-C, mg/dL) test relied on the Friedewald Equation as in [18]:

$$\text{LDL-C} = (\text{Total cholesterol} - \text{HDL}) - \text{Triacylglycerol}/5$$

Lipid peroxidation. We used the method described by Buege & Aust to analyze the lipid peroxidation (LPO) [19]. We dropped 1 mL sample and 2 mL TCA-TBA-HCL reagent (1:1:1) in a test tube. The tube was then submerged in boiling water for 15 min. After cooling down, the tubes were centrifuged at 5000 g for 10 min. The absorbance was measured at 532 nm. We expressed the lipid peroxidation in units/g of wet tissue using a 1.56×10^5 M^{-1} molar extinction co-efficient.

Albumin. According to Droumas *et al.*, the albumin content was defined based on its quantitative blending to 3′,3″,5′,5″-tetrabromo-m-cresol-sulphoepthalein (bromocresol green) [20]. We dispensed 3 mL bromocresol green in a labelled test tube. After that, we transferred 10 µL sample into the tubes and allowed them to settle for 5 min. The absorbance for spectrophotometry was measured at 580 nm.

Total protein. We used the Tietz technique to calculate the total protein [21]. Protein forms a blue-colored complex when treated with cupric ions in an alkaline solution. The blue color is proportional to the protein concentration. According to the procedure, we transferred 1 mL reagent to blank, test, and standard tubes. Then, we added 20 µL sample to the appropriate tubes. After 30 min, we measured the absorbance at 546 nm.

Statistical analysis. The data were subjected to descriptive statistics, and the results were shown as mean ± SD and mean bars. We used the ANOVA analysis of variance and the post hoc test to identify the significant differences between the groups. The statistical analysis relied on SPSS 22.0. A statistically significant difference between the test and control groups was defined as $p < 0.05$.

Table 1 Total cholesterol in rats administered cassava with and without crude oil and various *Monodora myristica* extracts

№	Groups	Total cholesterol, mg/dL	
		Serum	Liver
1	Non-contaminated cassava (normal control)	258.22 ± 30.13[a]	326.33 ± 23.44[a]
2	Contaminated cassava (experimental control)	317.45 ± 23.30[b]	374.32 ± 55.45[b]
3	Contaminated cassava + aqueous extract	289.11 ± 56.55[c]	367.77 ± 75.93[c]
4	Contaminated cassava + ethanol extract	278.87 ± 22.53[d]	341.13 ± 67.13[d]
5	Contaminated cassava + ether extract	241.27 ± 65.78[e]	317.47 ± 43.02[e]
6	Contaminated cassava + Tween 80	316.34 ± 23.44[b]	371.45 ± 46.37[b]

Values are presented as mean ± SD with n = 6. Values in the same column with various letter designations (a, b, c, d, and e) differ significantly ($p < 0.5$)

Table 2 Triacylglycerol in rats administered cassava with and without crude oil and various *Monodora myristica* extracts

№	Groups	Triacylglycerol, mg/dL	
		Serum	Liver
1	Non-contaminated cassava (normal control)	204.23 ± 34.13[a]	245.33 ± 33.33[a]
2	Contaminated cassava (experimental control)	305.44 ± 28.34[b]	343.44 ± 34.35[b]
3	Contaminated cassava + aqueous extract	275.43 ± 23.45[c]	312.93 ± 24.67[c]
4	Contaminated cassava + ethanol extract	256.45 ± 55.34[d]	293.35 ± 63.62[d]
5	Contaminated cassava + ether extract	225.67 ± 64.34[e]	263.24 ± 23.48[e]
6	Contaminated cassava + Tween 80	311.65 ± 32.34[b]	343.34 ± 34.67[b]

Values are presented as mean SD with n = 6. Values in the same column with different letter designations (a, b, c, d, and e) differ considerably ($p < 0.05$)

Table 3 High-density lipoprotein cholesterol (HDL-C) in rats administered cassava with and without crude oil and various *Monodora myristica* extracts

№	Groups	HDL-C, mg/dL	
		Serum	Liver
1	Non-contaminated cassava (normal control)	184.74 ± 13.35[a]	224.42 ± 28.25[a]
2	Contaminated cassava (experimental control)	145.47 ± 24.55[b]	182.26 ± 21.63[b]
3	Contaminated cassava + aqueous extract	153.34 ± 26.54[c]	196.46 ± 23.21[c]
4	Contaminated cassava + ethanol extract	164.25 ± 16.65[d]	202.03 ± 19.32[d]
5	Contaminated cassava + ether extract	182.46 ± 17.64[a]	222.16 ± 35.44[a]
6	Contaminated cassava + Tween 80	144.27 ± 24.60[b]	184.36 ± 36.12[b]

Values are presented as mean SD with n = 6. Values in the same column with different letter designations (a, b, c, and d) differ considerably ($p < 0.05$)

Table 4 Low-Density Lipoprotein Cholesterol (LDL-C) in rats administered cassava with and without crude oil and various *Monodora myristica* extracts

№	Groups	LDL-C, mg/dL	
		Serum	Liver
1	Non-contaminated cassava (normal control)	32.63 ± 17.42[a]	52.84 ± 10.22[a]
2	Contaminated cassava (experimental control)	110.89 ± 21.11[b]	123.37 ± 11.09[b]
3	Contaminated cassava + aqueous extract	80.68 ± 17.13[c]	108.72 ± 02.19
4	Contaminated cassava + ethanol extract	63.33 ± 14.22[d]	80.43 ± 09.23[d]
5	Contaminated cassava + ether extract	23.68 ± 07.56[e]	42.66 ± 06.39[e]
6	Contaminated cassava + Tween 80	109.74 ± 22.13[b]	118.42 ± 11.08[b]

Values are given in mean ± SD with n = 6. Values in the same column with different letter designations (a, b, c, d, and e) differ considerably ($p < 0.05$)

RESULTS AND DISCUSSION

Lipid profile of rats fed with cassava containing crude oil. Tables 1–4 illustrate the changes in serum and liver total cholesterol, triacylglycerol, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) in rats fed with cassava without crude oil, cassava with crude oil, and various *Monodora myristica* (Gaertn.) extracts. The serum and liver samples from Group 2 (cassava contaminated with crude oil) and Group 6 (cassava with crude oil plus 1 mL/kg of 5% Tween 80) were compared with Group 1. The samples demonstrated a significant ($p < 0.05$) increase in triacylglycerol, total cholesterol, and low-density lipoprotein cholesterol while the high-density lipoprotein cholesterol was significantly lower.

Group 3 (cassava with crude oil plus aqueous *M. myristica* extract), Group 4 (cassava with crude oil and ethanol *M. myristica* extract), and Group 5 (cassava with crude oil plus a diethyl ether *M. myristica* extract) were compared to Group 2. In contrast, they showed significantly lower levels of triacylglycerol, total cholesterol, and low-density lipoprotein cholesterol, whereas the level of high-density lipoprotein cholesterol increased.

Onuoha & Chukwuma reported that a higher lipid metabolism could induce generation of free radicals [3]. The cholesterol dropped due to the reduction in either the synthesis of cholesterol by hepatocytes or the fractional reabsorption in the small intestine [22].

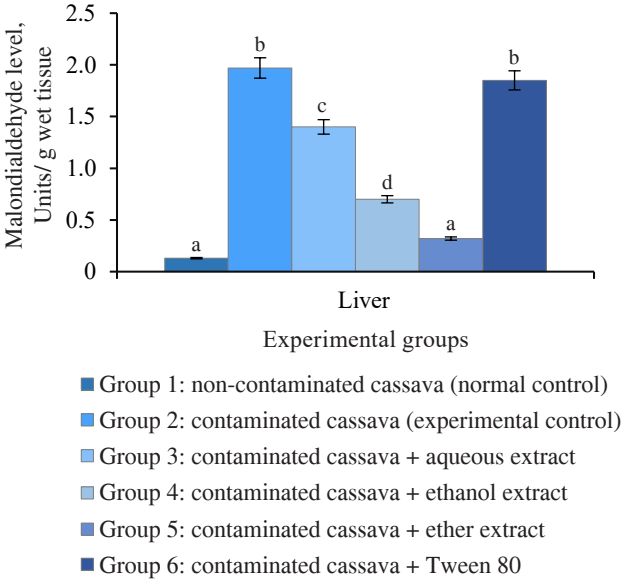
The oil-contaminated cassava might impose a reciprocal relationship between the low and high-density cholesterols in the serum and liver of albino rats. The condition when high-density lipoprotein cholesterol is low and low-density lipoprotein cholesterol is high is the primary risk factor for coronary heart disease [22].

In this study the extracts also reduced the levels of triacylglycerol, total cholesterol, and low-density lipoprotein cholesterol in rats fed with oil-contaminated cassava.

The low total cholesterol could be explained by the substantial amount of phytochemicals in *M. myristica* extracts: for instance, steroids are known to inhibit absorption of dietary cholesterol. Thus, the low total cholesterol in blood serum could arise from the lipid-lowering potential of *M. myristica*.

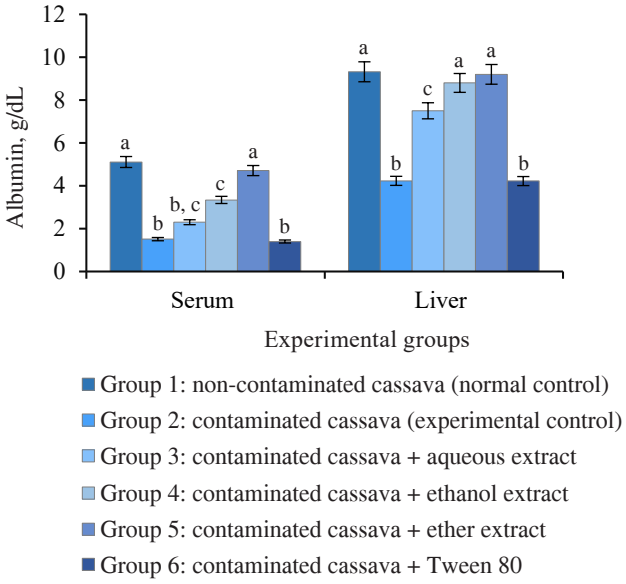
The hypolipidemic mechanism may be explained by the fact that the NADPH-dependent HMG-CoA reductase activity is inhibited in cholesterol biosynthetic pathway. This result is in line with Adeyanju *et al.*, who used *Sesamum indicum* to reduce hyperlipidemia in rats [23]. Likewise, Liu *et al.* reported that antioxidant spices enhanced the plasma lecithin cholesterol acyl transferase and hydroxyl methyl glutaryl CoA reductase, thus causing an improved degradation of cholesterol [22].

***M. myristica* effect on liver in cassava-fed rats.** Free radicals generated by crude petroleum oil have the potential to bind with various proteins or lipids and initiate lipid peroxidation [3, 24]. In the current study, rats fed with nothing but oil-contaminated cassava had significantly ($p < 0.05$) high levels of malondialdehyde in their liver compared to the control. The same was true for Group 6 fed with oil-contaminated cassava and Tween 80 (Fig. 1). The peroxyl radicals generated from crude petroleum oil seemed to initiate the degradation of membrane lipids. The process triggered the generation of lipid peroxides, which, in turn, caused lipid peroxida-



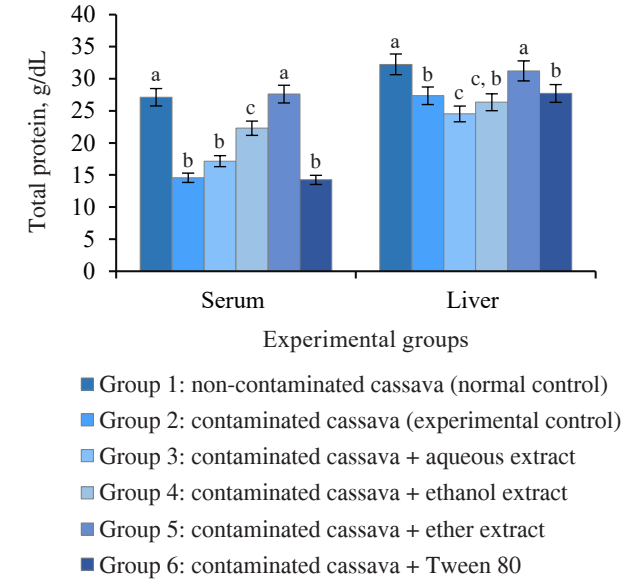
Bars represent mean values from six rats in each group. Bars with different superscripts (a, b, c, d) differed significantly ($p < 0.05$)

Figure 1 Malondialdehyde in the liver of rats fed cassava contaminated with crude oil and various *Monodora myristica* extracts



Bars with different superscripts (a, b, c, d) differed significantly ($p < 0.05$)

Figure 2 Albumin in rats administered cassava and without crude oil and various *Monodora myristica* extracts



Bars with different superscripts (a, b, c, d) differed significantly ($p < 0.05$)

Figure 3 Total protein in rats administered cassava with and without crude oil and various *Monodora myristica* extracts

tion that resulted in a loss of cell membrane integrity and liver injury [3]. The *M. myristica* extracts reduced the level malondialdehyde, i.e., the lipid peroxidation end product. Therefore, the *M. myristica* extracts could minimize lipid peroxidation induced by cassava contaminated with crude oil.

Protein in rats fed cassava with and without crude oil. Figures 2 and 3 show the albumin and total protein in the serum and liver tissue of rats that consumed cassava with and without crude oil. The groups that received oil-contaminated cassava demonstrated a significant decrease in albumin and total protein, compared to rats fed with non-contaminated cassava. The groups that dieted on contaminated cassava with *M. myristica* extracts demonstrated a significant increase in albumin and total protein when compared to the group that received oil-contaminated cassava without additives. The low level of total protein and albumin in serum may be a consequence of poor diet, crude petroleum oil, liver dysfunction, or abnormality in nutrient absorption [25]. Evidently, crude oil might contain toxic compounds such as polycyclic aromatic hydrocarbons, which are important constituent of crude oil [25]. Its effect on the liver could prevent it from synthesizing enough albumins to be released into the serum. Evidently, the extracts of *M. myristica* were able to reverse this effect and improve albumin synthesis.

CONCLUSION

A diet of cassava grown on soils contaminated with crude oil may caused the development of free radicals that coul be responsible for protein and lipid alterations in rats. The administration of various *Monodora myristica* (Gaertn.) extracts reversed the high levels of total cholesterol, triacylglycerol, and low-density lipoprotein cholesterol, thereby preventing chemically-induced dyslipidemia and oxidative damage to hepatocytes. Probably, the free radical scavenging properties of *M. myristica* could shift the demand for lipids as a substrate. Therefore, the inhibited lipid oxidation contributed to the hetoprotective effect against liver damage induced by oil-contaminated cassava. The diethyl ether extract of

M. myristica revealed a better antilipidemic potential than other extracts. Farmers should be warned against planting cassava and other crops in crude oil-contaminated areas because toxic compounds could permeate into the produce. In this research, the negative effect of crude oil-contaminated cassava could be reversed by *M. myristica* in the diet of albino rats.

CONTRIBUTION

J. Okpoghono designed and wrote the draft manuscript. J.K. Ukperegbulem performed the laboratory tests and generated the data. U.B. Igue analyzed the obtained data. The manuscript was approved by all named authors.

CONFLICT OF INTEREST

No conflicts of interest are disclosed by the authors.

DATA AVAILABILITY STATEMENT

The corresponding author will provide the datasets used and/or analyzed during the current work upon reasonable request.

AUTHORS' DECLARATION

The authors hereby declare that the work provided in this article is original and that they will bear all responsibility for any claims connected to its content.

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Effect of drying agents on quality parameters of lyophilized persimmon purée powder

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Abstract:
Persimmon juice has good nutritional composition and high antioxidant properties, however it requires more packing space because of large volume and has limited shelf-life. The research objective was to produce persimmon purée powder with prolonged shelf-life by using various concentrations of drying agents (maltodextrin and Arabic gum).
The control sample was persimmon purée powder without drying agents. Experimental samples included powders with maltodextrin (40, 45, and 50%), Arabic gum (25, 30, and 35%), and the mix of maltodextrin (40%) and Arabic gum (10%). All the experimental samples contained 1% of tri-calcium phosphate as an anti-caking agent. Tests were carried out according to the standard techniques.
The samples with 45 and 50% of maltodextrin had lower moisture, ash, redness (a^*), and hygroscopicity values. These powders demonstrated good yield, solubility, density, and color indices (L^*, b^*, C, H). The persimmon purée powders with 30 and 35% of Arabic gum showed an increase in ash content and total acidity. The samples with 30% of Arabic gum obtained the highest sensory evaluation scores.
The optimal results belonged to the samples of lyophilized persimmon purée powder with 45% of maltodextrin, which will have a longer shelf-life due to its low moisture content.

Keywords: Persimmon puree powder, lyophilization, maltodextrin, Arabic gum, chemical and physical indicators, color indicators

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INTRODUCTION

Fruits and vegetables are an important component of human diet as they are known to reduce the risk of some chronic diseases [1, 2]. Persimmon (*Diospyros kaki* L.) has excellent sensory and nutritional properties, is a source of minerals, carbohydrates, dietary fiber, ascorbic acid, and tannins, not to mention its powerful antioxidant activity [3].

Persimmon fruit contains 76.83% of moisture; its fiber content reaches 13.5% while fats and proteins stay between 0.05% and 1.32%. In addition, 100 g of permon contains 15.60 mg of ascorbic acid [4]. Persimsimmons are a rich source of sugars (12.5 g/100 g wet weight), represented by glucose, fructose, and sucrose [5]. Unfortunately, persimmon fruits have a short shelf-life and require sophisticated preservation techniques to reduce post-harvest losses and develop new products [6]. Nevertheless, persimmons have recently become a popular subject of food science because of their antioxidant, anti-atherosclerosis, and anti-tumor properties [7].

Persimmon juice powder is part of beverage formulations and can serve as a secondary ingredient in confectionary products or baby food. Contemporary studies feature a lot of fruit juice powders, e.g., concentrated apple juice [8]. Modern methods of fruit powder production, e.g., lyophilization, can open persimmon purée powder the way to the global food market.

Lyophilization is currently one of the most important drying methods because it requires low operating temperatures, which reduces thermal damage, thus preserving

the flavor and color indicators of the final product [9]. Lyophilization removes water by converting it first into ice crystals and then into steam. This process includes three stages: freezing, primary drying, and secondary drying. During the freezing stage, the fruit mass turns into solid ice crystals. At the primary drying stage, the ice is removed by sublimation. The secondary drying process means removing the bound and residual water until the required moisture content [10].

Lyophilization yields dried products of high nutritional quality, which are easy to transport, consume, and store. This method maintains high standards of reliability and quality, which makes it possible to preserve vitamins and antioxidants [11].

Dubkova *et al*. wrote that lyophilization products can be used in a wide range of food industries and in a variety of ways; e.g., dairy powders can be part of fast-soluble sauces and desserts due to their low weight and the small size of particles [12].

Fruit powders have many economic advantages over fresh juices, e.g., low weight and volume, less packing space, easy logistics, extended shelf-life, etc. [13].

However, the complex chemical composition of fruit juices limits their powdering options. The problem is that fruit juices contain organic acids, e.g., citric acid, malic acid, tartaric acid, etc., as well as low molecular weight sugars, e.g., glucose and fructose. As a result, they have a low glass transition temperature, which leads to high viscosity, extra hygroscopicity, and low solubility of the resulting powder because viscosity directly affects its fluidity and flowability. Drying agents, such as maltodextrin and gum, can solve this problem [14].

Arabic gum is a dried exudate obtained from acacia trees. The substance owes its wide pharmaceutical and industrial uses to its emulsifying properties. Arabic gum is a complex sugar composed of molecules of calcium, magnesium, and potassium with such sugars as arabinose and galactose. It has a mild taste and no smell [15].

Maltodextrin is a product of starch degradation, which consists of β-D-glucose units. It has high solubility and a pleasant flavor. Maltodextrin is cheap and commercially available, which explains its popularity in the food industry [16, 17].

The season of persimmon fruits is as short as three months, and fresh fruits are difficult to preserve [18]. Sucrose, glucose, and fructose have low molecular weight and low glass transition temperature of 62, 32, and 5°C, respectively [19]. These properties make it difficult to convert persimmons into a powder state. This research featured different concentrations of drying agents with a high glass transition temperature and their effect on powder viscosity, productivity, and agglomeration. We also revealed the quality standards of persimmon purée powder prepared by lyophilization method.

STUDY OBJECTS AND METHODS

The study involved fresh persimmons (*Diospyros kaki* L.). Their chemical composition was as follows: 81.31% of moisture, 0.11% total acidity, pH 6.17, 0.72% ash, and 12.64 g total sugar (100 g wet weight). The fruits were purchased at a local market in the city of Damascus. The research was conducted at the laboratories of the Department of Food Sciences, University of Damascus, in November and December during the ripening season of 2022. Each experiment included 2 kg of persimmon fruit.

Pre-treatment. After removing damaged fruits and impurities, we washed the persimmons in plain water and peeled them with a sharp knife. The peeled fruits were mashed in an electric mixer (Starway 4550D, China) for 3 min.

The persimmon purée was divided into several samples. Sample № 1 served as control and involved pure persimmon purée without any drying agents. Sample № 2 contained persimmon purée with maltodextrin at the concentrations of 40, 45, and 50% plus 1% tri-calcium phosphate as an anti-caking agent. Sample № 3 included persimmon purée with Arabic gum at the concentrations of 25, 30, 35% plus 1% tri-calcium phosphate as an anti-caking agent. Each sample was homogenized separately for 3 min using a mixer (Starway 4550D, China) [20, 21].

Freeze-drying. The persimmon purée mixes underwent drying by the freeze-drying method. After adding drying agents, each sample was frozen at –4°C, then processed in the laboratory lyophilization device at –60°C and a pressure of 0.11 mbar [22]. After that, we ground the dried samples in a mixer (Starway 4550D, China) for 3 min and stored the resulting powder in dark glass containers until further analysis.

Moisture content. The moisture content was determined in line with the method described in Article 925.09 of the Official Methods of Analysis of the Association of Official Analytical Chemists [23]. The samples were dried in a drying oven (Memmert D 91126, Germany) at 105°C until constant weight.

Ash content. We estimated the ash content in line with the method described in Article 923.03 of the Official Methods of Analysis of the Association of Official Analytical Chemists [23]. The samples were dried at 550°C until constant weight.

pH. The acidity was estimated using a pH-meter (Precisa PH-900).

Total acidity. The total acidity was measured by titration with 0.1 N sodium water in the presence of a phenolphthalein indicator [23].

Total sugars. Total sugars were defined according to the Lane-Enyon method as in [23].

Color indices. The color indices (L^*, a^*, b^*, C, H) were mapped using a Hunter Lab tool kit (Biobase, China): L^* (lightness) – the level of light to dark; a^* (red to green) – positive a^+ indicates red; negative a^- indicates green; b^* (yellow to blue) – positive b^+ indicates yellow; negative b^- indicates blue; C – color intensity; H (hue angle) – hue angles of 0, 90, 180, and 270° refer to pure red, pure yellow, pure green, and pure blue, respectively [24].

Process yield. The process yield, %, was determined in line with the equation described by Shuen *et al.* [25]:

$$\text{Yield} = \frac{\text{Dried powder}}{\text{Persimmon purée} + \text{Drying agents}} \quad (1)$$

Water solubility index. We defined the water solubility as in [26]: 1 g of persimmon purée and 10 mL of distilled water were mixed and incubated in a water bath at 37°C for 30 min, then centrifuged for 10 min. After that, we placed the floating part of the juice in Petri dishes of known weight and dried in a drying oven (Memmert D 91126, Germany) at 105°C until constant weight. The solubility of the powder was calculated as the difference in weights.

Hygroscopicity. We determined the hygroscopicity according to the method described by Šavikin *et al.* [27]. We put 1 g of persimmon purée in a petri dish with a saturated solution of sodium chloride with a relative humidity of 75.35% and stored the samples at room temperature for 7 days. The hygroscopicity was expressed as 1 g of absorbed moisture per 100 g dry solids.

Bulk and true density. We followed Igual *et al.* to measure the true and bulk density by placing 2 g of powder sample in a graduated cylinder [28]. The bulk density, g/cm^3, was obtained according to the following Eq. (2):

$$\text{Bulk density} = \frac{\text{Powder weight}}{\text{Powder volume}} \quad (2)$$

After determining the bulk density, the cylinder stayed on a vibrating device until the volume of the powder changed. The density (TRUE, g/cm^3) was calculated according to the following Eq. (3):

$$\text{TRUE} = \frac{\text{Powder weight}}{\text{True volume}} \quad (3)$$

Sensory evaluation. The sensory evaluation involved a group of panelists who used a five-point hedonic scale to evaluate the color, taste, aroma, appearance, and overall acceptability of the persimmon juice reconstituted from the powder samples [29]. The sensory tests featured only the samples with 45% of maltodextrin, 30% Arabic gum, and the mix of maltodextrin (40%) and Arabic gum (10%) because they had a better quality from a physical point of view, i.e., degree of solubility, hygroscopicity, yield, and density.

Statistical analysis. The analysis of variance involved the SPSS software, where the results were analyzed using the One-Way ANOVA test followed by the LSD test to determine the significant differences between the means at a 5% confidence level. The results were recorded as means ± standard deviation.

RESULTS AND DISCUSSION

Effect of drying agents on chemical indicators of lyophilized persimmon purée powder. Table 1 demonstrates the data obtained for moisture, ash, pH, total acidity, and content of total sugars. The measurements clearly indicate a significant effect of the drying agents on the moisture content in the powder. The experimental samples with high concentrations of maltodextrin and Arabic gum showed a significant decrease in the moisture content compared to the control. The lowest total moisture values belonged to the powders with 45 and 50% of maltodextrin, 30 and 35% Arabic gum, and the mix of maltodextrin (40%) and Arabic gum (10%). The moisture contents for these samples were as low as 10.36, 9.33, 10.37, 10.00, and 9.41%, respectively. The concentration of the drying agents added to the purée before the lyophilization increased the percentage of solids, which led to a decrease in the total volume of moisture to be evaporated [30]. The results are consistent with those obtained by Cid-Ortega *et al.*, who added Arabic gum and maltodextrin to powdered *Hibiscus sabdariffa* [31].

The samples with Arabic gum were significantly superior to those with maltodextrin in terms of ash content, which was in the range of 4.20–4.42 g/100 g dry weight, respectively. The ash contents in the samples with maltodextrin were from 1.45 to 2.40 g/100 g dry weight, respectively, while the indicator for the control sample was 2.99. Ali *et al.* reported that maltodextrin contained no minerals [32]. However, Kurniadi *et al.* mentioned that adding Arabic gum increased the ash content in the final product because of its high concentrations of calcium, magnesium, and potassium [33].

Table 1 also illustrates a drop in the pH of the powder with Arabic gum: the sample with 35% of Arabic gum had the lowest pH of 5.85. The samples with various

Table 1 Effect of drying agents on chemical indicators of lyophilized persimmon purée powder

Chemical composition		Moisture, %	Ash, g/100 g dry weight	pH	Total acidity, %	Total sugars, g/100 g dry weight
Control		13.49 ± 0.51^{b}	1.59 ± 0.25^{ab}	5.54 ± 0.02^{a}	0.37 ± 0.05^{ad}	78.19 ± 0.23^{f}
Persimmon puree with Arabic gum	25%	12.33 ± 0.33^{b}	4.35 ± 0.29^{c}	6.07 ± 0.01^{b}	0.31 ± 0.02^{ac}	57.07 ± 0.63^{d}
	30%	10.00 ± 0.57^{a}	4.42 ± 0.29^{c}	6.03 ± 0.02^{b}	0.35 ± 0.03^{a}	50.66 ± 0.33^{b}
	35%	10.37 ± 0.31^{a}	4.20 ± 0.19^{c}	5.85 ± 0.08^{c}	0.44 ± 0.03^{d}	47.66 ± 0.66^{a}
Persimmon puree with maltodextrin	40%	12.76 ± 0.24^{b}	2.40 ± 0.15^{ab}	6.35 ± 0.02^{d}	0.19 ± 0.02^{b}	63.06 ± 0.51^{e}
	45%	10.36 ± 0.31^{a}	2.08 ± 0.11^{ab}	6.33 ± 0.03^{d}	0.22 ± 0.03^{be}	55.32 ± 0.45^{c}
	50%	9.33 ± 0.72^{a}	1.45 ± 0.16^{a}	6.27 ± 0.03^{d}	0.28 ± 0.02^{ba}	49.54 ± 0.86^{b}
Persimmon puree with a mix of maltodextrin and Arabic gum	40:10%	9.41 ± 0.30^{a}	2.99 ± 0.27^{b}	6.25 ± 0.02^{d}	0.31 ± 0.06^{ae}	55.19 ± 0.26^{c}

Similar letters in the same column indicate that there were no significant differences at $p > 0.05$

concentrations of maltodextrin and the mix of maltodextrin and Arabic gum demonstrated no significant differences in pH values. In [34], Arabic gum also proved more effective in decreasing pH than maltodextrin because Arabic gum initially had a lower pH (4.64) than maltodextrin (5.16).

The total acidity was higher in the sample with 35% of Arabic gum, reaching 0.44%, while the samples with maltodextrin resulted in 0.19–0.28% total acidity, respectively.

The increasing concentration of both drying agents decreased the values for total sugars. Grabowski *et al.* reported a similar effect of maltodextrin on the total sugars in sweet potato powder [35].

Effect of drying agents on color indicators of lyophilized persimmon purée powder. Table 2 sums up the obtained values of color indicators. Lightness index *L** increased together with the concentration of the drying agent. The samples with high amounts of the drying agents, namely the powders with 50% of maltodextrin and 35% of Arabic gum demonstrated the highest lightness values, 50.33 and 46.56, respectively. The powders with maltodextrin had superior lightness values compared to those with Arabic gum because maltodextrin is white [36]. The obtained results for the lightness value were consistent with those reported by Suravanichnirachorn *et al.* [37].

As for indicator *a** (red to green), Table 2 shows a significant decrease in the samples with high concentrations of the drying agents. In the sample with 50% maltodextrin, *a** equaled 8.62 whereas the sample with 35% of Arabic gum had 9.05. Such results could be explained by the low concentration of persimmon purée [38].

The value of indicator *b** (yellow to blue) increased together with the concentration of maltodextrin: its highest value reached 45.21 in the sample with 50% maltodextrin. No significant differences in the *b** values were recorded for the samples with Arabic gum compared to the control.

Color intensity C increased following the concentration of maltodextrin, its highest value was in the sample with 50% ramaltodextrin (46.22). The C values decreased as the concentration of Arabic gum rose. The results are similar to those reported in [37].

Hue index H increased together with the concentration of the drying agent. The increase in the color gradient angle indicated that the red color of the powders turned yellow. The results were similar to those obtained by Shishir *et al.*, who reported an increase in the hue value at higher concentrations of maltodextrin [39].

Effect of drying agents on some physical parameters of lyophilized persimmon purée powder. The physical properties of powders depend on many factors, i.e., the drying method used, the drying temperature, and the type and concentration of the agent [40].

Table 3, which sums up the physical profiles of the persimmon powders, shows that a higher concentration of the drying agent caused a significant increase in the yield of persimmon purée powder. The highest yield belonged to the sample with 45 and 50% of maltodextrin: 28.93 and 28.86%, respectively. The sample with the mix of maltodextrin and Arabic gum reached a similar value of 29.33. Adetoro *et al.* also reported that maltodextrin had a greater effect on the yield rate than Arabic gum [41]. In general, powder yield is one of the most important physical indicators in terms of production process and economic efficiency [42].

The solubility index is an important property of powders because it is used as a standard to determine the solubility of a powder in water [43].

The solubility index of the powders rose together with the concentration of the drying agent. Its value increased from 90.92% in the control powder sample to 97.71 and 97.66% in the samples with 45 and 50% of maltodextrin, respectively. The samples with maltodextrin had a greater solubility compared to those with Arabic gum because maltodextrin is highly soluble in water [37].

Hygroscopicity is the ability of powder to attract water molecules from the environment. Fruit powders are highly hygroscopic because they contain glucose and fructose, which have polar ends. This property allows them to interact with water molecules, which increases the hygroscopicity of the product. Low hygroscopicity is important to maintain because it increases the stability of the final product [44].

In our case, the hygroscopicity index decreased when the concentration of the drying agent increased. It dropped to 5.19 and 5.51% in the samples with 45 and 50%

Table 2 Effect of drying agents on color indicators of lyophilized persimmon purée powder

Color		*L**	*a**	*b**	C	H
Control		20.51 ± 0.75^{a}	27.31 ± 0.36^{e}	24.39 ± 0.30^{a}	36.49 ± 0.26^{c}	41.55 ± 0.29^{a}
Persimmon puree with Arabic gum	25%	25.73 ± 0.16^{c}	16.64 ± 0.32^{bc}	23.28 ± 0.21^{a}	28.74 ± 0.37^{b}	53.91 ± 0.30^{e}
	30%	25.51 ± 0.33^{c}	17.60 ± 0.30^{c}	24.03 ± 0.57^{a}	29.83 ± 0.45^{b}	53.49 ± 0.36^{c}
	35%	46.56 ± 0.29^{d}	9.05 ± 0.15^{a}	24.89 ± 0.51^{ab}	26.30 ± 0.46^{a}	70.14 ± 0.48^{e}
Persimmon puree with maltodextrin	40%	22.92 ± 0.54^{b}	23.56 ± 0.29^{d}	26.66 ± 0.24^{b}	35.41 ± 0.30^{c}	47.62 ± 0.31^{b}
	45%	47.17 ± 0.59^{d}	17.01 ± 0.54^{bc}	41.52 ± 0.28^{d}	44.93 ± 0.56^{e}	67.72 ± 0.41^{d}
	50%	50.33 ± 0.33^{e}	8.62 ± 0.31^{a}	45.21 ± 0.38^{e}	46.22 ± 0.40^{e}	70.67 ± 0.30^{e}
Persimmon puree with a mix of maltodextrin and Arabic gum	40:10%	41.93 ± 0.58^{f}	15.77 ± 0.22^{b}	35.55 ± 0.33^{c}	38.90 ± 0.34^{d}	66.71 ± 0.25^{d}

Similar letters in the same column indicate that there were no significant differences at $p > 0.05$

Table 3 Effect of drying agents on some physical indicators of lyophilized persimmon purée powder

Physical indicator		Yield, %	Water solubility, %	Hygroscopicity, %	Bulk density, g/cm^3	True bulk density, g/cm^3
Control		10.88 ± 0.20^a	90.92 ± 0.30^a	15.33 ± 0.33^c	0.25 ± 0.02^a	0.33 ± 0.05^a
Persimmon puree with Arabic gum	25%	20.33 ± 0.33^b	94.50 ± 0.28^b	13.14 ± 0.45^d	0.29 ± 0.03^a	0.33 ± 0.03^a
	30%	26.30 ± 0.65^c	93.97 ± 0.20^b	10.33 ± 0.33^c	0.44 ± 0.02^b	0.48 ± 0.01^{ab}
	35%	26.33 ± 0.33^c	94.33 ± 0.33^b	7.33 ± 0.33^b	0.44 ± 0.02^b	0.48 ± 0.02^a
Persimmon puree with maltodextrin	40%	21.33 ± 0.42^b	94.58 ± 0.34^b	8.46 ± 0.29^b	0.27 ± 0.01^a	0.33 ± 0.03^b
	45%	28.93 ± 0.54^d	97.71 ± 0.28^c	5.19 ± 0.42^a	0.47 ± 0.01^b	0.55 ± 0.06^b
	50%	28.86 ± 0.50^d	97.66 ± 0.33^c	5.51 ± 0.28^a	0.46 ± 0.05^b	0.54 ± 0.02^b
Persimmon puree with a mix of maltodextrin and Arabic gum	40:10%	29.33 ± 0.33^d	96.98 ± 0.56^c	4.50 ± 0.28^a	0.43 ± 0.03^b	0.48 ± 0.05^{ab}

Similar letters in the same column indicate that there were no significant differences at $p > 0.05$

Table 4 Effect of drying agents on sensory properties of juices reconstituted from lyophilized persimmon purée powder

	Taste	Color	Aroma	Appearance	Overall acceptability
Control	2.04 ± 0.40^a	1.33 ± 0.33^a	2.03 ± 0.22^a	1.33 ± 0.33^a	1.80 ± 0.19^a
Persimmon puree with 45% maltodextrin	3.34 ± 0.33^{bc}	3.02 ± 0.28^b	3.23 ± 0.42^a	2.42 ± 0.29^b	3.01 ± 0.15^b
Persimmon puree with 30% Arabic gum	4.33 ± 0.33^c	4.57 ± 0.29^c	3.26 ± 0.33^a	4.09 ± 0.20^c	4.42 ± 0.29^c
Persimmon puree with a mix of maltodextrin and Arabic gum (40:10%)	2.71 ± 0.45^{ab}	2.42 ± 0.25^b	2.96 ± 0.50^a	2.33 ± 0.33^b	2.07 ± 0.30^a

Similar letters in the same column indicate that there were no significant differences at $p > 0.05$

of maltodextrin, respectively, and to 4.50% in the mix of maltodextrin and Arabic gum, compared to the control, where it was 15.33%. The powders with maltodextrin had lower hygroscopicity values compared to those with Arabic gum, because maltodextrin has a higher glass transition temperature [41]. In addition, Arabic gum contains free hydroxyl groups that can bind water molecules more easily [45].

Bulk density is one of the most important physical properties for storage, packaging, and transportation of food powders. Powders with high density are more commercially advantageous because they reduce shipping and storage costs [19].

The drying agents had a significant effect on the bulk density of the persimmon powders. The samples with 45 and 50% of maltodextrin had the highest bulk density values, which amounted to 0.47 and 0.46 g/cm, respectively. No significant differences in the bulk density were registered between the samples with 30 and 35% of Arabic gum, 45 and 50% of maltodextrin samples, and the mix of maltodextrin and Arabic gum. The results are consistent with those obtained by Nthimole *et al.* [43].

Table 3 demonstrates a significant effect of the drying agents on the true density values of the persimmon powders. The samples with 45 and 50% of maltodextrin showed the highest true density values.

Effect of drying agents on sensory properties of lyophilized persimmon purée powder. Table 4 summarizes the results of the sensory evaluation of persimmon juice reconstituted from the lyophilized powders. The type of drying agent had a significant effect on the taste of the final product. The juice prepared from the powder with 30% of Arabic gum had the best score for taste, followed by juice samples prepared from the maltodextrin samples. The lowest score belonged to the samples prepared from the powder with the mix of maltodextrin and Arabic gum and the control sample. In terms of color, appearance, and overall acceptability, the juice prepared from powder with 30% of Arabic gum achieved the highest scores for acceptance, followed by the sample with 45% of maltodextrin. The scores for aroma demonstrated no significant differences between the control and the experimental samples: the drying agents had no aroma which could affect the sensory profile of the final product [46].

CONCLUSION

Drying agents with a high glass transition temperature proved effective in the production of high-quality persimmon purée powder.

The lyophilized powders with 45 and 50% of maltodextrin had a much lower moisture content, redness (a^*), and hygroscopicity, as well as greater color indicators L^*, b^*, C, and H. These samples also demonstrated good yield rate, solubility index, and true and apparent density. The samples with 30% of Arabic gum had a high ash content and better sensory characteristics compared to the other samples.

Maltodextrin at a concentration of 45% with 1% anticaking agent could be recommended for commercial use because it improved the physical properties of persimmon purée powder and reduced its moisture content, thus increasing its shelf-life.

CONTRIBUTION

All authors participated equally to the research and the manuscript.

CONFLICT OF INTEREST

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

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Cardiorenal protective effects of extracts of bitter leaf (*Vernonia amygdalina* L.) in animal model of metabolic syndrome

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Abstract:
Every year, metabolic syndrome and cardiorenal diseases cause many deaths worldwide. African bitter leaf (*Vernonia amygdalina* L.) is known for its numerous therapeutic effects. Potentially, it can lower plasma lipid and glucose levels, which, in turn, may improve the condition of patients with the abovementioned diseases. This research featured the antihyperlipidemic and antihyperglycemic effects of methanol extract of *V. amygdalina* in an animal model of metabolic syndrome.
Twenty albino rats were divided into four groups. Groups A to C were orally administered with ghee (3 mL/kg) + high-cholesterol diet (500 mg/kg) + high-sugar diet (10 mL/kg) to induce metabolic syndrome. Group A served as negative control and received no treatment with bitter leaf methanol extract. Groups B and C received 200 and 400 mg/kg of *V. amygdalina* methanol extract, respectively. Group D received no administration. The cardiorenal injuries and alterations in blood lipids and sugar levels were assessed via various biochemical analyses.
The combination of ghee + high-cholesterol diet + high-sugar diet triggered a significant elevation of creatine kinase myocardial band, lactate dehydrogenase, aspartate aminotransferase, triglycerides, total cholesterol, low density lipoprotein-cholesterol, glucose, urea, creatinine, and potassium levels. The histopathological results agreed with the biochemical findings. However, the treatment with 200 and 400 mg/kg of *V. amygdalina* methanol extract was able to inhibit the adverse alterations causing a dose-dependent significant antihyperlipidemic and antihyperglycemic effect ($p < 0.05$).
Bitter leaf (*V. amygdalina*) demonstrated cardiorenal protective effects and may be used to manage metabolic syndrome.

Keywords: Bitter leaf, *Vernonia amygdalina*, methanol extract, metabolic syndrome, animal model, hyperlipidemia, hyperglycemia

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INTRODUCTION

Metabolic syndrome is an umbrella term for such disorders as hyperlipidemia, hyperglycemia, hypertension, and obesity, rather than a single condition [1]. As a result, it is among the biggest causes of morbidity and mortality worldwide [2]. In economically developing countries like Nigeria, inadequate early diagnosis and poor management often lead to a sequence of complications and death. Metabolic syndrome triggers a group of risk factors that, if not addressed properly, can lead to more serious metabolic problems, such as Type II diabetes and nonalcoholic fatty liver disease [3–5].

Sedentary lifestyle and consumption of low-fiber, high-fat food products are two primary contributors to this condition [2]. However, the pathogenic processes behind metabolic syndrome remain unclear and understudied. For instance, scientists cannot explain whether its numerous symptoms are evidence of separate diseases or of a single, shared pathogenic mechanism. The enormous geographic heterogeneity of metabolic syndrome highlights the extent to which social variables, including consuming too many calories and not exer cising enough, contribute to its etiology [6]. Most of the mechanisms involved in metabolic syndrome are primarily triggered by visceral fat, which means that a high calorie intake is a primary cause of disease [7]. Out of all the potential mechanisms, chronic inflammation, neurohormonal activation, and insulin resistance seem

to be the key elements in the onset, development, and transformation of metabolic syndrome into cardiovascular diseases (Fig. 1) [8].

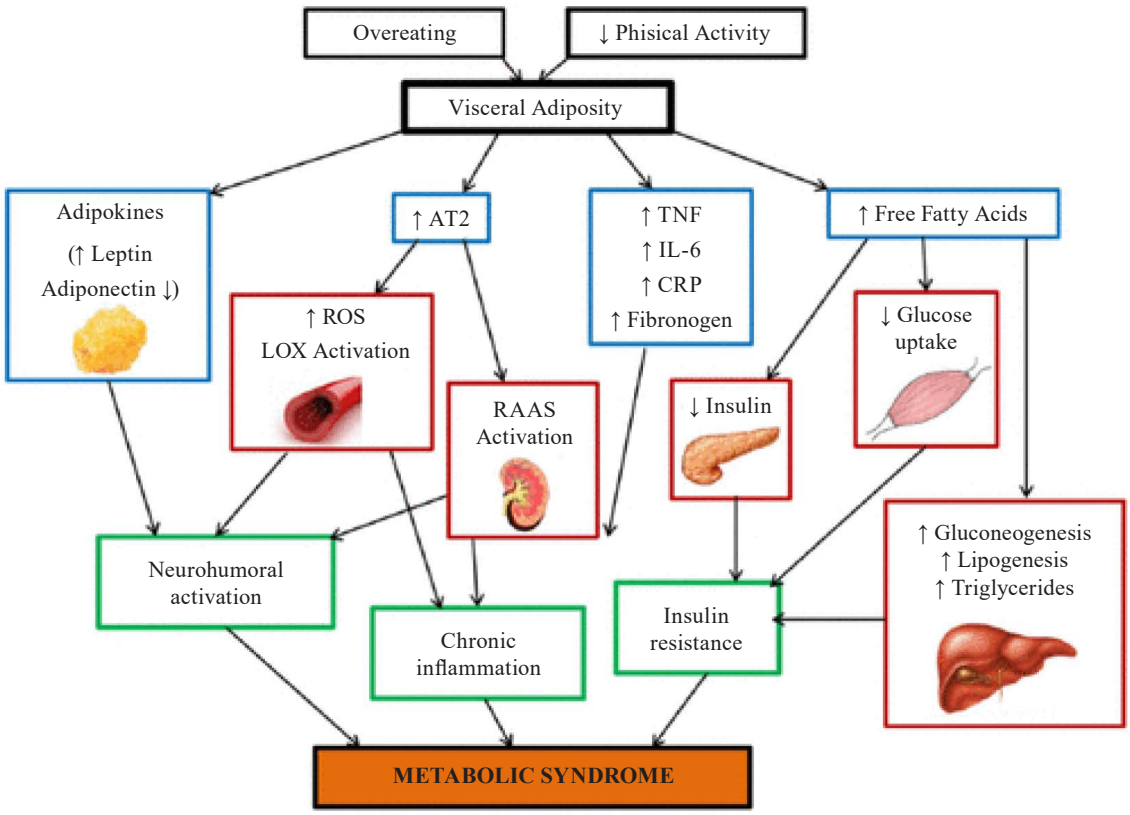
Numerous studies have established that metabolic syndrome can cause alterations in the cardiovascular system and renal structure [10]. Cardiovascular and kidney diseases are among the foremost causes of mortality in the world, despite the rapid progress in medical care [11]. Heart failure, hypertension, chronic kidney disease, high cholesterol, Type II diabetes, and cardiorenal metabolic disease are interconnected medical conditions [12, 13]. All these illnesses are associated with systemic inflammation, central obesity, and insulin resistance. Diets that are rich in fat and sugar cause degeneration in heart, kidney, and liver tissues [3, 14].

According to established scientific principles, a medical food is defined as a food prescribed by a physician and prepared to be eaten or delivered enterally. This food is designed specifically for dietary treatment of a particular medical condition or disease for which scientifically-proven dietary requirements are available [15].

Extracts of bitter leaf (*Vernonia amygdalina* L.) are known to have nutritional and therapeutic benefits that depend on its constituents [16–18]. Bitter leaf is a shrub or a small tree that can grow to be 23 feet tall when fully developed. Bitter leaf has flaking and gritty grey or brown bark. *V. amygdalina* is a native African plant that can be found throughout Sub-Saharan Africa. Bitter leaf has long been used as a medicinal herb against renal toxicity and oxidative stress [18]. In addition, it contains vitamins and trace elements that minimize oxidative stress and promote healing. Cu, Fe, and Zn values in *V. amygdaline* were reported as 0.0375, 0.5352, and 0.1916 mg/kg, respectively [19]. African bitter leaf is a popular food component in eastern Nigeria. Tradition has it that bitter leaf is good for hyperglycemic patients due to its bitter taste, or that it can reduce cholesterol and relieve fever, joint pain, intestinal colic, stomach ache, and malaria. The total effect of the bitter leaf can be determined experimentally by analyzing various biochemical parameters.

This research featured the lipid profile and glucose level in a rat model with hyperlipidemia and hyperglycemia after treatment with *V. amygdalina* extract. We defined metabolic syndrome as three abnormal outcomes from five components: increased triglycerides, increased blood pressure, decreased high-density lipoprotein-cholesterol, increased fasting plasma glucose, and increased waist circumference [20].

We believe that a locally available herbal product derived from bitter leaf may be an effective solution to the metabolic syndrome epidemic, which affects population of all age in a country where most people cannot afford drugs required for the treatment and control of obesity, hypertension, and other complications.



CRP – C-reactive protein; ROS – reactive oxygen species; IL-6 – interleukin 6; LOX – lectin-like oxidized low-density lipoprotein; AT2 – angiotensin II type 2 receptor; RAAS – renin-angiotensin-aldosterone system; TNF – tumor necrosis factor

Figure 1 Pathophysiology of metabolic syndrome as adapted from [9]

STUDY OBJECTS AND METHODS

Plant raw materials. Fresh *Vernonia amygdalina* L. leaves were purchased on the Ogbete market, Enugu, Nigeria. A consulting taxonomist verified their authenticity. A voucher specimen (no. PCG/UNN/030) was deposited at the Department of Pharmacognosy and Environmental Medicines, University of Nigeria, Nsukka.

Chemicals and reagents. Vegetable oil ghee, coconut oil, sucrose, and cholesterol powder were acquired from the Ogbete Market, Enugu, Nigeria. All reagents were obtained from Randox Laboratory Ltd., UK.

High-fat, high-sucrose diet for inducing metabolic syndrome. We mixed vegetable oil ghee and coconut oil in a 3:1 (v/v) ratio to create a high-fat diet which we administered orally to rats at a dose of 3 mL/kg per day for 28 days. The high-sugar diet of 30% sucrose solution was given orally to rats at a dose of 10 mL/kg per day for 28 days. The high-cholesterol diet involved 50 g of readily available cholesterol powder plus 9 g of sodium deoxycholate with bile salt to improve bioavailability. The components were mixed together and dissolved in coconut oil before being diluted to 200 mL with coconut oil to produce 250 mg/mL. The rats received it at the amount of 500 mg/kg per day for 28 days.

Laboratory animals. Twenty adult albino Wistar rats (110 ± 10 g) were procured from the College of Veterinary Medicine, University of Nigeria. The rats lived in a metallic cage with a regular temperature of 22 ± 3°C and a 12-h light-dark cycle. The animals were monitored for 14 days prior to the experiment date in order to allow them to acclimatize to the environment. The experimental design and management complied with the institutional guidelines for the care and use of laboratory vertebrates published by the American Physiological Society [21].

Preparing the plant extracts. A 400-g fresh plant of *V. amygdalina* was cleaned, cut into little pieces, and fully homogenized in a Waring blender. The final mix was immersed in 2 L of 80% methanol followed by mixing at room temperature for 24 h while being shaken intermittently. We used a low-pressure rotary evaporator to concentrate the filtrate to dryness at 40°C after filtration. After that, we extracted the residue once more in line with the same method. The entire amount of methanol was extracted from the collected filtrates using a rotary evaporator, and then the resulting crude extract was made and stored at 4°C until use.

Phytoconstituents analyses of bitter leaf. The sample of *V. amygdalina* was analyzed for flavonoids, glycosides, saponins, tannins, steroids, proteins, carbohydrates, and terpenoids at the Department of Pharmacognosy, University of Nigeria, Nsukka. We followed the methodology developed by Trease & Evans [22].

Acute toxicity. Tijjani *et al.* wrote that the intraperitoneal LD50 of ethanolic *V. amygdalina* leaf extract was 3721 mg/kg [23]. They reported no deaths after administering 5000 mg/kg extract orally, which means that the extract was deemed safe to use. In the current study, we applied methanol extract of bitter leaf as high and low doses of 400 and 200 mg/kg, respectively.

Experimental design. We divided 20 albino rats into four groups (A–D) of five rats in each. They received the following treatments for 28 days:

1. Group A (negative control) received ghee (3 mL/kg) + high-cholesterol diet (500 mg/kg) + sucrose solution (10 mL/kg), orally;

2. Group B ras were given ghee (3 mL/kg) + high-cholesterol diet (500 mg/kg) + sucrose solution (10 mL/kg),and low dose of *V. amygdalina* methanol extract (200 mg/kg), orally;

3. Group C rats were subjected to oral administration of ghee (3 mL/kg) + high-cholesterol diet (500 mg/kg) + sucrose solution (10 mL/kg), and high dose of *V. amygdalina* methanol extract (400 mg/kg); and

4. Group D (normal control) received no special treatment.

Sample collection. Blood samples for biochemical analysis were taken from the left ventricle of the heart under chloroform anesthesia. Heart and kidney tissues were excised for histopathological analyses.

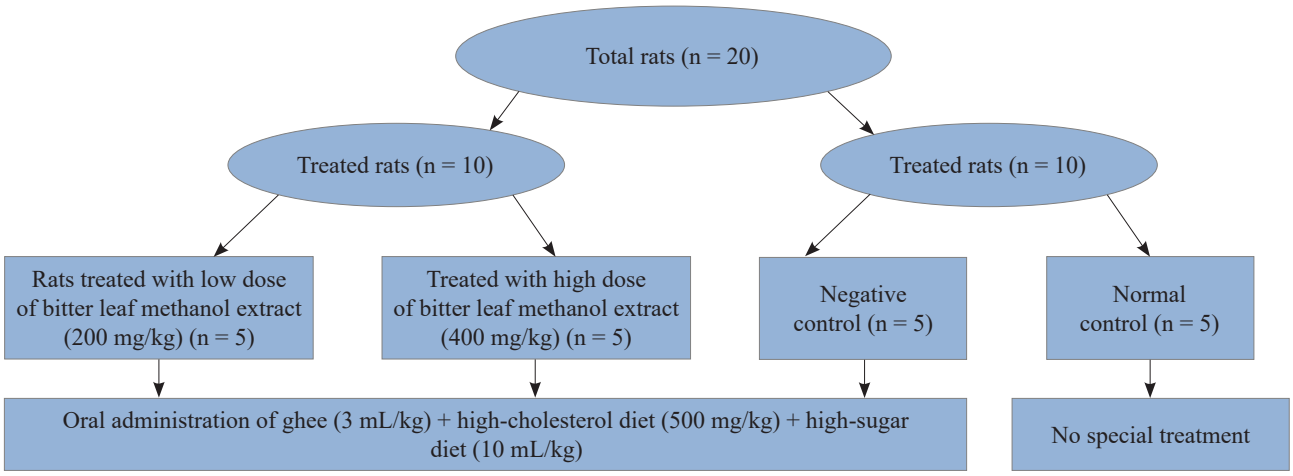


Figure 2 Research design: flow chart

Biochemical analyses. ***Serum lipid profile.*** We followed the cholesterol oxidase method described by Fredrickson *et al.* to measure total cholesterol [24]. The precipitation technique to determine high-density lipoprotein-cholesterol was described by Albers *et al.* [25]. The triglyceride test was in line with the glycerol phosphate oxidase method specified in [26]. The low-density lipoprotein-cholesterol was calculated using the Friedewald formula: Low-density lipoprotein-cholesterol = Total cholesterol − (Very low-density lipoprotein-cholesterol + High-density lipoprotein-cholesterol) [27].

Plasma glucose. The blood glucose levels were determined using the glucose oxidase method as in [28].

Measuring the cardiac biomarkers. The level of creatine kinase myocardial band was determined in line with the kinetic colorimetric technique [29]. A Randox kit made it possible to measure the lactate dehydrogenase. We adopted Reitman & Frankel's colorimetric approach to determine the aspartate transaminase [30].

Measuring the renal function biomarkers. Electro lyte, urea, and creatinine levels in the blood were measured to assess the renal function. A Perlong Medical PL1000A electrolyte analyzer served to study serum K^+ and Na^+. The urea level was estimated using diacetyl monoxime technique with protein precipitation, while the creatinine level was estimated by the Jaffe creatinine method as described in [31, 32].

Histopathological analysis. The paraffin wax embedding method was employed to prepare the removed heart and kidney tissues. The sections of 5 μm were subjected to the hematoxylin and eosin staining and examined under an Olympus™ light microscope [33].

Statistical analysis. The data analysis involved Graph Pad Prism 7.0 (San Diego, CA, USA). The results of the biochemical experiments were presented as mean ± SEM (standard error of mean). We used the one-way analysis of variance (ANOVA) to determine the degree of significance with probability levels below 0.05 ($p < 0.05$) as significant.

RESULTS AND DISCUSSION

Phytochemical results. Table 1 illustrates the phytochemical examination of *Vernonia amygdalina* L. methanol extract, which appeared to be rich in alkaloids. Carbohydrates, glycosides, saponins, and tannins were present in moderate amounts. Flavonoids and resins were detected in trace amounts while proteins, acidic compounds, oils, terpenoids, and steroids were found absent.

Biochemical results. This experiment featured the antihyperlipidemic and antihyperglycemic benefits of bitter leaf extract in albino Wistar rats with high-fat and high-glucose metabolic syndrome. The negative control rats that received ghee (3 mg/kg), high-cholesterol diet (500 mg/kg), and 30% sucrose solution (10 mg/kg) had the greatest levels of blood triglycerides, total cholesterol, and low-density lipoprotein (Table 2). This combination was able to induce significant dyslipidemia, thus giving the rats metabolic syndrome.

Low and high doses of bitter leaf methanol extract reduced triglyceride, total cholesterol, and low-density lipoprotein compared to the normal control group. Our results were comparable with those reported by Ogunrinola *et al*., who detected a significant ($p < 0.05$) hypolipidemia in rats treated with bitter leaf extract at all doses [34]. Ogbuabu *et al.* also posited that *V. amygdalina* leaves could substantially reduce triglyceride and total cholesterol, with no effect on high-density lipoprotein-cholesterol [35].

The negative control rats that received a combination of ghee + high-cholesterol diet + high-sugar diet showed the highest plasma glucose levels (Fig. 3). Therefore, the treatment with ghee, high-cholesterol, and sucrose solution induced hyperglycemia, i.e., a metabolic syndrome condition, in the rats.

Table 1 Phytochemical analysis of *Vernonia amygdalina* L.

Compounds	Indication
Saponins	Moderately present
Reducing sugar	Absent
Alkaloids	Abundantly present
Tannins	Moderately present
Carbohydrates	Moderately present
Glycosides	Moderately present
Resins	Present (in trace amounts)
Flavonoids	Present (in trace amounts)
Steroids	Absent
Terpenoids	Absent
Oils	Absent
Acidic compounds	Absent
Proteins	Absent

Table 2 Serum lipid profile parameters of all experimental groups vs. normal control

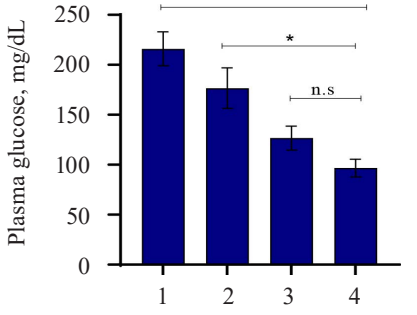
Groups	Triglyceride, mg/dL	Total cholesterol, md/dL	High-density lipoprotein, mg/dL	Low-density lipoprotein, mg/dL
Ghee + high-cholesterol diet + high-sugar diet (negative control)	131.00 ± 9.74**	74.13 ± 5.59*	20.50 ± 1.31*	27.43 ± 6.28**
Ghee + high-cholesterol diet + high-sugar diet + bitter leaf extract (low dose)	122.25 ± 8.31*	70.63 ± 6.55	21.25 ± 1.75	24.93 ± 5.96*
Ghee + high-cholesterol diet + high-sugar diet + bitter leaf extract (high dose)	105.63 ± 8.43	67.13 ± 5.17	24.25 ± 2.66	18.55 ± 7.94
Normal control	70.50 ± 7.21	57.75 ± 4.53	25.00 ± 2.39	17.90 ± 4.82

Compared to the normal control, ** ($p < 0.01$) or * ($p < 0.05$) were significant

In comparison with the normal control, the rats treated with low or high doses of bitter leaf extract demonstrated non-significant and significant declines in plasma glucose levels, respectively. This result also corroborates with other publications that reported the hypoglycemic properties of bitter leaf extract [36]. For instance, Olooto *et al.* studied the hypoglycemic potential of *V. amygdalina* in albino rats fed with a high-sucrose diet. In their study, the mean plasma glucose level was significantly elevated ($p < 0.05$) in the rats that followed a high-sucrose diet while those treated with bitter leaf demonstrated a small decrease [37]. Bawa & Ayobola proved the antidiabetic and anti-hyperlipidemic properties of *V. amygdalina* methanol extract [38]. Probably, that is why bitter leaf has historically been used to combat diabetes mellitus and its complications [39]. This beneficial effect may be attributed to such bioactive phytoconstituents as alkaloids, tannins, saponin, glycosides, and flavonoids [40].

Figure 3 clearly demonstrates that the bitter leaf extract was able to reduce blood glucose levels in a dose-dependent manner.

The negative control rats that received ghee + high-cholesterol diet + sucrose solution showed a statistically significant ($p < 0.05$) elevated levels of creatine kinase



1 – Chee + HCD + SS
2 – Chee + HCD + SS + MEVA (Low dose)
3 – Chee + HCD + SS + MEVA (High dose)
4 – Normal Control

MEVA – *V. amygdalina* methanol extract; HCD – high-cholesterol diet; SS – sucrose solution, i.e., high-sugar diet.

** ($p < 0.01$) or * ($p < 0.05$) were significant

Figure 3 Plasma glucose in experimental groups vs. normal control

myocardial band, low-density cholesterol, and aspartate transaminase when compared to the high-dose treatment group and the normal control, separately. Evidently, the combination of ghee, high-cholesterol diet, and sucrose solution could induce cardiac muscle damage in the rats. Both the low and high dose treatments caused a significant reduction of serum creatine kinase myocardial band, low-density cholesterol, and aspartate transaminase, compared to the negative control (Table 3).

Our finding is consistent with that made by Wijaya *et al.*, who also reported that rats treated with 500 mg/kg of ethanolic extract of *V. amygdalina* had the lowest levels of creatine kinase myocardial band and low-density cholesterol [41]. Syahputra *et al.* similarly observed that rats treated with *V. amygdalina* had lower creatine kinase myocardial band, low-density cholesterol, and aspartate transaminase [42]. The cardioprotective effect of bitter leaf ethanol extract could be attributed to luteolin. This flavonoid inhibits apoptosis, improves systolic and diastolic function of the heart, and potentiates nitric oxide synthesis, thereby improving the overall function of the heart [43].

The negative control rats that received a combination of ghee + high-cholesterol diet + sucrose solution for 28 days showed a significant ($p < 0.05$) increase in blood urea nitrogen, creatinine, and potassium (K^+), compared to the high-dose treatment group and the normal control, separately. Obviously, the combination of ghee, high-cholesterol diet, and sucrose solution was able to induce renopathy secondary to metabolic syndrome in the rats. When compared to the negative control, the treatment with both low and high doses of bitter leaf extract lowered the levels of blood urea nitrogen, creatinine, and potassium (K^+) (Table 4).

Our renal assessment was similar to that published by Barnes *et al.*, who fed renal dysfunctional rats with *V. amygdalina* extract for 3 weeks to achieve normal restoration of creatinine and urea level [44]. Onwubiko *et al.* also treated rats with 400 mg/kg of *V. amygdalina* extract and reported lower urea and creatinine levels [45].

Histopathological results. In this research, metabolic syndrome damaged cardiac muscles, which could potentially lead to a variety of cardiovascular diseases. Figure 4 shows the photomicrograph of heart sections in the experimental groups following the treatments. The rats that received no special treatment showed

Table 3 Cardiac biochemical markers of damage parameters in experimental groups vs. normal control

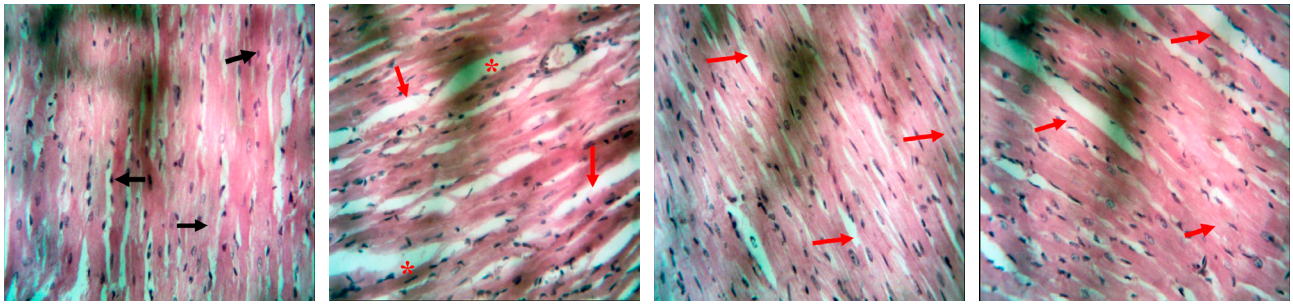
Groups	Creatine kinase myocardial band, U/L	Low-density cholesterol, U/L	Aspartate transaminase, U/L
Ghee + high-cholesterol diet + high-sugar diet	216.89 ± 17.87**	269.87 ± 15.01**	115.69 ± 9.26**
Ghee + high-cholesterol diet + high-sugar diet + bitter leaf methanolic extract (low dose)	169.37 ± 4.59*	219.12 ± 9.03	82.72 ± 9.58
Ghee + high-sugar diet + bitter leaf methanolic extract (high dose)	156.01 ± 7.71	211.24 ± 11.12	72.71 ± 4.62
Normal control	146.24 ± 11.05	208.45 ± 14.78	67.59 ± 7.13

Compared to the normal control, * ($p < 0.05$) and ** ($p < 0.01$) were significant

Table 4 Serum renal biochemical parameters in experimental groups vs. normal control

Groups	Blood urea nitrogen, mg/dL	Creatinine, mg/dL	K^+, mmol/L	Na^+, mmol/L
Ghee + high-cholesterol diet + high-sugar diet	37.92 ± 4.21	1.62 ± 0.04	7.68 ± 0.09	135.41 ± 0.23
Ghee + high-cholesterol diet + high-sugar diet + bitter leaf methanolic extract (low dose)	21.75 ± 3.48*	1.04 ± 0.05*	5.92 ± 0.24	140.02 ± 3.10
Ghee + high-sugar diet + bitter leaf methanolic extract (high dose)	21.68 ± 1.09*	0.92 ± 0.19*	6.19 ± 0.76*	143.88 ± 0.35*
Normal control	20.62 ± 1.31*	0.89 ± 0.30*	5.83 ± 0.12*	145.25 ± 1.42*

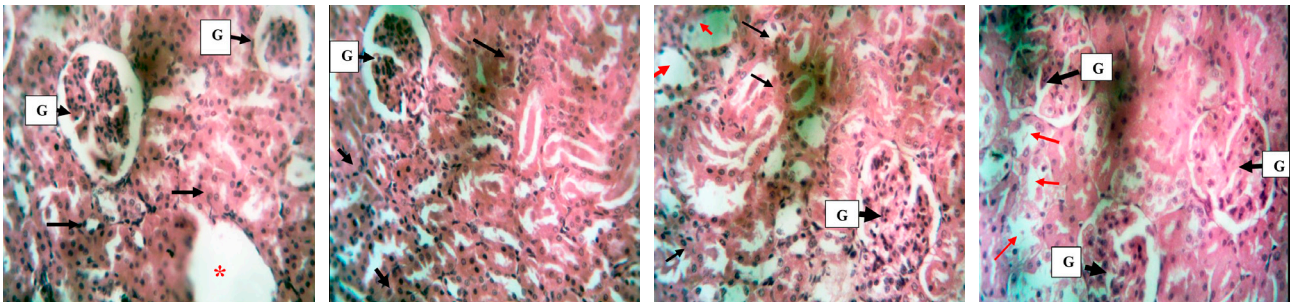
Compared to the group of ghee + high-cholesterol diet + high-sugar diet, *($p < 0.05$) was significant



a b c d

* – shows the evidence of oedema. The black arrows point at myocardial fibers undergoing degenerative changes while the red arrows mark undamaged tissues

Figure 4 Heart tissues of rats in different experimental groups (A–D): a representative micrograph (staining: hematoxylin and eosin; magnification 400×)



a b c d

* – marks mildly constricted or eroded glomeruli (G). The black arrows point at normal tubules or those with intraluminal eosinophilic casts. The red arrows mark tubules with degenerated epithelia

Figure 5 Kidney tissues of rats in different experimental groups (A–D): a representative micrograph (staining: hematoxylin and eosin; magnification 400×)

unaffected myocardial fibers (Group D). The myocardial fibers of the negative control rats that received oral administration of ghee + high-cholesterol diet + sucrose solution showed some degenerative changes, e.g., poor striations (Group A). However, the rats subjected to the low dose of *V. amygdalina* treatment demonstrated normal myocardial fibers (Group B) with some evidence of edema seen as increased spaces between the fibers. Furthermore, the rats that received the high dose of *V. amygdalina* extract had normal-looking myocardial fibers (Group C).

The histopathological findings were in tandem with the biochemical results. This result was similar to the research conducted by Syahputra *et al.*, where *V. amygdalina* extract also reduced cardiac degeneration [42].

In Fig. 5, the glomeruli appeared normal while some tubules seemed to have slightly degenerated epithelia in the untreated rats (Group D). The kidney section of the negative control rats that received ghee + high-cholesterol diet + sucrose solution showed some constricted or eroded glomeruli while the tubules appeared normal (Group A). However, the rats that were treated with the low-dose of bitter leaf extract demonstrated standard glomeruli while occasional tubules looked mildly eroded, some with intraluminal eosinophilic casts (Group B). The high-dose group had normal

glomeruli while the tubules appeared eroded with occasional intraluminal eosinophilic casts (Group C).

This histopathological finding showed a dose-dependent effect, with the high dose having a better renal protective function than the low one. This result was in tandem with the study published by Barnes *et al*., where *V. amygdalina* extract improved the renal tissues of rats damaged by heavy metals [44]. The positive effect could be attributed to vitamins, antioxidants, and minerals in the plant.

CONCLUSION

The methanol extract of *Vernonia amygdalina* L. reduced hyperlipidemia and hyperglycemia in rats with metabolic syndrome caused by increased glucose and fat intake. In the test group, the bitter leaf extract ameliorated the negative effects. Its anti-cardiotoxic properties provided important protection against cardiovascular diseases. In sufficient doses, *V. amygdalina* demonstrated a renoprotective effect.

CONTRIBUTION

The authors are equally liable for any plagiarism because they all contributed equally to writing the content.

CONFLICT OF INTEREST

There are no disclosed conflicts of interests regarding the publication of this article.

ETHICAL CONSIDERATIONS

Ethical approval was issued by the Ethics Committee of the Department of Veterinary Medicine, University of Nigeria (Approval number: UNN/eTC/14/67485).

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The elemental profile of ciders made from different varieties of apples

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Abstract:
Macro- and microelements are vital components of the nutrient profile of apples and apple juice. Although the mineral composition of apple juices has been well studied, there is a lack of research into the elemental profile of ciders. We aimed to determine the concentrations of macro- and microelements in various samples of ciders.
We studied 25 experimental ciders from apple juice of direct extraction (fresh must) and 4 commercial ciders purchased from a retailer in Krasnodar. Mass concentrations of metal cations were determined by high-performance capillary electrophoresis, atomic absorption spectrometry with electrothermal atomization, and atomic emission spectrometry with inductively coupled plasma.
The concentrations of macroelements in the ciders from fresh must depending on the variety varied significantly in the following ranges (mg/L): 696–1920 for potassium; 6.7–26.8 for sodium; 4.3–35.5 for calcium; and 10.2–36.8 for magnesium. The commercial ciders had significantly lower concentrations of macroelements. The content of iron ranged from 0.86 to 2.26 mg/L. Among microelements, copper cations were detected in the range from 31.0 to 375 μg/L. The concentrations of toxic elements did not exceed the maximum permissible values in any of the samples, including the commercial ones. Finally, ranges of variation were established in the concentrations of macro- and microelements depending on the varietal characteristics of apples.
The pomological varieties of apples used in the study were grown under the same agrotechnical conditions. Therefore, the differences revealed in the elemental profile of the ciders were assumingly due to the genetic characteristics of the respective variety.

Keywords: Apple varieties, microelements, macroelements, concentration ranges, cider

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INTRODUCTION

According to numerous studies, the nutrient profile of apples and apple juice contains macroelements (potassium, calcium, magnesium, and sodium) and microelements (iron, zinc, copper, iodine, manganese, molybdenum, and fluorine). Their concentrations vary depending on the variety and location of the apple tree [1–4]. A number of studies also report the presence of so-called ultraelements in apples, including selenium, cobalt, and chromium [5, 6].

Fully or partially fermented apple juice is used to produce ciders – pleasantly refreshing beverages with a low alcohol content. Recent years have seen a growing consumer interest in cider in many European countries (France, Spain, Austria, Germany, Switzerland) and America. During alcoholic fermentation, the juice's profile undergoes significant changes in the concentrations of some elements, which is associated with the use of clay minerals for physical and chemical treatments [7–11]. Ciders are rich in various micro- and macro elements that come from apples, which, in their turn, obtain them directly from the soil. Therefore, their diversity largely depends on the mineral composition of the soil. Apple trees growing in calcic or siliceous soils contain calcium or silicon, which can sometimes be felt in the taste of cider.

Ciders also vary depending on the maturity of apples (more mature fruits are richer in microelements), the growing method (with or without mineral fertilizers), and processing technology. Although apple juices have been widely studied for their mineral composition, there has been insufficient research into the elemental composition of ciders [3, 12, 13]. Yet, it is important to know metal concentrations in cider not only to assess potential health risks for consumers, but also to evaluate the quality, origin, and authenticity of ciders [14–19].

Minerals have a high nutritional value in addition to their effect on the taste and various biochemical processes in ciders. For the human body, minerals are the most important components taking part in the main physiological processes in cells, organs, and tissues. Ciders contain so-called "essential elements" whose deficiency in the body disrupts its normal activity, development, and reproduction. They are iron, copper, zinc, cobalt, chromium, molybdenum, selenium, and manganese. Conditionally essential elements include actinium, boron, bromine, lithium, nickel, silicon, vanadium, and others.

We aimed to study the macro- and microelemental profile in ciders and to establish ranges of their mass concentrations.

STUDY OBJECTS AND METHODS

We studied apples of domestic and foreign selection, including those produced by the North-Caucasian Federal Scientific Center of Horticulture, Viticulture and Winemaking. The apples of various ripening stages were provided by the Tsentralnoye Experimental Farm (Krasnodar, Russia). The ploidy was 2n = 2x for most varieties and 2n = 3x for the Soyuz, Ekzotika, and Dzhin varieties. The apples typical in shape, color, and degree of maturity were collected from different sides of 3–5 trees for each variety according to the generally accepted method [14].

To produce ciders, the fruits were crushed in a homogenizer using the same processing mode. The must was fermented under laboratory conditions with the *Fruit* yeast race (genus *Saccharomyces cerevisiae*, Erbsle Geisenheim, Germany) at 18 ± 1°C. The ciders clarified spontaneously by settling, followed by sediment separation and filtration. The control samples were a semi-sweet carbonated pasteurized cider "Strongbow Rose" (Heineken United Breweries, St. Petersburg, Russia) prepared from fermented reconstituted apple juice; a carbonated sweet cider "Chester's" (Agroservis, Ramenskoye, Russia) from fresh apples grown in the Lipetsk region; as well as a semi-sweet "Greenvill Natural" (Kazakhstan) and "Cidre Royal" (Belarus) both produced from directly extracted juice.

Mass concentrations of potassium (K), calcium (Ca), sodium (Na), and magnesium (Mg) were determined by high-performance capillary electrophoresis according to the method developed by Scientific Center of Winemaking and the Instrumental and Analytical Center for Collective Use at the North-Caucasian Federal Scientific Center of Horticulture, Viticulture and Winemaking (certificates No. 61-10 dated 01.01.2010 and No. 60-10 dated 10.20.2010) using a Kapel-105M apparatus (Lumeks, Russia). This method is based on the separation of ions due to their different electrophoretic mobility during migration through a quartz capillary in an electrolyte under the influence of an electric field, followed by detection in the ultraviolet region at 254 nm.

Mass concentrations of copper (Cu), zinc (Zn), manganese (Mn), molybdenum (Mo), rubidium (Rb), and cesium (Cs) were determined by atomic absorption spectrometry with electrothermal atomization on a Kvant-Z.ETA spectrometer (KORTEK, Russia) according to State Standard R 51309-99. The method measures the absorption of resonance wavelength radiation by the atomic vapor of an element resulting from the electrothermal atomization of the sample under analysis in the graphite furnace of the spectrometer.

Mass concentrations of nickel (Ni), titanium (Ti), tin (Pn), cadmium (Cd), lead (Pb), arsenic (As), and mercury (Hg) were determined by inductively coupled plasma atomic emission spectrometry using an iCAP 7400 spectrometer (Thermo Scientific, USA) according to State Standard 30178-96. This method measures the intensity of the atomic radiation of the elements being determined, when the sample under analysis is sprayed into argon plasma inductively excited by a radio-frequency electromagnetic field. The samples were prepared by acid (nitric and hydrochloric) mineralization at elevated pressure according to State Standard R 53150–2008. For calibration, state standard reference samples of aqueous solutions were used with certified nominal values of mass concentrations and a relative error under ± 1% at $P = 0.95$. The sensory characteristics of the samples were evaluated on a 100-point scale by a panel at the Vinodelie (Winemaking) Scientific Center at the North-Caucasian Federal Scientific Center of Horticulture, Viticulture and Winemaking. Microsoft Excel 2019 and Statistika V.10.1 were used for statistical data processing by the analysis of variance.

RESULTS AND DISCUSSION

Table 1 presents experimental data on the concentrations of macroelements in ciders, which usually include cations of alkaline elements and alkaline earth elements.

We found significant differences in the concentrations of all macronutrients, including iron, depending on the variety of apples used to make the cider. The maximum and minimum concentrations of each cation differed several times: 2.6 times for potassium, magnesium, and iron, 6.6 times for sodium, and 8.3 times for calcium. Since the apples were grown in similar soils and using similar cultivation methods, such differences can only be due to their genetic characteristics and the ability of the trees to interact with the soil components.

Potassium was over 1 g/dm^3 in the ciders from the following varieties of apples (in descending order): Virginia (crab apple), Orfey, Ketni (crab apple), Persikovoye, Dzhin, Bagryanets Kubani, Margo, Florina, Zolotoye Letneye, and Enterprise. Its lowest concentration

Table 1 Mass concentrations of macroelements in ciders

No.	Name of sample	Mass concentration of alkaline and alkaline earth metal cations, mg/L			
		K	Na	Mg	Ca
		Varietal ciders from different pomological varieties of apples			
1	Ekzotika	895 ± 90	21.7 ± 2.4	23.6 ± 2.6	17.8 ± 1.8
2	Dzhin	1350 ± 135	31.3 ± 3.4	36.8 ± 4.0	35.5 ± 3.6
3	Enterprise	1090 ± 109	25.6 ± 2.8	17.8 ± 2.0	10.7 ± 1.1
4	Liberti	969 ± 97	16.1 ± 1.8	15.0 ± 1.7	7.2 ± 0.7
5	Karmen	760 ± 76	13.6 ± 1.5	19.1 ± 2.1	13.8 ± 1.4
6	Renet Platona	792 ± 79	6.7 ± 0.7	25.2 ± 2.8	9.9 ± 1.0
7	Bagryanets Kubani	1120 ± 112	18.0 ± 2.0	18.0 ± 2.0	13.3 ± 1.3
8	Soyuz	874 ± 87	8.3 ± 0.9	27.4 ± 3.0	11.8 ± 1.2
9	Ligol	704 ± 70	9.7 ± 1.1	17.5 ± 1.9	19.9 ± 2.0
10	Lyubimoye Dutovoy	696 ± 70	19.7 ± 2.2	20.6 ± 2.3	26.7 ± 2.7
11	Azimut	915 ± 92	21.3 ± 2.3	18.1 ± 2.0	16.8 ± 1.7
12	Margo	1120 ± 112	10.3 ± 1.1	15.8 ± 1.7	11.3 ± 1.1
13	Persikovoye	1370 ± 137	38.8 ± 4.3	17.7 ± 1.9	10.4 ± 1.0
14	Virginia	1920 ± 192	44.1 ± 4.9	33.7 ± 3.7	27.7 ± 2.8
15	Amulet	989 ± 99	34.3 ± 3.8	18.3 ± 2.0	9.8 ± 1.0
16	Orfey	1830 ± 183	42.3 ± 4.7	28.2 ± 3.1	13.9 ± 1.4
17	Ketni	1380 ± 138	41.0 ± 4.5	25.8 ± 2.8	34.0 ± 3.4
18	Champion	747 ± 75	26.5 ± 2.9	16.3 ± 1.8	15.5 ± 1.6
19	Zolotoye Letneye	1010 ± 101	36.6 ± 4.0	20.5 ± 2.3	10.1 ± 1.0
20	Prikubanskoye	847 ± 85	20.7 ± 2.3	14.7 ± 1.6	14.4 ± 1.4
21	Florina	1120 ± 112	25.4 ± 2.8	22.7 ± 2.5	16.7 ± 1.7
min		696 ± 70	6.7 ± 0.7	14.7 ± 1.6	9.8 ± 1.0
max		1920 ± 192	44.1 ± 4.9	36.8 ± 4.0	35.5 ± 3.6
		Varietal ciders from various apple tree forms			
22	12/1-20-16	1270 ± 127	26.8 ± 2.9	15.4 ± 1.7	5.5 ± 0.6
23	12/2-21-15	1060 ± 106	9.1 ± 1.0	26.4 ± 2.9	42.3 ± 4.2
24	12/2-21-36	900 ± 90	22.7 ± 2.5	10.2 ± 1.1	4.3 ± 0.4
25	12/3-2-6	853 ± 85	21.1 ± 2.3	10.7 ± 1.2	6.3 ± 0.6
min		696 ±70	9.1 ± 1.0	10.2 ± 1.1	4.3 ± 0.4
max		1270 ± 127	26.8 ± 2.9	26.4 ± 2.9	26.7 ± 2.7
		Commercial ciders			
26	Strongbow Rose	375 ± 38	138 ± 15	26.1 ± 2.9	24.2 ± 2.4
27	Chester’s	520 ± 52	114 ± 13	36.3 ± 4.0	62.0 ± 6.0
28	Greenvill Natural	580 ± 58	92 ± 10	46.3 ± 5.1	55.0 ± 6.0
29	Cidre Royal	610 ± 61	68 ± 7	43.1 ± 4.7	64.0 ± 6.0
min		375 ± 38	68 ± 7	26.1 ± 2.9	24.2 ± 2.4
max		610 ± 61	138 ± 15	46.0 ± 5.1	64.0 ± 6.0

(700–800 mg/L) was found in the ciders from Ligol, Lyubimoye Dutovoy, Champion, Karmen, and Renet Platona. In the commercial ciders, potassium varied in the range from 375 to 610 mg/L. This difference can be explained by the production technology: a greater content of potassium was in the ciders made from directly extracted juice.

Sodium concentrations, on the contrary, were higher in the ciders from juice concentrates reconstituted with softened water. It might be that sodium-cation exchange resins were used in the sample under analysis to regulate the hardness, resulting in calcium and magnesium ions being replaced with sodium ions. The highest sodium content (> 40 mg/L) was found in the ciders from Virginia, Orfey, and Ketni varieties, while the lowest content (< 10 mg/L) was found in the samples from Renet Platona, Soyuz, Ligol, and from the 12/2-21-15 apple tree.

Calcium and magnesium cations play an important role in plant development, particularly in the functioning of the root system [18]. Calcium enhances plant metabolism, affects the conversion of nitrogenous substances, and accelerates the breakdown of storage proteins. In addition, it is essential for cell membranes and a good acid-base balance in plants. Magnesium, the central molecule of chlorophyll, is involved in photosynthesis and is part of pectin and phytin. Magnetosynsium deficiency decreases the content of chlorophyll in the green parts of the plant.

Many researchers believe that calcium and magnesium concentrations depend on the ability of the plant to absorb these elements from the soil. With other conditions

being equal (e.g., the place of growth, cultivation method, or fertilizers), calcium and magnesium concentrations in the fruits and processing products may differ depending on the genetic characteristics of the variety, the development of its root system, and the plant's metabolism. According to our experimental data (Table 1), the largest amount of magnesium cations (> 25 mg/L) was in the ciders from Dzhin, Virginia, Orfey, Ketni, Renet Platona, and Soyuz varieties. The highest content of calcium (> 5 mg/L) was found in the ciders from Dzhin, Ketni, and Virginia.

Iron is involved in such vital processes as DNA synthesis, respiration, and photosynthesis [19]. It participates in various biochemical reactions catalyzed by enzymes, being their non-protein part (catalase, peroxidase). Due to its redox properties, iron is involved in the transfer of electrons and enzymes. According to Fig. 1, the maximum concentrations of iron (> 2 mg/L) were found in the samples from the following varieties: Virginia, Ketni, Soyuz, Liberti, and the 12/1-20-16 apple tree. This might be the reason why the juices from these varieties quickly changed their color from light golden to light brown during processing.

The commercial ciders had significantly lower concentrations of iron than the experimental samples prepared without technological treatments. This difference can be explained by the fact that in manufacturing facilities cider blends are demetallized to prevent ferric tannate haze.

The contents of microelements in the ciders from different apple varieties are shown in Table 2. Considering that neither juices nor ciders were subjected to any treatment in addition to alcoholic fermentation, which underwent under the same conditions, we can assume that the identified trends are also characteristic of apple fruits. Most microelements ensure normal growth and development of plants. They are involved in such vital processes as photosynthesis (manganese, iron, copper), respiration (manganese, iron, copper, zinc, copper), as well as carbohydrate, fat, and protein metabolism and the formation of organic acids and enzymes (manganese, copper, nickel, molybdenum, zinc). Microelements also bind free nitrogen (molybdenum, manganese, iron), convert nitrogen and phosphorus compounds (zinc, copper, manganese, molybdenum), participate in the development of nodule bacteria (copper, molybdenum), and catalyze various biochemical reactions (iron, manganese, molybdenum, copper, zinc, etc.) [20, 21]. Copper, cobalt, molybdenum, and zinc protect frost-resistant and drought-resistant plant species and contribute to a high level of protein synthesis. Zinc and manganese provide plant resistance to sudden temperature fluctuations. Molybdenum slows down water movement in plants during the day and accelerates it in the morning. It also increases the content of bound water and reduces the daytime depression of photosynthesis. Zinc and copper make plants more frost-resistant.

Microelements are mainly accumulated in the roots and their concentrations in fruits may indicate the intensity of metabolic processes in the root system. We found significant differences in the concentrations of microelements. In particular, the minimum and maximum concentrations differed 5.4 times for copper, 1.8 times for zinc, 4.6 times for manganese, 18 times for molybdenum, 1.9 times for rubidium (Fig. 2), 11.2 times for cesium, 14.8 times for nickel, 5.4 times for titanium, 5.9 times for lead, and 2.4 times for cadmium.

In the commercial samples, especially those made from directly extracted juice, the concentration of copper varied in about the same range as in the experimental samples, with much lower concentrations of other microelements. The ciders made from concentrated apple juice did not contain any manganese, molybdenum, or nickel, and the concentration of rubidium in them was 5–8 times lower compared to the ciders from directly extracted juice. Thus, the concentrations of microelement cations can be indicative of a relationship betweenthe cider and the composition of the soil on which the apple trees were grown. They can also be used as a marker of the beverage's origin.

According to our comparative analysis, the highest concentration of copper (> 200 μg/L) was found in the samples from Ekzotika, Dzhin, Amulet, and Prikubanskoye varieties, as well as from the 12/2-21-36 apple tree. The highest content of zinc (180–213 μg/L) was recorded in the ciders from Soyuz, Liberti, Ekzotika, and Bagryanets Kubani, as well as from the 12/2-21-36 tree. Manganese was abundant (> 90 μg/L) in the samples

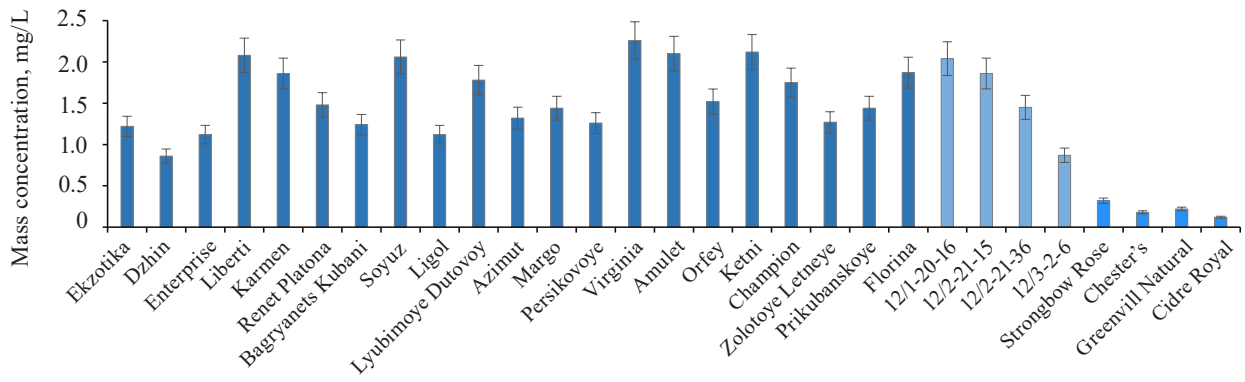

Figure 1 Mass concentrations of iron cations in ciders

Table 2 Mass concentrations of microelements in ciders

No.	Name of sample	Mass concentration of microelements, µg/dL								
		Cu	Zn	Mn	Mo	Cs	Ni	Ti	Pb	Cd
Varietal ciders from different pomological varieties of apples										
1	Ekzotika	376 ± 56	190 ± 25	197 ± 35	11.3 ± 1.7	1.7 ± 0.3	10.9 ± 1.6	1.9 ± 0.3	8.4 ± 1.5	8.4 ± 1.3
2	Dzhin	222 ± 33	113 ± 15	213 ± 38	53.1 ± 8.0	0.2 ± 0.1	12.5 ± 1.9	0.5 ± 0.1	14.6 ± 2.6	6.2 ± 0.9
3	Enterprise	188 ± 28	162 ± 21	87 ± 16	34.1 ± 5.1	0.5 ± 0.1	32.4 ± 4.9	1.1 ± 0.2	17.6 ± 3.2	7.6 ± 1.1
4	Liberti	158 ± 24	196 ± 26	113 ± 20	27.5 ± 4.1	0.4 ± 0.1	27.4 ± 4.1	0.5 ± 0.1	21.3 ± 3.8	10.2 ± 1.5
5	Karmen	125 ± 19	157 ± 20	68 ± 12	20.5 ± 3.1	1.5 ± 0.3	16.6 ± 2.5	0.5 ± 0.1	18.7 ± 3.4	7.3 ± 1.1
6	Renet Platona	117 ± 18	175 ± 23	90 ± 16	17.9 ± 2.7	2.5 ± 0.5	2.4 ± 0.4	1.4 ± 0.2	12.7 ± 2.3	5.2 ± 0.8
7	Bagryanets Kubani	71 ± 11	195 ± 25	57 ± 10	14.5 ± 2.2	1.4 ± 0.2	6.8 ± 1.0	1.5 ± 0.2	16.5 ± 3.0	6.6 ± 1.0
8	Soyuz	100 ± 15	200 ± 26	70 ± 13	13.4 ± 2.0	1.6 ± 0.3	27.1 ± 4.1	1.9 ± 0.3	22.6 ± 4.1	8.6 ± 1.3
9	Ligol	129 ± 19	128 ± 17	112 ± 20	12.5 ± 1.9	1.2 ± 0.2	8.3 ± 1.3	1.5 ± 0.2	16.1 ± 2.9	11.2 ± 1.7
10	Lyubimoye Dutovoy	51 ± 8	88 ± 12	212 ± 38	9.0 ± 1.4	1.9 ± 0.3	12.8 ± 1.9	1.4 ± 0.2	5.6 ± 1.0	4.8 ± 0.7
11	Azimut	121 ± 18	165 ± 21	154 ± 28	7.2 ± 1.1	1.6 ± 0.3	18.4 ± 2.8	1.7 ± 0.3	3.8 ± 0.7	7.6 ± 1.1
12	Margo	175 ± 26	149 ± 19	56 ± 10	12.2 ± 1.8	2.2 ± 0.4	15.2 ± 2.3	2.1 ± 0.3	14.2 ± 2.6	7.3 ± 1.1
13	Persikovoye	97 ± 15	152 ± 20	55 ± 10	10.9 ± 1.6	0.8 ± 0.2	11.1 ± 1.7	2.4 ± 0.4	11.8 ± 2.1	5.9 ± 0.9
14	Virginia	108 ± 16	157 ± 20	117 ± 21	8.9 ± 1.3	1.6 ± 0.3	34.2 ± 5.1	2.3 ± 0.4	19.4 ± 3.5	11.7 ± 1.8
15	Amulet	201 ± 30	170 ± 22	174 ± 31	30.8 ± 4.6	2.8 ± 0.5	21.4 ± 3.2	2.0 ± 0.3	14.2 ± 2.6	9.5 ± 1.4
16	Orfey	82 ± 12	168 ± 22	63 ± 11	20.6 ± 3.1	2.9 ± 0.5	36.8 ± 5.5	2.4 ± 0.4	11.8 ± 2.1	8.3 ± 1.2
17	Ketni	71 ± 11	114 ± 15	141 ± 25	15.2 ± 2.3	1.1 ± 0.2	26.5 ± 4.0	2.5 ± 0.4	18.3 ± 3.3	9.6 ± 1.4
18	Champion	74 ± 11	193 ± 25	146 ± 26	13.2 ± 2.0	1.4 ± 0.3	31.4 ± 4.7	1.3 ± 0.2	17.8 ± 3.2	10.7 ± 1.6
19	Zolotoye Letneye	90 ± 14	175 ± 23	165 ± 30	12.0 ± 1.8	2.6 ± 0.5	19.2 ± 2.9	0.6 ± 0.1	14.6 ± 2.6	5.5 ± 0.8
20	Prikubanskoye	201 ± 30	170 ± 22	174 ± 31	30.8 ± 4.6	2.7 ± 0.5	16.5 ± 2.5	2.8 ± 0.4	9.2 ± 1.7	6.4 ± 1.0
21	Florina	82 ± 12	168 ± 22	63 ± 11	20.6 ± 3.1	2.9 ± 0.5	28.5 ± 4.3	1.5 ± 0.2	14.6 ± 2.6	8.9 ± 1.3
min		71 ± 11	88 ± 15	55 ± 10	7.2 ± 1.1	0.3 ± 0.1	2.4 ± 0.4	0.5 ± 0.1	3.8 ± 0.7	4.8 ± 0.7
max		376 ± 56	200 ± 30	213 ± 38	53.0 ± 8.0	2.9 ± 0.5	36.8 ± 5.5	2.8 ± 0.4	22.6 ± 4.1	11.7 ± 1.8
Varietal ciders from various apple tree forms										
22	12/1-20-16	36.7 ± 5.5	159 ± 21	46.1 ± 8.3	6.3 ± 0.9	1.5 ± 0.3	23.1 ± 3.5	1.1 ± 0.2	4.6 ± 0.8	6.4 ± 1.0
23	12/2-21-15	109 ± 16	156 ± 20	192 ± 35	9.8 ± 1.5	2.2 ± 0.4	8.7 ± 1.3	2.2 ± 0.3	3.8 ± 0.7	8.0 ± 1.2
24	12/2-21-36	35.6 ± 5.3	214 ± 28	75 ± 14	8.0 ± 1.2	1.8 ± 0.3	14.1 ± 2.1	1.9 ± 0.3	4.4 ± 0.8	7.3 ± 1.1
25	12/3-21-6	31.1 ± 4.7	138 ± 18	71.1 ± 13.0	6.8 ± 1.0	1.1 ± 0.2	21.3 ± 3.5	0.9 ± 0.1	4.5 ± 0.8	5.2 ± 0.8
min		31.1 ± 4.7	138 ± 18	46.1 ± 8.3	6.3 ± 0.9	1.1 ± 0.2	8.7 ± 1.3	0.9 ± 0.1	3.8 ± 0.7	5.2 ± 0.8
max		109 ± 16	214 ± 28	192 ± 35	9.8 ± 1.5	2.2 ± 0.4	23.3 ± 3.5	2.2 ± 0.3	4.6 ± 0.8	8.0 ± 1.2
Commercial ciders										
26	Strongbow Rose	210 ± 32	10.2 ± 1.3	< 1.0*	< 1.0*	0.2 ± 0.1	< 1.0*	0.2 ± 0.1	1.2 ± 0.2	2.1 ± 0.3
27	Chester's	252 ± 38	45.3 ± 5.9	86 ± 16	14.3 ± 2.0	0.3 ± 0.1	4.6 ± 0.7	0.5 ± 0.1	1.8 ± 0.3	4.6 ± 0.7
28	Greenvill Natural	371 ± 56	54 ± 7	73 ± 13	18.4 ± 2.8	0.3 ± 0.1	11.3 ± 1.7	0.4 ± 0.1	2.4 ± 0.4	5.8 ± 0.9
29	Cidre Royal	311 ± 47	60 ± 8	113 ± 20	16.4 ± 2.5	0.5 ± 0.1	13.6 ± 2.0	0.3 ± 0.1	3.6 ± 0.6	6.2 ± 0.9
min		210 ± 32	10.2 ± 1.3	< 1.0*	< 1.0*	0.2 ± 0.1	< 1.0*	0.2 ± 0.1	1.2 ± 0.2	2.1 ± 0.3
max		371 ± 56	60 ± 8	113 ± 20	18.4 ± 2.8	0.5 ± 0.1	13.6 ± 2.0	0.5 ± 0.1	3.6 ± 0.6	6.2 ± 0.9

*The value is below the lower limit of the detection range

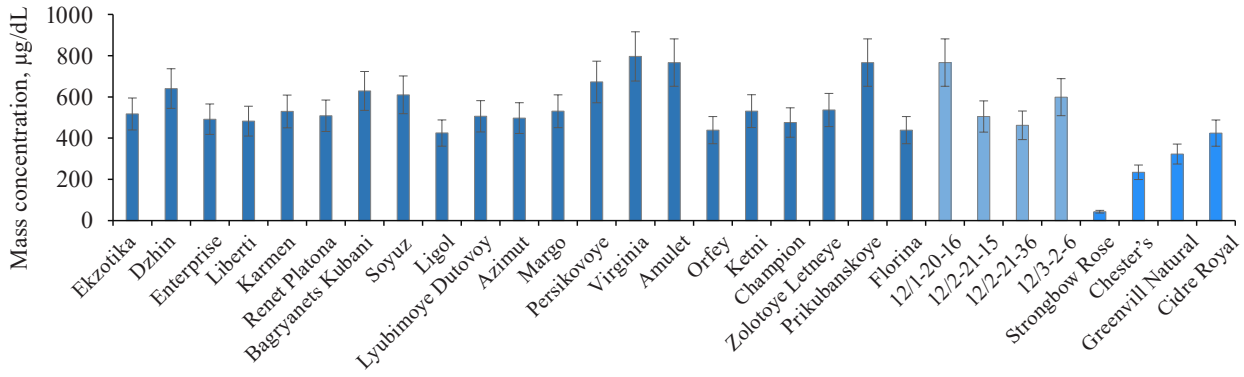

Figure 1 Mass concentrations of iron cations in ciders

from Dzhin, Ekzotika, and Lyubimoye Dutovoy varieties, as well as from the 12/2-21-15 apple tree. Also high were the contents of molybdenum (> 50 μg/L) in the Dzhin ciders; rubidium (> 750 μg/L) in the Virginia, Amulet, and Prikubanskoye ciders; cesium (> 2.5 μg/L) in the Florina, Orfey, Prikubanskoye, Renet Platona, Amulet, and Zolotoye Letneye samples; nickel (> 30 μg/L) in the ciders from Orfey, Virginia, and Enterprise; and titanium (> 2.0 μg/L) in the Prikubanskoye, Ketni, Virginia, Margo, Persikovoye, and Orfey ciders.

Titanium and cesium are often present simultaneously, and their concentrations correlate with each other. This can be explained by the fact that titanium salts have a high sorption capacity for cesium ions, including in soils [22, 23]. Ligands formed by titanium attract cesium ions through electrostatic mechanisms. Our study did not reveal any significant relationships between the concentrations of these cations. Yet, they depended on the varietal characteristics of apples. The highest concentrations of titanium were found in the ciders from Prikubanskoye, Ketni, Orfey, Virginia, and Margo varieties, while cesium was most abundant in the Florina, Prikubanskoye, Orfey, Amulet, and Zolotoye Letneye ciders.

The concentrations of heavy metals or toxic elements are legislatively regulated worldwide. In Russia, they are governed by the Technical Regulations of the Customs Union TR TS 021/2011 [24, 25]. Such microelements are widely used in microfertilizers, but in high concentrations they can disrupt biological cycles, suppress plant growth, and sometimes even cause plants to die. Especially toxic for living organisms are high concentrations of tin, cadmium, copper, zinc, and nickel [26, 27]. Therefore, although microfertilizers are highly effective, they should not exceed the recommended concentrations of heavy metals to prevent them from accumulating in the soil and getting into fruits and their products, including ciders. Excessive amounts of microelements, just as their deficiency, can cause metabolic disorders in the development of apple trees.

It is important to know the concentrations of toxic elements in cider not only to assess potential health risks, but also to evaluate its quality, origin, and authenticity.

In our study, the contents of mercury and arsenic were below the lower limits of the range established by the method we used – none of the samples exceeded their maximum permissible concentrations. Therefore, they are not presented in Table 2. Neither were the conrations of cadmium and lead exceeded in the samples. However, we found statistically significant differences depending on the variety of apples. In particular, the highest concentration of lead (> 20 μg/L) was found in the Soyuz cider, while the highest content of cadmium (10–11 μg/L) was detected in the ciders from Champion, Virginia, Liberti, and Ligol varieties.

The commercial samples had somewhat lower concentrations of cadmium and lead than the experimental samples, which is associated (just as with other elements) with their exposure to various technological treatments.

The experimental ciders were evaluated for their sensory characteristics (Table 3). All the samples had clean aroma and taste without any off-flavors. Some of the samples made from fresh must were opalescent, since they were not additionally clarified. The ciders only clarified by settling, followed by sediment separation and

Table 3 Sensory evaluation of ciders

No.	Name of sample	Sensory characteristics
		Varietal ciders from different pomological varieties of apples
1	Ekzotika	Transparent, of a golden color with a greenish tint. Bright aroma with tones of exotic fruits, green apple, and quince. Fresh and clean taste.
2	Dzhin	Opalescent, of a golden color. Bright aroma with tones of fresh apple and dried fruit. Full taste with cream-cheese hints.
3	Enterprise	Transparent, of a golden yellow color. Complex aroma with tones of apple, banana, and mango. Full, tannic taste.
4	Liberti	Opalescent, of a golden color. Bright aroma with tones of peach, pineapple, and mango. Full taste with cream-cheese hints.
5	Karmen	Opalescent, of a golden-orange color. Bright aroma with tones of fruit stones, dried fruits, and citrus fruits. Fresh, full, tannic taste.
6	Renet Platona	Opalescent, of a golden-brown color. Complex aroma with tones of honey, flowers, and citrus fruits. Full, tart, fresh taste.
7	Bagryanets Kubani	Opalescent, of a straw-golden color. Bright aroma with tones of green apple and citrus fruits. Fresh, full taste with creamy tones in the aftertaste.
8	Soyuz	Opalescent, of a golden-brown color. Clean aroma with tones of fresh apple, caramel, and dried fruit. Full, tannic taste.
9	Ligol	Opalescent, of a straw color. Complex aroma with tones of berries, apples, dried fruits, and flowers. Full, harmonious taste.
10	Margo	Transparent, of a golden color. Bright aroma with floral and fruity tones. Clean, fresh, and full taste.
11	Persikovoye	Opalescent, of a golden-orange color. Complex aroma with tones of plum, cherry plum, peach, and caramel. Full and fresh taste.
12	Virginia	Opalescent, of a golden-orange color. Complex aroma with dried fruit hints. Full, tart, and fresh taste.
13	Amulet	Opalescent, of a golden-brown color. Complex aroma with tones of fresh and baked apple. Full, fresh taste with tones of dried fruits.

Continuation of Table 3

No.	Name of sample	Sensory characteristics
		Varietal ciders from different pomological varieties of apples
14	Orfey	Transparent, of a straw color. Clean, bright aroma with hints of green apple and exotic fruits (mango). Clean, full, harmonious taste.
15	Ketni	Opalescent, of an orange-golden color. Clean aroma with tones of dried fruits, banana, and quince. Full, tannic, and tart taste.
16	Champion	Transparent, of a straw-golden color. Fruity aroma with cider (apples and fermentation) tones. Full, tannic, and tart taste.
17	Zolotoye Letneye	Opalescent, of a golden-orange color. Fruity aroma with tones of undergrowth, fresh apple, and dried fruits. Simple, flat taste.
18	Prikubanskoye	Opalescent, of a golden-brown color. Fruity aroma with tones of fresh fruits (apples, pears, quince). Full, fresh taste.
19	Florina	Opalescent, of a bright yellow color. Clean aroma with tones of fresh apple and fruity-floral nuances. Full, tart, slightly bitter taste.
20	Lyubimoye Dutovoy	Opalescent, of a golden color. Clean aroma with tones of fresh apple and dried fruit. Simple taste with low tannins.
21	Azimut	Opalescent, of a golden-orange color. Bright aroma with tones of plum, cherry plum, and citrus. Full, fresh, and tart taste.
		Varietal ciders from various apple tree forms
22	12/1-20-16	Opalescent, of a golden-brown color. Complex aroma with tones of wild rose and dried fruits. Full, fresh, and tart taste
23	12/1-21-36	Opalescent, of an orange-golden color. Complex aroma with tones of rotten foliage and dried fruits. Flat, watery, simple taste with low tannins.
24	12/2-21-15	Opalescent, of a light straw color. Fruity aroma with tones of fresh apple and fruit stones. Full taste with tones of dried fruits.
25	12/3-21-6	Opalescent, of a golden-orange color. Bright aroma with tones of plum, cherry plum, and citrus. Full, fresh, and tart taste.
		Commercial ciders
26	Strongbow Rose	Transparent, of a pink color. Clean, bright, and fruity-floral aroma with tones of apple. Clean and harmonious taste with tones of fermentation.
27	Chester's	Transparent, of a straw color with a greenish tint. Clean aroma with hints of apple and caramel. Fully developed, fresh taste with tones of fermentation.
28	Greenvill Natural	Transparent, of a pink color. Clean aroma with tones of fresh apple. Full, harmonious, rounded taste with well-pronounced tones of fermentation.
29	Cidre Royal	Transparent, of a straw color with a yellow tint. Bright aroma with tones of fresh apple. Full, harmonious, rounded taste with well-pronounced tones of fermentation.

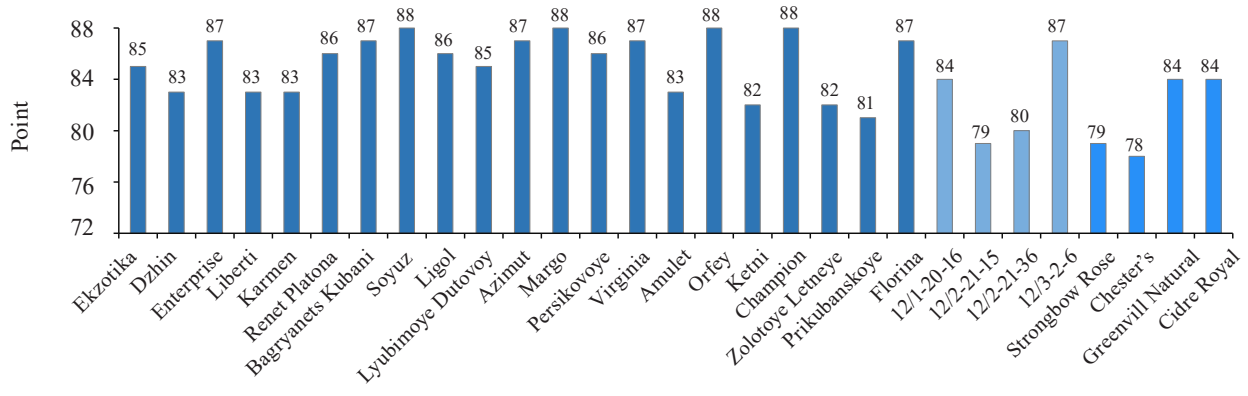

Figure 3 Sensory scores for ciders

filtration through filter sheets. The sensory scores were in the range of 79–88 points (Fig. 3).

The highest sensory scores were given to the experimental ciders from Soyuz, Virginia, Champion, and Prikubanskoye apple varieties (88 points) and the samples from Enterprise, Bagryanets Kubani, Margo, Persikovoye, Orfey, and Azimut varieties, as well as the 12/3-21-6 apple tree (87 points). These ciders had a bright aroma with various hints and a harmonious, full taste. The commercial samples received 78–84 points. They had a clean apple aroma and a taste with pronounced fermentation tones.

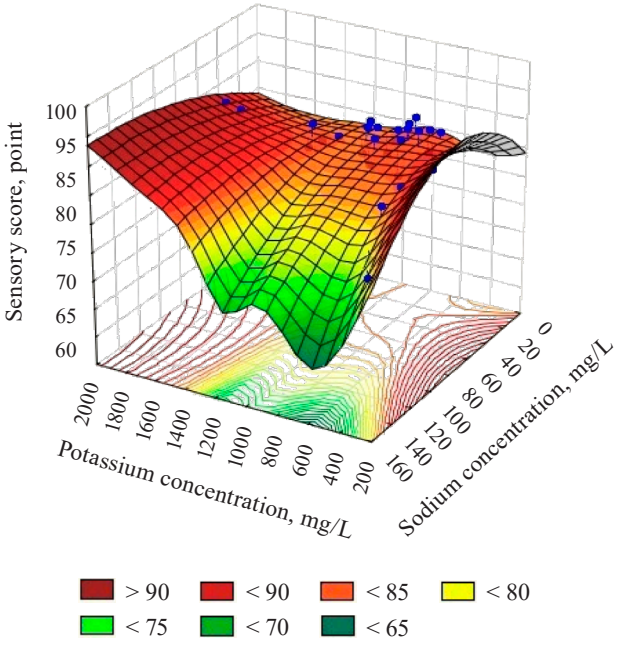

Figure 4 Effect of potassium and sodium cations on the sensory evaluation of ciders

The experimental data were statistically processed to establish a relationship between the concentrations of macroelements in the ciders and their sensory scores. As a result, we found a statistically significant positive relationship between the potassium content ($r = 0.4$) and the sensory score, as well as a negative relationship between the sodium content ($r = –0.4$) and the sensory score. The highest scores were given to the cider samples with potassium ranging from 850 to 1900 mg/L and sodium ranging from 100 to 10 mg/L (Fig. 4).

CONCLUSION

Thus, we found significant differences in the concentrations of all metal cations depending on the pomological variety of apples. If the varieties under study were grown in different soils and climatic conditions, with the use of different fertilizers and crop protection products, we might assume that the differences in the elemental composition of the ciders were down to the growing conditions, agricultural practices, and other technogenic and anthropogenic factors. However, the varieties we used were grown under the same agrotechnical conditions. Therefore, we concluded that the differences in the elemental profile of the ciders were determined by the genetic characteristics of the respective varieties. As a result, we constructed the following elemental profiles of the ciders depending on the concentration of metal cations: K > Na > Ca = Mg > Fe for macronutrients and Rb > Cu > Mn = Zn > Ni > Cs > Ti for microelements.

The commercial samples of ciders, especially those obtained from directly extracted juice, had similar concentrations of copper to those in the experimental ciders and much lower concentrations of other microelements. The cider made from concentrated apple juice did not contain any manganese, molybdenum, or nickel, and its concentration of rubidium was 5–8 times lower than in the ciders made from fresh juice. The revealed concentrations of microelement cations provide information on the relationship between the cider and the composition of the soil on which the apple trees were grown. Therefore, they can be used as a marker of the origin of the beverage.

CONTRIBUTION

N.M. Ageyeva and E.V. Ulyanovskaya developed the research concept and design. A.A. Khrapov and E.A. Chernutskaya collected and analyzed the material. N.M. Ageyeva, A.A. Shirshova, and E.V. Ulyanovskaya wrote and edited the manuscript. L.E. Chemisova processed the data statistically; and all the authors approved the final version of the article.

CONFLICT OF INTEREST

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

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SNP-based genetic signatures revealed breeding effects in indigenous Livni compared with Landrace and Large White breeds

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Abstract:
Livni is one of the Russian local pig breeds. We previously reported that this breed was more distinct from Duroc breed than from Landrace and the Large White breeds, which participated in the Livni breed creation. The aim of the study was to determine the SNP-based genetic signatures in fat-type Livni breed shared with commercial Landrace and the Large White breeds, and ones that are affected by putative selection.
The genome-wide SNP genotyping was carried out using the Porcine GGP HD BeadChip, which contains ~ 80 000 SNPs.
Obtained breed relationship and admixture results indicated the insignificant participation of the Landrace and the Large White breeds in the formation of the modern allelofund of Livni pigs. 238 candidate genes were found in the genomic regions with selection signatures, 182 genes with described functions were identified. In the Livni and Landrace breeds, 35 common genes were detected which formed one cluster with enrichment coefficient = 4.94 and predominant *HOXD* genes. In the Livni and Large White breeds, the largest amounts of common genes were detected (62 in average), which formed two clusters. Cluster 1, with enrichment coefficient = 2.11, was characterized with genes involved in glucose metabolism. Cluster 2, with enrichment coefficient = 1.60, demonstrated helicase genes. Annotated clusters were not determined for the Livni breed. However, 50 candidate genes were specific to Livni pigs and associated with various growth, carcass and reproductive traits, essential for thermoregulation.
Results revealed common SNP-based genetic signatures and breeding effects in indigenous Livni compared with Landrace and Large White breeds.

Keywords: Livni breed, animal genetic resources, SNPs, pig, carcass, traits

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INTRODUCTION

The pig is a major livestock species, and the global pork production primarily relies on the use of a limited number of international commercial breeds, specifically Duroc, Large White, and Landrace [1]. Intensive implementation of commercial hybrid breeds characterized by high production standards led to an impoverishment of genetic resources which in the past had a fair distribution [2]. However, recently a strong attention has been attracted to local breeds for improving genetic diversity and conservation of genetic resources. Local breeds are valued not only by adaptive traits, but also by the unique functional characteristics and intensively studied in Asia, Europe, Africa, as well as North and Latin America [3–14].

Twenty-two local breeds were recorded in the Soviet Union in 1980, which were generated by crossing of native breeds adapted to the local climate and having appropriate constitution and disease resistance with highly-productive improved European breeds [15, 16]. As a result of the interbreeding of the imported breeds and crossing them with the native animals, many pig breeds were created during 1920–1990. For example, Ukranian White Steppe was created in Askania Nova and approved in 1932; North Siberian – in Novosibirsk and approved in 1942, Urzgum – in the Kirov region

and approved in 1957 – by crossing native pigs with the Large White boars. Breeds, as Kemerovo (approved in 1961), Breitov (approved in 1948), Latvian White and Lithuanian White (both approved in 1967), Semirechensk (1978), Mirgorod (1940), Tsivilsk (a cross of native Chuvash pigs with Large White boars, the breed is not approved), Mangalitsa, Altai (approved in 2015), and others were created by multiple crossbreeding procedures. [15]. According to the Department of Livestock and Breeding of the Ministry of Agriculture of the Russian Federation data, 98% of the total pig purebred population in 2020 included four breeds – Large White (66%), Landrace (15%), Yorkshire (13%) and Duroc (4%) [17]. Other breeds' share was about 2%. Pavlova *et al.* consider 0.56% of the total pig population in the RF are of the local breeds – Livni, Altai, Tsivilsk, as of January 1, 2022. Four breeds make 99.46% of the RF pig herd, namely 56.9% Large White, 18.52% – Yorkshire, 18.18% – Landrace, and 5.83% – Duroc breed [18]. The dramatically reduction of local pig breeds during last 30 years finally led to remaining only Livni, Altai Meat-type, Short-Eared White, and Tsivislk. The authentic Kemerovo breed has also been mentioned for a number of years. However, according to the Yearbook on breeding work in pig husbandry in establishment of the Russian Federation for 2021, the last time a breeding farm certificate for the Kemerovo purebred was issued in 2019 [19]. It should be noted, that the certificate for the Tsivislk breed was last issued in 2021.

Livni is one of the Russian local pig breeds approved in 1949. Pigs of the Livni breed are large, white, black-mottled, black and red. At present, only a small population of Livni pigs is kept in a single farm in the Oryol region [20]. According to the Yearbook on breeding work, in pig husbandry in establishment of the Russian Federation for 2021 one certificate is issued annually for the Livni purebred, but the total number of the Livni pigs is steadily declining. At the beginning of 2022, 547 heads were purebreds, including 348 sows with the share in the total livestock of 0.24% [19]. For comparison, in 1949 the Livni livestock was 6757 purebreds (1334 sows), while in 1980 it was 27 200 purebreds (5500 sows) [21]. It is noteworthy that at the age of 6 months, Livni correspond to the bacon (meat) type. Then the active accumulation of fat begins and at the age of 10 months Livni pigs are already belong to meat-and-fat type, and with further fattening lead to fat type [21].

We previously reported that Livni breed is characterized the highest level of genetic diversity compared with commercial breeds. The neighbor-joining tree showed that this breed was the most distinct from Duroc breeds, but formed the knot bounding the branches corresponding to the Landrace and the Large White breeds. This observation confirmed the participation of these two breeds in the Livni breed creation. The aim of our study was to determine the SNP-based genetic signatures in Livni breed common with Landrace and the Large White breeds, and ones that are affected by putative selection in the genome of Livni breed and could be associated with fatty tissue formation and breed specificity.

STUDY OBJECTS AND METHODS

Samples and genotyping. For the study, we used samples (ear tissue) of Livni pigs (n = 35). Only purebred animals registered in Russian swine herdbook were selected, the origin of which is confirmed by both the pedigree data and DNA analysis. For genotyping, we selected the most unrelated individuals. Samples of all breeds were sent to the Ernst Federal Research Center for Animal Husbandry. A parentage and breed assignment of those breeds were confirmed based on the microsatellites in the laboratory of the Ernst Federal Research Center for Animal Husbandry, which has a certificate of 2020–2021 ISAG Pig STR Comparison Test (2020–2021) and has a special license issued by the Russian Ministry of Agriculture. Commercial breeding farms and the Ernst Federal Research Center for Animal Husbandry collaborate based on the contracts. In the contract, a clause states the consent of the owners (breeding farms) to use the samples with research purpose.

Moreover, the study did not involve any endangered or protected animal and all procedures were conducted according to the ethical guidelines of the L.K. Ernst Federal Science Center for Animal Husbandry. The Commission on the Ethics of Animal Experiments of the L.K. Ernst Federal Science Center for Animal Husbandry approved the protocol No. 6 of May 10, 2021. The ear tissues were collected by trained personnel under strict veterinary rules in accordance with the rules for conducting laboratory research (tests) in the implementation of the veterinary control (supervision) approved by Council Decision Eurasian Economic Commission № 80 (November 10, 2017).

Genomic DNA was extracted using the DNA Extran 2 kit (ZAO Sintol, Moscow, Russia) according to the manufacturer's instructions. Concentrations of dsDNA solutions were determined using a Qubit 1.0 fluorometer (Invitrogen, Life Technologies, Waltham, Massachusetts, USA). The OD260/280 ratio was determined using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

The genome-wide SNP genotyping was carried out using an iScan microarray scanner (Illumina Inc., Singapore) using the Porcine GGP HD BeadChip (Illumina Inc., San Diego, CA, USA), which contains ~ 80 000 SNPs. In our study, we used all the capital equipment required for SNP genotyping by Illumina SNP arrays. The equipment belongs to the Center for Collective Use "Bioresources and Bioengineering of Agricultural Animals" of the Ernst Federal Research Center for Animal Husbandry (https://www.vij.ru/infrastruktura/ckp, accessed on 10 May 2021). The SNPs genotypes of Large White (n = 53) and Landrace (n = 50) breeds were included in the data set and obtained from Center for Collective Use "Bioresources and Bioengineering of

Agricultural Animals" of the Ernst Federal Research Center for Animal Husbandry.

Quality control. Using PLINK 1.9 software, the SNP quality control was performed [22, 23]. All samples were subjected to filtering for genotyping efficiency (--mind 0.2). The SNPs genotyped in less than 90% of the samples (--geno), minor allele frequencies below 0.01 (--maf 0.01), and *p*-values below 10^{-6} for Hardy-Weinberg equilibrium were excluded from the analysis. The final data set used for analysis included 51 912 autosomal SNPs. Additional filters for linkage disequilibrium (LD) with r2 every 50kb (--indep-pairwise) were performed, amount of SNP passes LD-filtration amounted 24 861.

Genetic diversity, PCA, Neighbor-Net and Admixture. To assess the within-population genetic diversity, the observed (H_O) and unbiased expected ($_UH_E$) heterozygosity, the rarefied allelic richness (A_R), and the unbiased in-breeding coefficient ($_UF_{IS}$) were estimated using the R package, diveRsity [24]. Additionally, we computed the genomic inbreeding coefficient based on runs of homozygosity (ROH, F_{ROH}) as the ratio of the sum of the length of all ROHs per animal to the total autosomal SNP coverage; for ROH estimation, see the "*Runs of Homozygosity Estimation*" Section below). PCA was performed using PLINK v1.9 software. An R package, ggplot2, was used to visualize the results [25]. Pairwise FST values were calculated in the R package, diveRsity, and used for the construction of the Neighbor-Net tree in SplitsTree software (version 4.14.5) [24, 26, 27]. Admixture software (version 1.3.0) was employed for genetic admixture analysis and an R package, pophelper, was used for plotting the results [28, 29]. A cross-validation (CV) procedure was used to calculate the number of ancestral populations (k) from one to five using Admixture software (version 1.3.0).

Selection signature analysis. Three different statistics were used for detecting the signatures of selection in the genome of pigs: the calculation of F_{ST} values for each SNP when comparing pairs of breeds, the estimation of the ROH islands, which were overlapped among different animals within each breed, and hapFLK analysis.

F_{ST} analysis. F_{ST} values for all SNPs were estimated for pairs of breeds using PLINK 1.9 [24]. Minor allele frequencies were below 5% (--maf 0.05) [30]. The top SNPs corresponding to 0.1% of F_{ST} values were used to represent a selection signature, according to Kijas *et al.* and Zhao *et al.* [31, 32].

Runs of homozygosity estimation. Runs of homozygosity were detected according to the window-free method for consecutive SNP-based detection using the R package, detectRUNS [33]. One SNP with a missing genotype and up to one possible heterozygous genotype in one run were allowed to avoid the underestimation of the number of ROHs that were longer than 8 Mb [34]. The minimum ROH length was set to 500 kb to exclude the common ROHs. To minimize false-positive results, the minimum number of SNPs was calculated as it was proposed by Lencz *et al.* and later modified by Purfield *et al.* [35, 36].

Putative ROH islands were defined as overlapping homozygous regions in analyzed individuals within each breed. A threshold of 50% (the minimum proportion of animals within the breed in which overlapping ROH were detected) was selected, as this was suggested in other studies [37, 38]. We applied the threshold of 0.1 Mb for the minimal overlapping length size and 5 SNP for minimum number in ROH island.

HapFLK analysis. In this study, a hapFLK analysis was performed to detect the selection signatures through haplotype differentiation among the studied breeds using hapFLK software (version 1.4.) [39]. The number of haplotype clusters per chromosome was calculated in fast-PHASE by using cross-validation and was set to 35 [40]. For detailed analyses, the hapFLK regions containing at least one SNP with a *p*-value threshold of 0.01 ($-\log10(p) > 2$) were selected.

Identification of candidate genes. For candidate gene mining in the genomic regions under putative selection, the genomic localization of the regions as detected by three different statistics was used, i.e., the FST, ROH, and hapFLK methods. Regions that were overlapped and revealed by at least two different techniques were prioritized. Borders of these regions according to the 10.2 genome assembly were converted to genome assembly 11.1. Genes located on the selected regions were obtained from the Ensembl Genes Release 103 database based on the *Sus scrofa* gene sequence assembly [41].

Functional enrichment analysis. To understand the biological functions of the candidate genes, the Database for Annotation, Visualization, and Integrated Discovery (DAVID) was used for enrichment analysis [42]. Significant annotation clusters of enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology were selected using an enrichment score of more than 1.3 and a *p*-value of < 0.05. To learn the biological functions of annotated genes and genes not included in clusters, a comprehensive literature search including information from other species was carried out.

RESULTS AND DISCUSSION

Genetic diversity. The Livni pigs were characterized by higher level of genetic diversity assessed by the levels of observed heterozygosity, unbiased expected heterozygosity, and allelic richness as compared to the Landrace and Large White breeds. The negative value of the inbreeding coefficient $_UF_{IS}$ indicates an excess of heterozygotes from the Hardy–Weinberg equilibrium in all the breeds (Table 1). In commercial breeds, the excess of heterozygotes was more significant compared to the Livni breed.

Breed relationship and admixture. The PCA-plot (Fig. 1a), the neighbor-joining tree (Fig. 1b) and cluster structure (Fig. 1c) showed the breed-specific distribution of individuals for all of the studied breeds. Obtained distribution indicated the insignificant participation of

Table 1 Summary of genetic diversity statistics calculated in studied pig breeds

Breed	*n**	H_O (M ± SE)	${}_UH_E$ (M ± SE)	${}_UF_{IS}$ [CI 95%]	A_R (M ± SE)
Livni	35	0.416 ± 0.001	0.411 ± 0.001	–0.011 [–0.013; –0.009]	1.998 ± 0
Landrace	50	0.373 ± 0.001	0.360 ± 0.001	–0.032 [–0.034; –0.030]	1.969 ± 0.001
Large White	53	0.351 ± 0.001	0.339 ± 0.001	–0.032 [–0.034; –0.030]	1.941 ± 0.001

* n – number of individuals; H_O – observed heterozygosity; M – mean value; SE – standard error; ${}_UH_E$ – unbiased expected heterozygosity; A_R – rarefied allelic richness; ${}_UF_{IS}$ – unbiased inbreeding coefficient [CI 95%, range variation of ${}_UF_{IS}$ coefficient at a confidence interval of 95%]

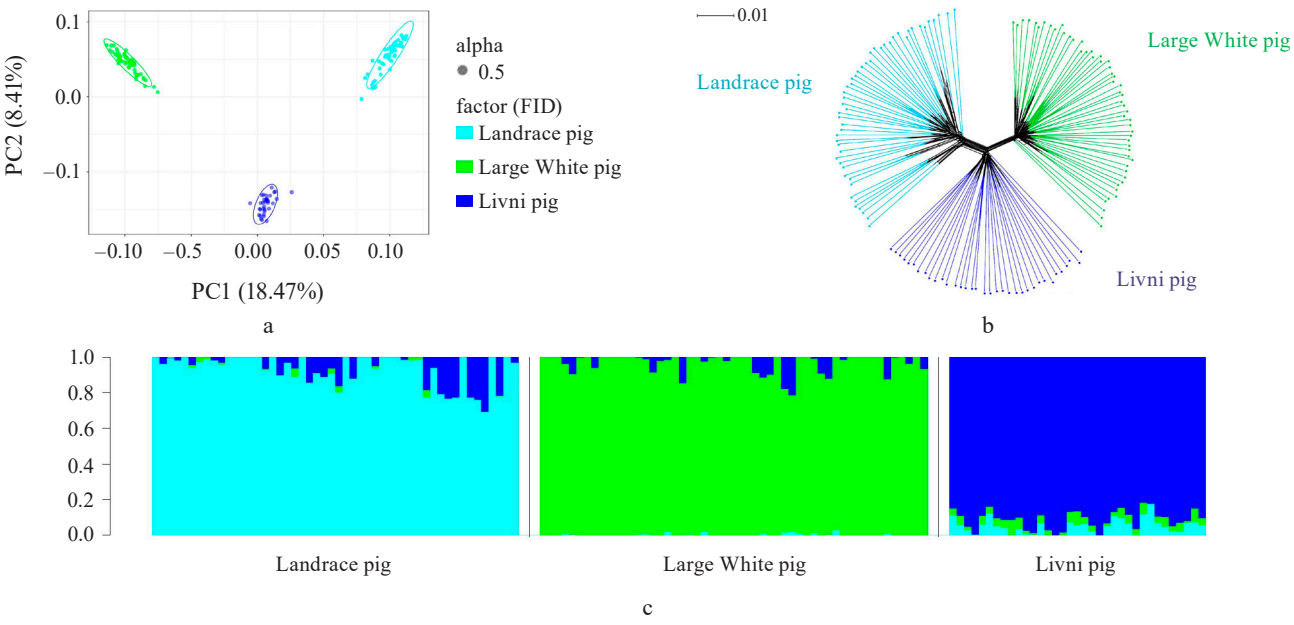

Figure 1 Genetic relationships between Landrace, Large White and Livni pig populations: (a) Principal component analysis (PCA) plot showing the distribution of Landrace, Large White and Livni individuals in two-dimensional coordinate system, i.e., the first (PC1; X-axis) and second (PC2; Y-axis) principal components, with percentage of total genetic variability, which can be explained by each of the two components, being indicated within the parentheses; (b) Neighbor-Net tree constructed based on the IBS-distances among the studied populations; (c) Admixture plot representing cluster structure of the studied populations if the number of clusters K = 3

the Landrace and Large White breeds in the formation of the modern allelofund of Livni pigs and demonstrated that sampling is suitable for searching for loci under selection pressure in the studied pig breeds and their subsequent structural annotation.

Selection signature detection. SNPs with F_{ST}-values beyond the cut-off (top 0.1%) were distributed among all autosomes, excepting SSA18. Most of these SNPs were specific to breed pairs. Six SNPs were found in SSA4 (1 SNP), SSA6 (2 SNP), and SSA11 (3 SNP), which were common for Livni – Landrace and Landrace – Large White, and seven SNPs on SSA5 (5 SNP), SSA8 (1 SNP), and SSA12 (1 SNP), for Livni – Large White and Landrace – Large White (Fig. 2).

The distribution of ROH island number and length in chromosomes is presented in Table 2. Forty-two ROH islands were detected in the Livni breed, which covered 34.415 Mb of the genome, while for Landrace and Large White, 126 and 224 ROH islands covered 161.792 and 282.402 Mb of the genome, respectively. The average length of the ROH island in Livni breed was significantly lower than that of pigs of commercial breeds: 2.868 ± 0.822 Mb versus 8.988 ± 2.185 (Landrace) and 15.689 ± 2.770 Mb (Large White), respectively ($p < 0.001$).

Eighteen common ROH islands were detected in the Large White and Landrace breeds, which were identified in ten autosomes: SSA1 (4 ROH islands), SSA4 (3 ROH islands), SSA5, SSA6 (3 ROH islands), SSA8, SSA9, SSA11, SSA13, SSA14, SSA16, and SSA17. Eight common ROH islands were detected in the Livni and Large White breeds, which were identified in six autosomes, namely SSA1, SSA2, SSA7, SSA12, SSA14 (3 ROH islands), and SSA15. Eight common ROH islands were detected in the Livni and Landrace breeds, which were identified in five autosomes: SSA1, SSA3 (2 ROH islands), SSA4, SSA6, SSA11 (2 ROH islands), and SSA15. Five ROH islands detected in SSA1, SSA6, SSA11, SSA14, and SSA15 were common for three breeds (Table 3).

The hapFLK analysis resulted in the identification of 13 putative regions affected by the selection (Fig. 3). These regions were distributed among 10 autosomes, including regions on SSA1, SSA3, SSA14, and SSA13 with

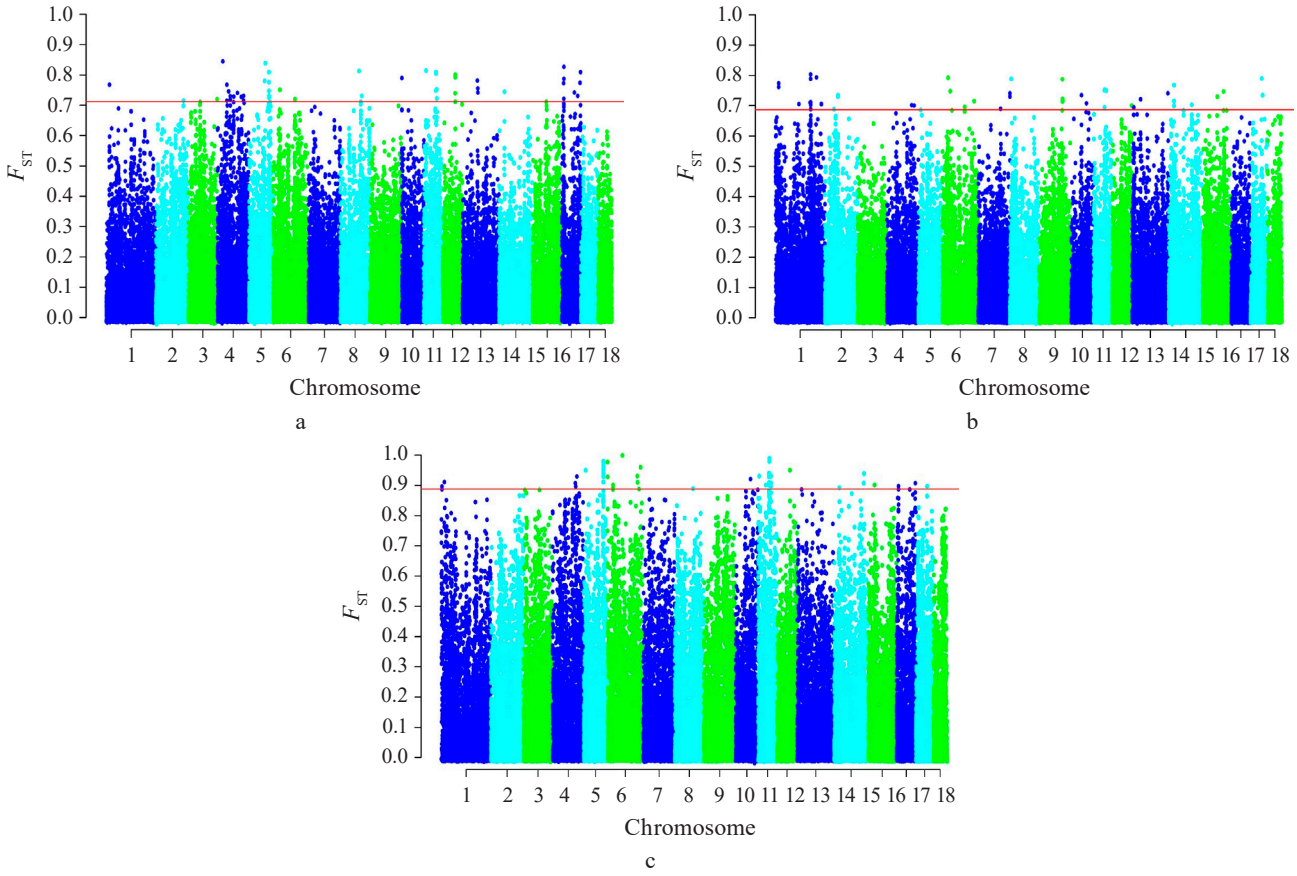

Figure 2 Genomic distribution of F_{ST} values estimated between the breeds: (a) Livni – Landrace; (b) Livni – Large White; (c) Landrace – Large White. Values for the X-axis are pig autosomes (the breadth of autosomes corresponds to their length); and those for the Y-axis are F_{ST} values. SNPs were plotted relative to their positions within each autosome. The threshold, which was estimated as the top 0.1% for F_{ST} values, is indicated by a horizontal line

Table 2 The distribution of ROH island number and length in chromosomes

SSA*	Livni breed				Landrace breed				Large White breed			
	50%		70%		50%		70%		50%		70%	
	n#	Length, Mb	n	Length, Mb	n	Length, Mb	n	Length, Mb	n	Length, Mb	n	Length, Mb
1	8	6.027			13	40.348	4	3.574	25	35.475	2	2.942
2	3	3.596	1	0.181	1	1.991			12	13.752	4	1.387
3	4	1.748			7	5.025			13	25.441	2	3.136
4	1	0.611			11	11.232			30	44.178	5	9.820
5					3	4.004	1	1.907	14	13.519	2	1.819
6	2	1.322	1	0.700	14	12.401	1	0.497	21	31.490	6	5.046
7	1	0.365			6	4.943	1	0.618	12	12.894	1	0.681
8	1	0.706			4	5.273			10	10.570	2	2.155
9					13	18.110	1	0.127	14	15.720	2	0.846
10					5	4.054			6	3.227	1	0.494
11	5	6.298	1	4.116	5	10.478	3	4.824	7	11.946	4	5.088
12	1	0.562			3	1.115			6	2.886		
13	3	1.478			10	8.386	1	0.485	11	13.026		
14	9	9.187	2	1.748	15	17.094			13	18.220	7	8.033
15	4	2.516	2	1.183	5	7.820	3	4.719	13	17.427	4	3.230
16					5	3.429			8	6.952	1	0.777
17					4	2.838	1	0.607	6	3.558		45.455
18					2	3.251			3	2.120		3.247 ± 0.762
SUM	42	34.415	7	7.928	126	161.792	16	17.358	224	282.402	43	45.455
Average		2.868 ± 0.822		1.586 ± 0.684		8.988 ± 2.185		1.929 ± 0.643		15.689 ± 2.770		3.247 ± 0.762

* SSA – *Sus scrofa* autosomes; # *n* – number of SSA

a statistical significance of $p < 0.001$. Four regions were Large White-specific, three – Landrace-specific, three – Large White and Landrace-specific, three – Livni and Large White-specific (Table 4).

Comparing the genomic localization of the regions under putative selection detected by three different statistics (F_{ST}, ROHs, and hapFLK) revealed the presence of 13 overlapping regions, which were identified by at least two different methods (Table 5); 7 regions corresponded to the Large White breed, 2 corresponded to the Landrace breed, 2 were common to Large White and Landrace breeds, and 2 were common to Large White and Livni breeds. Additionally, in the list of genes for structural and functional annotation, we included ROH islands identified only in Livni pigs, as well as common ROH islands identified in the Livni and one or two compared breeds. Thus, 16 Livni-specific regions, and 39 regions, which are common for both two and more breeds were selected for the structural and functional annotation.

Candidate gene determination and functional enrichment determination. The structural annotation of these regions revealed the presence of 238 candidate genes: 50 genes were specific to Livni pigs, 62 to Livni

Table 3 Common ROH islands identified in genome of two or three studied breeds

SSA*	Livni breed			Landrace breed			Large White breed		
	SNP#	Position&	Mb	SNP	Position	Mb	SNP	Position	Mb
1	17	65.16–65.97	0.811				20	65.10–65.97	0.868
				15	82.43–83.14	0.709	14	82.43–83.12	0.687
	22	83.26–84.22	0.964	17	83.23–84.06	0.827			
				18	94.66–96.30	1.647	22	94.37–96.30	1.936
				34 (31)	216.94–222.84 (218.18–222.84)	5.901 (4.657)	38 (20)	215.75–221.94 (219.60–221.43)	6.190 (1.833)
	19	241.90 242.96	1.052	219 (180)	223.97–245.52 (228.65–245.52)	21.551 (16.864)	22 (21)	241.90–243.21 (241.90–243.01)	1.303 (1.109)
				24	265.78–266.65	0.873	25	265.78–266.71	0.928
2	41 (5)	44.46–46.37 (45.09–45.27)	1.909 (0.181)				21	44.46–45.27	0.804
3	19	28.95–29.45	0.493	8	29.18–29.62	0.441			
	9	111.31–111.65	0.340	13	111.31–111.81	0.499			
4				20	13.87–14.59	0.715	20	13.87–14.59	0.715
				37	49.18–52.60	3.428	98 (48)	48.13–61.32 (48.38–53.11)	13.189 (4.735)
				20	84.63–85.69	1.065	6	85.11–85.40	0.291
	10	107.67–108.28	0.611	33	107.09–108.28	1.194			
5				21	67.31–67.88	0.570	45	66.43–67.68	1.251
6				24 (18)	14.62–15.26 (14.65–15.14)	0.635 (0.497)	74	14.18–16.13	1.947
				16	18.83–19.35	0.525	25	18.39–19.00	0.615
				20	19.66–20.42	0.761	38	19.11–20.64	1.526
	18	71.44–72.06	0.622	13	71.88–72.48	0.601			
	19 (18)	88.24–88.94 (88.24–88.94)	0.700 (0.700)	13	88.24–88.62	0.373	63 (19)	88.05–91.08 (88.24–88.94)	3.026 (0.700)
7	9	36.20–36.57	0.365				30	35.88 37.26	1.380
8				23	34.62 35.34	0.715	15	34.81 35.22	0.405
9				18	83.56 84.66	1.099	30	83.40 85.15	1.751
11	6	7.81–8.06	0.249	19 (10)	7.55–8.45 (7.55–8.06)	0.900 (0.504)	75 (64)	6.48–10.17 (6.48–9.59)	3.686 (3.112)
	29 (23)	34.82–39.79 (35.23–39.35)	4.966 (4.116)	45 (23) (16)	34.72–40.9 (34.72–37.40) (38.52–40.16)	6.246 (2.683 (1.637)			
	6	45.77–46.05	0.284	20	45.77–46.69	0.920			
	11	46.24–46.64	0.407						
				24	59.35–61.04	1.684	32	59.82–62.09	2.270
12	20	1.56–2.12	0.562				24	1.56–2.25	0.694
13				8	95.33–96.75	1.421	18	94.50–97.50	3.004
14				30	47.72–48.97	1.255	64	47.65–50.41	2.752
		51	49.26–51.17		1.911				

Continuation of Table 3

SSA*	Livni breed			Landrace breed			Large White breed		
	SNP#	Position&	Mb	SNP	Position	Mb	SNP	Position	Mb
14	40	71.65–73.67	2.023				58 (40)	70.89–74.21 (71.65–73.67)	3.325 (2.023)
	37 (20)	75.38–76.74 (75.38–76.01)	1.354 (0.624)				82 (36)	75.38–78.86 (75.45–76.74)	3.474 (1.291)
	34 (25)	76.89–78.43 (77.05–78.17)	1.545 (1.124)				31	76.89–78.22	(1.325)
	7	78.49–78.69	0.199						
	7	94.10–94.94	0.846				29	93.46 95.28	1.818
	6	95.18–95.40	0.226						
	24	98.02–99.36	1.341	24	98.02–99.36	1.341	28 (14)	97.62–98.92 (98.02–98.73)	1.300 (0.707)
15	5	84.30–84.56	0.262				55 (18)	84.37–87.85 (84.70–85.83)	3.476 (1.135)
	19 (5)	90.46–91.44 (91.14–91.44)	0.983 (0.297)	11	90.76–91.44	0.675			
	24 (19)	92.81–93.87 (92.86–93.75)	1.059 (0.886)	33 (26)	92.40–93.87 (92.69–93.87)	1.469 (1.180)	21	92.86–93.77	0.902
16				5	5.85–6.00	0.151	44	5.43–6.42	0.990
17				30	8.32–9.16	0.839	6	8.63–8.78	0.153

* SSA – *Sus scrofa* autosomes; # SNP – number of SNP in ROH island; & position – start and end of ROH island in accordance with genome assembly 10.2, information about ROH islands detected in more than 70% of animals is presented in brackets

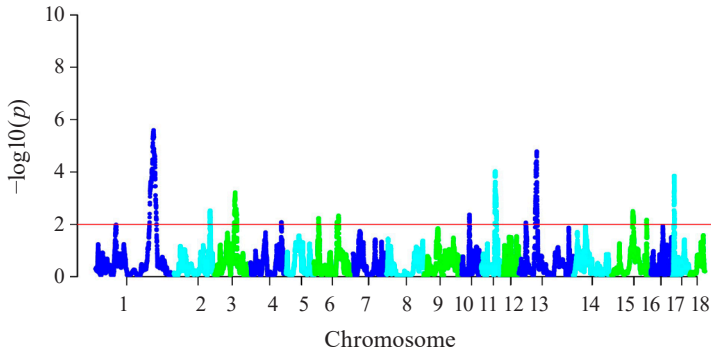

Figure 3 Signatures of selection in the genomes of the studied breeds based on the hapFLK statistics. Values for the *X*-axis are pig autosomes, and those for the *Y*-axis are values of statistical significance (−log10 *p*-values). The red line indicates the threshold of significance at $p < 0.01$ (i.e., $-\log10(p) > 2$)

Table 4 HapFLK regions identified in the genome of the studied breeds

SSA*	Breed	Position of Region		Amount of SNP in Region	Length, Mb	The Most Significant SNP	*p*-Value
		Start	End				
1	Landrace	216 939 236	244 858 851	245	27.92	232 953 425	2.44E-06
2	Large White	143 991 472	146 088 237	78	2.10	144 881 039	2.99E-03
3	Large White	79 798 323	89 895 102	92	10.10	83 233 266	5.92E-04
4	Large White	124 675 286	124 955 662	10	0.28	124 832 207	8.14E-03
6	Landrace, Large White	18 888 120	20 189 159	38	1.30	19 601 974	5.73E-03
6	Livni, Large White	92 938 033	100 318 389	20	7.38	99 706 201	4.56E-03
10	Landrace	30 140 466	31 750 116	36	1.61	31 038 967	4.14E-03
11	Landrace, Large White	54 362 880	61 415 073	110	7.05	56 397 482	9.27E-05
13	Landrace	27 775 922	27 893 903	7	0.12	27 823 032	8.43E-03
13	Livni, Large White	64 933 643	74 382 805	122	9.45	71 775 121	1.62E-05
15	Livni, Large White	84 301 944	88 728 150	80	4.43	85 769 904	3.14E-03
15	Large White	140 660 077	141 264 578	30	0.60	140 897 463	6.65E-03
17	Landrace, Large White	6 263 548	8 746 763	71	2.48	7 572 534	1.40E-04

* SSA – *Sus scrofa* autosomes

Table 5 Overlapped genomic regions and/or SNPs under putative selection identified by at least two different statistics in the Duroc and Livni breeds

SSA*	F_{ST} [a]		ROH[b]		hapFLK[c]	
	Breed	Position	Breed	Position	Breed	Position
1	Landrace/Livni	9 951 603	Landrace	9.87–10.52		
		10 051 445				
		10 070 322				
1	Landrace/Livni	226 429 888–226 458 237	Landrace	216.94–222.84	Landrace	216.94–244.86
		230 057 074		218.18–515.22		
		231 262 134–231 476 049		223.97–245.52		
		232 259 626		228.65–245.52		
1	Livni/Large White	145 137 405	Large White	143.69–145.62	Large White	143.99–146.09
				144.50–144.81		
3			Large White	78.28–91.19	Large White	79.80–89.90
				80.19–81.59		
4	Livni/Large White	31 519 009–31 637 170	Large White	30.71–33.25		
5	Landrace/Large White	94 308 964–94 995 044	Large White	94.26–95.05		
	Livni/Large White	94 408 638–94 822 437				
6			Large White	19.11–20.64	Landrace, Large White	18.89–20.19
			Landrace	19.66–20.42		
6			Large White	94.45–96.28	Large White	92.94–100.32
11	Landrace/Large White	54 595 810	Large White	54.83–57.35	Large White	54.36–61.42
		54 768 013				
		54 829 740		56.40–56.90		
		55 413 895				
		56 258 459–56 663 342				
13	Livni/Large White	68 207 174	Large White	71.91–72.12	Livni, Large White	64.93–74.38
		71 946 567				
		72 118 478				
15	Livni/Large White	84 696 087	Livni	84.30–84.56	Livni, Large White	84.30–88.73
			Large White	84.37–87.85		
				84.70–85.83		
15			Large White	140.12–142.04	Large White	140.66–141.26
17			Large White	6.68–7.82	Landrace, Large White	6.26–8.75
			Landrace	8.32–9.16		
			Large White	8.63–8.78		

* SSA – *Sus scrofa* autosomes. Methods used for defining the signatures of selection: [a]F_{ST} – top 0.1% SNPs by the F_{ST} value at pairwise population comparison; [b]ROH – ROH segments distributed in more than 70% of animals; and [c]hapFLK – regions identified by hapFLK analysis at $p < 0.001$

and Large White pigs, 35 to Livni and Landrace pigs, 36 to all studied breeds, and 55 were specific to Large White and Landrace pigs (Table 6).

Using the DAVID web tool and a list of 238 candidate genes found in the genomic regions with selection signatures, 182 genes with described functions were identified. The significant clusters are shown in Table 7. Annotated clusters with an enrichment coefficient – log10(p) > 1.3 (corresponds to $p < 0.05$) were not determined for the Livni breed and all three studied breeds (Livni, Large White and Landrace). Two reliably annotated clusters were identified for the Livni and Large White, Large White and Landrace breeds, and one annotated cluster for Livni and Landrace. For the list of Livni and Large White genes, the presence of two annotated clusters was revealed. Cluster 1 (enrichment coefficient = 2.11) included *G6PC2*, *HKDC1*, *HK1* genes involved in carbohydrate metabolism. Cluster 2 (enrichment coefficient = 1.60) included *SUPV3L1*, *SLC25A16*, *HKDC1*, *DDX21*, *PIK3C2A*, *MAP3K7*, *DDX50*, and *HK1* genes involved in the processes of DNA replition and repair. For Livni and Landrace one reliable cluster (enrichment coefficient = 4.49) was determined, including the genes *CIART*, *HORMAD1*, *HOXD3*, *HOXD4*, *HOXD8*, *HOXD9*, *HOXD10*, *HOXD12*, *HOXD13*, *EVX2*, *NR2E1*, and *PLEKHO1*. Genes under selection pressure in commercial pig breeds (Large White and Landrace) were combined into two reliable clusters. Cluster 1 (enrichment co-efficient = 1.74) combined the genes *KCNA1*, *KCNA6*, *KV1.5*, and *SLC30A9* involved in the regulation of ion transmembrane transport, mainly potassium. The *IBTK*, *KCNA1*, *KCNA6*, and *ZBTB10* genes regulating transcription repression and interaction with components of histone deacetylase co-repressor complexes were localized in cluster 2 (enrichment coefficient = 1.54).

Specific and overlapping sites in the genome of Livni, Large White and Landrace breeds that are under

Table 6 Genes within the overlapped genomic regions affected by putative selection

SSA	Region (Mb)			Genes[a]
	Livni	Landrace	Large White	
1	4.31–4.80			*PDE10A*, *C6orf118*
1	71.81–72.72			*FHL5*, *GPR63*, *NDUFAF4*, *KLHL32*
1	87.60–87.85			*U6*, *WISP3*, *TUBE1*, *FAM229B*
1	204.60–205.65			*WDHD1*, *SOCS4*, *MAPK1IP1L*, *LGALS3*, *DLGAP5*, *ATG14*, *TBPL2*, *U4*, *KTN1*
1	299.94–300.44			*U5*, *PBX3*
2	98.53–99.63			*7SK*, *ssc-mir-9-2*, *MEF2C*
2	118.05–118.64			–
3	29.62–30.60*			*PARN*, *BFAR*, *ssc-mir-365-1*, *CCDC12*, *ERCC4*
8	102.43–103.14			*C4orf33*, *JADE1*
11	42.95–43.35			–
13	36.28–36.92			*MAPKAPK3*, *CISH_TV2*, *DOCK3*, *SNORD22*, *RBM15B*, *MANF*, *VPRBP*
13	60.38–61.29*			*PDZRN3*
14	74.80–75.20			–
14	94.10–95.53**			*WAPAL*, *OPN4*, *LDB3*, *C14H10orf116*, *SNCG*, *BMPR1A*, *GLUD1*
14	100.10–101.35			*ZNF239*, *ZNF32*, *TFAM*, *RPL37A*
15	91.54–91.76			–
1	65.16–65.97		65.10–65.97	*MAP3K7*
2	44.46–46.37		44.46–45.27	*MYOD1*, *OTOG*, *SNORD89*, *USH1C*, *ABCC8*, *KCNJ11*, *NUCB2*, *PIK3C2A*, *RPS13*, *SNORD14*, *U1*, *PLEKHA7*, *C11orf58*
7	36.20–36.57		35.88–37.26	*DEF6*, *ZNF76*, *FKBP5*, *ARMC12*, *CLPSL2*, *CLPS*, *LHFPL5*
12	1.56–2.12		1.56–2.25	*CHMP6*, *NPTX*, *RNF213*
14	71.65–73.67		70.89–74.21	*NRBF2*, *JMJD1C*, *ssc-mir-1296*, *REEP3*
14	75.38–78.69**		75.38–78.86	*LRRTM3*, *DNAJC12*, *SIRT1*, *HERC4*, *MYPN*, *ATOH7*, *PBLD*, *HNRNPH3*, *RUFY2*, *SLC25A16*, *CCAR1*, *STOX1*, *SNORA70*, *DDX50*, *DDX21*, *KIAA1279*, *SRGN*, *VPS26A*, *SUPV3L1*, *HKDC1*, *TACR2*, *HK1*, *COL13A1*
14	94.10–95.40*		93.46–95.28	*WAPAL*, *OPN4*, *LDB3*, *U3*, *C14H10orf116*, *SNCG*, *BMPR1A*
15	84.30–84.56		84.37–87.85	*NOSTRIN*, *SPC25*, *G6PC2*, *ABCB11*
1	83.26–84.22	83.23–84.06		*SEC63*, *GL*, *NR2E1*, *SNX3*, *FOXO3A*
3	28.95–29.45	29.18–29.62		*ABCC1*, *U6*, *SNORA70*, *CPPED1*
3	111.31–111.65	111.31–111.81		–
4	107.67–108.28	107.09–108.28		*HORMAD1*, *GOLPH3L*, *ENSA*, *MCL1*, *ADAMTSL4*, *ECM1*, *TARS2*, *RPRD2*, *PRPF3*, *CIART*, *PLEKHO1*, *VPS45*
6	71.44–72.06	71.88–72.48		*MINOS1*, *HTR6*, *TMCO4*
11	34.82–39.79	34.72–40.96		*SNORA31*
11	45.77–46.64*	45.77–46.69		*KLHL1*
15	90.46–91.75*	90.76–91.44		*EVX2*, *HOXD13*, *HOXD12*, *HOXD10*, *HOXD9*, *HOXD8*, *ssc-mir-10b*, *HOXD4*, *HOXD3*
1	241.90–242.96	223.97–245.52	241.90–243.21	*MLANA*, *ERMP1*, *RIC1*, *U6*, *SNORA19*, *PDL1*, *PLGRKT*, *RLN*, *INSL6*, *JAK2*
6	88.24–88.94	88.24–88.62	88.05–91.08	*PABPC4*, *SNORA55*, *U6*, *HEYL*, *NT5C1A*, *HPCAL4*
11	7.81–8.06	7.55–8.45	6.48–10.17	*HSPH1*, *U6*, *B3GALTL*
14	98.02–99.36	98.02–99.36	97.62–98.92	*CHAT*, *C10orf53*, *OGDHL*, *PARG*, *NCOA4*, *MSMB*, *ZFAND4*, *MARCH8*, *ALOX5*, *ZNF22*, *C10orf10*
15	92.81–93.87	92.40–93.87	92.86–93.77	*RBM45*, *U1*, *SNORD112*, *OSBPL6*, *PRKRA*, *DFNB59*, *FKBP7*, *PLEKHA3*
1		82.43–83.14	82.43–83.12	*SOBP*
1		94.66–96.30	94.37–96.30	*TPBG*, *IBTK*, *SNORD112*, *FAM46A*
1		216.94–222.84	215.75–221.94	*TEK*, *IFT74*, *LRRC19*, *PLAA*, *CAAP1*, *U6*, *TUSC1*, *IZUMO3*, *ELAVL2*
1		265.78–266.65	265.78–266.71	*ZCCHC7*, *GRHPR*, *POLR1E*, *U6*, *FRMPD1*, *TRMT10B*, *EXOSC3*, *DCAF10*
4		13.87–14.59	13.87–14.59	*FAM84B*, *U6*, *5S_rRNA*
4		49.18–52.60 60.52–61.40	48.13–61.32	*OTUD6B*, *TMEM55A*, *NECAB1*, *CALB1*, *DECR1*, *NBN*, *OSGIN2*, *CU607036.1*, *RIPK2*, *5S_rRNA*, *PAG1*, *ZNF704*, *ZBTB10*
4		84.63–85.69	85.11–85.40	*ST18*, *PCMTD1*

Continuation of Table 6

SSA	Region (Mb)			Genes[a]
	Livni	Landrace	Large White	
5		67.31–67.88	66.43–67.68	*KV1.5*, *KCNA1*, *KCNA6*
6		14.62–15.26	14.18–16.13	*HP*, *ZFHX3*
6		18.83–19.35	18.39–19.00	–
6		19.66–20.42	19.11–20.64	–
8		34.62–35.34	34.81–35.22	*TMEM33*, *SLC30A9*, *BEND4*, *U6*
9		83.56–84.66	83.40–85.15	*SDHAF3*
11		59.35–61.04	59.82–62.09	*SLITRK1*
13		95.33–96.75	94.50–97.50	*7SK*, *ZIC1*, *ZIC4*
14		47.72–1.17*	47.65–50.41	*MN1*, *PITPNB*, *TTC28*, *U1*
16		5.85–6.00	5.43–6.42	–
17		8.32–9.16	8.63–8.78	–

[a] Candidate genes. *2 closely located ROH islands, **3 closely located ROH islands

selection pressure have been identified. Positional candidate genes were identified and their annotation was performed. In the current study, three pig breeds were examined and compared. We previously reported that Livni breed is characterized the highest level of genetic diversity compared with commercial breeds. The neighbor-joining tree showed that this breed was the most distinct from Duroc but formed the knot bounding the branches corresponding to the Landrace and the Large White breeds. This observation confirmed the participation of these two breeds in the formation of the Livni breed during it artificial selection. We observed the highest level of genetic diversity in Livni pigs compared to commercial breeds (Table 1), which may be a consequence of the participation of various breeds in the development of the Livni breed, including Large White and Landrace. However, results of breed relationship and admixture revealed distribution indicated the insignificant participation of the Landrace and Large White breeds in the formation of the modern allelofund of the Livni pigs.

Using three different statistics (top 0.1 F_{ST} at pairwise breed comparison, ROH islands and hapFLK analysis), we selected 13 overlapping regions, which were identified by at least two different methods (Table 2); 7 regions corresponded to the Large White breed, 2 corresponded to the Landrace breed, 2 were common to Large White and Landrace breeds, and 2 were common to Large White and Livni breeds. Among 238 candidate genes, which were localized within selected genomic regions (Table 3), 182 genes had the described functions in GO-terms; among them, 50 genes were specific to Livni pigs, 62 were specific to Livni and Large White pigs, 35 were specific to Livni and Landrace pigs, 36 were specific to all studied breeds, and 55 were specific to Large White and Landrace pigs (Table 3).

Among common genes for three studied breeds, *MLANA* and *JAK2* were previously observed in Livni and Duroc breeds and involved in adipogenesis [20, 43]. It was reported that *FKBP7* is highly expressed in subcutaneous adipose tissue of mature Erhualian pig, while *CHAT* is essential for macrophages as a source of acetylcholine for the regulation of adaptive thermogenesis [44, 45]. *HSPH1* is a known marker of both human and mouse brown adipocytes and was upregulated in young and old brown adipocytes after acute cold exposure [46]. *HSPA1L* were found to be differently expressed between the low and high drip loss groups in the Duroc pigs [47]. *NCOA4* may play a role in early events of adipocyte differentiation and were found in Pudong White pigs [48, 49]. *PLGRKT* coordinately regulates multiple aspects of adipose function and was found to be related to obesity [50, 51]. According to Gene Ontology terms, *ALOX5* is strongly associated with immunity, lipid metabolism and fat cell differentiation, insulin secretion, and oxidative stress. Interestingly, this gene was also very highly significantly associated with feet and leg structure soundness traits in pigs [52]. *OSBPL6* linked with lipid and sterol transport and encoded by *miR-33*, which may also regulate adaptive thermogenesis [53]. *PLEKHA3* is also associated with lipid metabolism, and mutations were identified for this gene in the Puławska pig breed, which is characterized by thicker backfat and better meat quality values [54]. *PARG* is linked with carbohydrate metabolic process and could be involved in lipid metabolism [55]. According to Gene Ontology terms, *ERMP1* involved incellular response to oxidative stress, *HEYL* – in skeletal muscle cell differentiation. *INSL6* was linked with male fertility in Enshi pigs and reproduction in Anhui pigs [56, 57]. *MSMB* was closely related to body weight, body height, abdominal circumference, and chest depth in Xiangsu hybrid pigs [58]. *OGDHL* was up-regulated in the liver in pigs with higher backfat thickness of Songliao black female pig population [59]. Although *PRKRA* is strongly associated with immune response, including piglets, this gene plays unexpected role in the regulation of mitochondrial biogenesis and energetics in cells and brown adipocytes [60, 61]. *ZFAND4* gene encodes stress proteins and was detected in Pudong White pigs, as well as *ZNF22* [49, 62]. *C10orf10* is involved in adipose tissue thermogenesis and was observed in heavy Iberian

Table 7 Functional Gene Ontology terms enriched with candidate genes

Claster	Category	Term	P	Genes
Livni and Large White				
Cluster 1, enrichment coefficient = 2.11	KEGG_PATHWAY	ssc00052: galactose metabolism	0.002	*G6PC2*, *HKDC1*, *HK1*
		ssc00500: starch and sucrose metabolism	0.003	
		ssc04973: glucose digestion and absorption	0.005	
		ssc00010: glycolysis/gluconeogenesis	0.009	
		ssc04910: insulin signaling pathways	0.040	
Cluster 2, enrichment coefficient = 1.60	UP_KW_MOLECULAR_FUNCTION	KW-0347 ~ helicase	0.002	*SUPV3L1*, *SLC25A16*, *DDX21*, *DDX50*
	GOTERM_MF_DIRECT	GO:0003724 ~ activity of RNA helicase	0.009	*SUPV3L1*, *DDX21*, *DDX50*
	UP_KW_LIGAND	KW-0067 ~ ATP binding	0.010	*SUPV3L1*, *SLC25A16*, *HKDC1*, *DDX21*, *PIK3C2A*, *MAP3K7*, *DDX50*, *HK1*
		KW-0547 ~ nucleotide binding	0.013	
	UP_SEQ_FEATURE	DOMAIN: C-terminal helicase	0.025	*SUPV3L1*, *DDX21*, *DDX50*
	INTERPRO	IPR001650: C-terminal helicase	0.029	
	SMART	SM00490: HELICc	0.043	
Livni and Landrace				
Cluster 1, enrichment coefficient = 4.94	GOTERM_MF_DIRECT	GO:0000981 ~ transcription factor activity of RNA polymerase II, sequence-specific DNA binding	0.001	*HOXD13*, *HOXD4*, *HOXD12*, *HOXD3*, *EVX2*, *HOXD10*, *HOXD8*
		GO:0000978 ~ sequence-specific DNA binding of the proximal promoter region of RNA polymerase II	0.001	*HOXD13*, *HOXD4*, *NR2E1*, *HOXD3*, *EVX2*, *HOXD10*, *HOXD9*
		GO:0001228 ~ transcription activator activity, sequence-specific binding of the transcriptional regulatory region of RNA polymerase II	0.002	*HOXD13*, *HOXD4*, *NR2E1*, *HOXD10*, *HOXD8*
		GO:0005634 ~ nucleus	0.028	*PLEKHO1*, *HOXD4*, *HORMAD1*, *NR2E1*, *HOXD12*, *HOXD3*, *EVX2*, *HOXD10*, *HOXD9*, *HOXD8*, *CIART*
Large White and Landrace				
Cluster 1, enrichment coefficient = 1.74	UP_KW_MOLECULAR_FUNCTION	KW-0631 ~ potassium channel	0.003	*KV1.5*, *KCNA1*, *KCNA6*
		KW-0851 ~ voltage-controlled ion channels	0.007	
	UP_KW_BIOLOGICAL_PROCESS	KW-0633 ~ potassium transport	0.007	
	UP_SEQ_FEATURE	DOMAIN: ion transport	0.007	
	GOTERM_MF_DIRECT	GO:0005249 ~ activity of the voltage-controlled potassium channel	0.008	
		GO:0008076 ~ voltage-controlled complex of potassium channels	0.008	
		GO:0034765 ~ regulation of ion transmembrane transport	0.025	
	INTERPRO	IPR005821: ion transport domain	0.020	
	UP_KW_BIOLOGICAL_PROCESS	KW-0406 ~ ion transport	0.030	*SLC30A9*, *KV1.5*, *KCNA1*, *KCNA6*
	UP_KW_LIGAND	KW-0630 ~ potassium	0.032	*KV1.5*, *KCNA1*, *KCNA6*
Cluster 2, enrichment coefficient = 1.54	UP_SEQ_FEATURE	DOMAIN: BTB	0.005	*IBTK*, *KCNA1*, *KCNA6*
	INTERPRO	IPR000210: BTB/POZ-like	0.046	*IBTK*, *KCNA1*, *ZBTB10*

Pigs [63, 64]. *PDL1* was determined as candidate biomarkers for predicting residual feed intake in Yorkshire pigs, as well as *U1* [65, 66]. *RLN* is a candidate gene for reproductive traits in pigs and was found to regulate adipose tissue development through stimulating adipogenesis and modulating adipocyte metabolism [67–69]. *SNORA19* could be involved in body temperature regulation [70]. *U6* was associated with litter traits in Yorkshire and Landrace pigs and was a selection signature gene in Meishan population [71, 72].

In the Livni and Landrace breeds, 35 common genes were detected, which formed one cluster with enrichment coefficient = 4.94 and predominant *HOXD* genes. According to Gene Ontology terms, *HOXD10* and *HOXD9* are involved in various developmental processes, such as single fertilization, skeletal muscle tissue development, adult locomotory behavior, embryonic skeletal system morphogenesis, peripheral nervous system neuron development, neuromuscular process, etc. *HOXD10* is required systemically for secretory activation in lactation [73]. Expression level of *HOXD10* was increased in animals with high marbling [74]. *HOXD9* and *HOXD10* are associated with such traits as growth, body weight and composition, abdominal fat, organogenesis, and feed intake and consumption [75]. They also play an active role in chondrogenesis and the development of adipose depots [76, 77]. *HOXD3*, *HOXD8*, *HOXD12*, and *HOXD13* are also associated with skeletal system development. *HOXD12* is differently expressed between large and small piglet size [78]. *HOXD3* is also associated with nervous system development, considered as predictors for feed efficiency traits [79, 80]. It was reported that *HOXD4* and *HOXD8* are up-regulated in differentiated adipocytes [81]. *HOXD8* gene is involved in patterning the lower thoracic and lumbar vertebrae, in the urogenital tract development, also of mesoderm origin [82, 83]. *HTR6*, associated with nervous system, was identified as interesting candidate genes involved in axonogenesis and synapsis in Iberian breed [84]. *ADAMTSL4* was found to evolve under positive selection and exhibited significant down-regulated mRNA expression in the Tibetan pigs [85]. *ABCC1* is expressed in adipose and skeletal muscle, up-regulated in obesity, and involved in the embryo development of pig; it was also detected in Northeast wild boar [86–89]. *HORMAD1* is linked with embryo development and productivity. Z. Zeng *et al.* noted *HORMAD1* to belong to growth-related Meishan pig genes [90]. *HORMAD1* was under heavy selection based on runs of homozygosity in a Large White pig population and associated with obesity [91]. *SEC63* was determined as candidate genes for estimated breeding values feed conversion ratio in Maxgro boars [92]. It was found an association between the *CIART* genotype and backfat thickness in Duroc pigs, and its expression is affected by food intake [93, 94]. According to Gene Ontology terms, *ENSA* is associated with regulation of insulin secretion and related to adipocyte development [95]. *ECM1* is involved in immunity and bone development. It was reported to be an important gene highly expressed in subcutaneous white adipose tissue (sWAT) as compared to brown adipocytes, and was determined in Korean Wild Boar, up-regulated in Congjiang Xiang pigs with large litter size and in testis tissue from Duroc boars [96–99]. *KLHL1* could be linked with Landrace and Yorkshire pig backfat thickness in Korea and involved in environmental adaptation [100, 101]. *NR2E1* is involved in developmental processes and linked with environmental adaptation concerning behavioral defense response in Xiang pigs [102]. It showed significant associations with feed conversion efficiency and growth rate in pigs [92]. *PRPF3* gene is differentially expressed in the *Longissimus dorsi* muscle being more abundant in Large White than in Wujin pigs [103]. *VPS45* could be linked with growth trait [104]. *FOXO3A* promotes metabolic adaptation and stress resistance in hypoxia, associated with carcass length, backfat thickness and drip loss, related to muscle development in Iberian pigs [105–107]. *FOXO3A* could promote lipid accumulation as well [108]. *Ssc-mir-10b* was downregulated in Tibetan pigs, related to hypoxia adaptation, play important roles in fat-related processes in adipose tissue, had been frequently reported highly expressed in skeletal muscle during porcine prenatal and postnatal developmental stages and abundantly expressed in subcutaneous adipose tissue in pigs [109–113].

In the Livni and Large White breeds the largest amount of common genes was detected and averaged 62, which formed two clusters. Cluster 1, with enrichment coefficient = 2.1, was characterized with genes involved in glucose metabolism. Among them, *G6PC2*, *HKDC1* and *HK1* are critical for glucose homeostasis. *HK1* effects on growth and meat quality in Polish Landrace [114]. It is important for sperm motility in Duroc, enriched in brown adipocytes of aged mice, up-regulated by severe cold and essential for brown adipocytes thermogenesis [115–118]. Cluster 2, with enrichment coefficient = 1.60, demonstrated helicase genes. *DDX21* is associated with immunity and belongs to the top 4 lymphocyte associated genes in pigs [119]. *SUPV3L1* is important for the maintenance of the skin barrier and related to percentage of certain fiber types [120, 121]. *MAP3K7* is also linked with immunity and strongly associated with neuropsychiatric processes [122]. It was reported to be associated with growth traits and adipocyte differentiation [100, 123]. *PIK3C2A* gene is related to hepatic insulin resistance and steatosis, average daily gain and lean meat percentage, intramuscular fat and backfat thickness in two Duroc populations, being under positive selection in all high-altitude species [124–127]. According to Gene Ontology terms, *ABCB11* is associated with fatty and bile acid metabolic process and could be involved in gene networks for intramuscular fatty acid composition in porcine [128, 129]. *ABCC8* was reported to be selection region for intramuscular fat and backfat thickness in two Duroc populations, and the most down-regulated genes in the group with higher backfat thickness in Yimeng black pigs [126, 130]. *DEF6*

is linked with average backfat thickness [131]. *FKBP5* is associated with immunity, backfat thickness and leaf fat weight, significantly contributed to residual feed intake [79, 131–133]. Expression of this gene is inversely associated with the expression of lipolytic, lipogenic and adipogenic genes [134]. According to Gene Ontology terms, *LDB3* is associated with heart development and muscle structure development, related to muscle growth traits in pig and may have potential roles in environmental adaptation [135, 136]. *ARMC12* regulates spatiotemporal mitochondrial dynamics during spermiogenesis and is required for male fertility [137]. *BMPR1A* is associated with numerous developmental processes, identified as a novel candidate gene affecting the number of thoracic vertebrae in pigs, and regulates the development of hypothalamic circuits that are critical to the feeding behavior [138, 139]. Additionally, *BMPR1A* is important in brown fat development and involved in browning of white adipose tissue [140, 141]. *CCAR1* positively regulates adipocyte differentiation [142]. *CLPSL2* and *CLPS* are linked with digestion, lipid catabolic process, and response to food. *CLPSL2* could be involved in the regulation of acrosomal integrity, spermatozoa motility, and male fertility, while *CLPS* demonstrated effect on some characteristics connected with lean content of the carcass and fat content and affected intramuscular fat content [143–145]. It may be associated with former selection toward reduced fat content in carcass [114]. According to Gene Ontology terms, *COL13A1* is associated with skeletal system development, was under significant positive selection Yorkshire pigs and associated with fat deposition, as well as *HNRNPH3* [146–148]. *JMJD1C* is potentially associated with cold adaptation [149, 150]. It demonstrates the positive selection in regulation of various reproductive traits in pigs [151–154]. *JMJD1C* was identified in Tibetan pigs that are well adapted to the high altitude [155]. On the other hand, this gene have been associated with white blood cells in Large White pigs, identified as a novel regulator of adipogenesis and contributed to browning [156–158]. According to Gene Ontology terms, *MYOD1* and *MYPN* are strongly involved in skeletal muscle tissue development. It was reported about potential role of *MYOD1* in body-fat distribution regulation [159]. Mutations in the *MYOD1* gene show a significant effect on the pork meat quality and single nucleotide polymorphisms in the porcine *MYOD1* affected on meat quality traits and carcass traits in heavy pigs [160–162]. *MYPN* is related to body composition and can be considered as candidate for meat and carcass traits in pigs [163–165]. *NRBF2* is linked with energy metabolism and was specific selective for Tibetan pig [155]. *NUCB2* is expressed in fat depots of the pig and that level of expression is sensitive to stimulation of appetite-regulating pathways in the hypothalamus [166]. It plays an important role in whole-body energy homeostasis and body weight at puberty by regulation of appetite of Jinhua Pigs [126]. *NUCB2* is also involved in cold adaptation, indicating that central nesfatin-1 regulates thermogenesis [167, 168]. *REEP3* mediates adipogenic differentiation [169]. According to Gene Ontology terms, *SIRT1* is linked with regulation of lipid storage, white and brown fat cell differentiation, adipose tissue development, etc. It is implied in the browning of white adipose tissue, promotes lipid metabolism and mitochondrial biogenesis in adipocytes and coordinates abiogenesis by targeting key enzymatic pathways [170, 171]. Apart from that, it negatively correlates with intramuscular fat content and demonstrates protective role in skeletal muscle's adaptation to cold stress [172, 173]. *SNCG* controls metabolic functions in fat cells and belongs to white adipose tissue-selective genes [174, 175]. *ZNF76* is very close to peroxisome proliferative activated receptor delta (PPARD) at 35 Mb, which is a positional and physiological candidate for affecting backfat thickness [176]. *RNF213* is involved in adipogenesis and emerged as a link between obesity, inflammation, and insulin resistance [177, 178]. *SNORD14* were more expressed in Large White heavy pigs with high intramuscular fat content [179]. *U3* was identified as a promising candidate gene for average backfat thickness in multiple pig breeds and populations [180].

Annotated clusters with an enrichment coefficient $-\log10(p) > 1.3$ (corresponds to $p < 0.05$) were not determined for the Livni breed. However, 50 candidate genes were specific to Livni pigs. *DLGAP5* is a stillbirth associated gene involved in lipid deposition-related pathways and significantly associated with intramuscular fat content [181–183]. *ERCC4* is also associated with intramuscular fat content, presented in Tibetan wild boar and related to "response to UV" [151, 184, 185]. *GPR63* has been identified as a receptor for intercellular lipid messengers and associated with reproduction traits [186, 187]. According to Gene Ontology terms, *LDB3* is involved in heart and muscle structure development, while *PBX3* – in various important developmental processes. *PDZRN3* and *ATG14* could affect intramuscular fat content in Suhuai pigs [183, 184]. They are involved in adipocyte differentiation, demonstrating negatively influence [188, 189]. *RBM15B* is linked with average daily gain in Italian Large White pigs, while *TBPL2* – with fertility [190, 191]. *WDHD1* is associated with stillbirth in Large White sows and residual feed intake [79, 181]. According to Gene Ontology terms, *BMPR1A* is associated with immunity, bone, lung and heart development. *BMPR1A* is reported to be associated with obesity and important for brown adipocytes, candidate gene affecting the number of thoracic vertebrae in a Large White × Minzhu intercross pigs [138, 192–194]. *DOCK3* is linked with fatness and growth in Huainan pigs [195]. *LGALS3* is linked with immunity, sensitive to cold exposure, associated with stillbirth, and involved in adipogenesis [181, 196–198]. *GLUD1* is an important gene for metabolic process, increased by cold exposure and essential for brown adipocytes [199, 200]. *MANF* positively regulates thermogenesis, resists obesity, as well as regulates hypothalamic control of food intake and body weight [201–203]. *MAPK1IP1L* and *SOCS4*

are likely candidate genes for stillborn [181, 204]. According to Gene Ontology terms, *MEF2C* is involved in numerous developmental processes, may be a key gene in insulin-induced adipocyte differentiation, involved in fat deposition in pigs, important for foetal developmental, and associated with total number born and number born alive [205–207]. *WISP3* is linked with sketetal and muscular development [208]. *PDE10A* is associated with chest circumference in Yorkshire Pigs, back fat thickness at 100 kg in Landrace pigs, and contributes to the regulation of energy homeostasis [209–211]. *PARN* was identified as candidate genes associated with age at 100 kg in Large White pigs [212]. *TFAM* promotes mitochondrial DNA content, which necessary for increased fusion during cold adaptation [213]. Its amount significantly elevated after cold exposure and essential for thermoregulation [214, 215]. Mutation in the *TFAM* gene effects on fattening and carcass traits in commercial pig populations [216]. *TFAM* gene expression abundance in particular tissues such as liver and *L. dorsi* revealed some strong correlations with carcass and meat quality traits including marbling [217]. *SNORD22* is associated with trimmed thigh weight in Italian crossbred pigs [218]. *U4* and *ssc-mir-9-2* were previously determined in pigs [219, 220]. Genes associated with reproductive, meat and fat quality, carcass, and immunity traits in pigs were found in genomic regions affected by putative selection. Along with fatting genes, ones linked with thermogenesis were unexpectedly detected which oppositely should led to fat reduction. However, pigs could not have brown adipocytes but could have beige ones, which are very important for maintaining alternative mechanisms of thermoregulation in pigs that possibly avoid fat reduction [221–224].

CONCLUSION

The dramatic reduction of local pig breeds during last 30 years finally led to 0.56% of the total pig population in the RF, mainly Livni, Altai, and Tsivilsk breeds. There are several reasons for the reduction of local pig breeds: a trend to the reduction the total amount of fat on pork carcass and in meat and the aggressive implementation of the Western commercial breeds. Commercial breeds were bred without taking into account Russia environment, the quality and composition of feed and drinking water. Local pigs bred in the USSR are characterized by unpretentiousness to feed, stress and cold resistance, as well as precocity and high productivity. Livni is one of the Russian local pig breeds. Landrace and the Large White breeds participated in creation of the Livni breed, but obtained breed relationship and admixture results indicated the insignificant participation of these breeds in the formation of the modern allelofund of Livni pigs. The largest amount of common genes was detected between the Livni and Large White breeds. Genes involved in glucose metabolism, namely *G6PC2*, *HKDC1*, and *HK1* are critical for glucose homeostasis, which could effect on the growth and meat quality traits, as well as on thermogenesis. Other genes were associated with immunity, related to percentage of certain fiber types, growth traits, average daily gain and lean meat percentage, intramuscular fat and backfat thickness, etc. Among 35 common genes of the Livni and Landrace breeds, enrichment with *HOXD* genes was observed. *HOXD* genes are involved in various developmental processes, such as single fertilization, skeletal muscle tissue development, adult locomotory behaviour, embryonic skeletal system morphogenesis, lipid metabolism, etc., and are associated with traits such as growth, body weight and composition, fat development, organogenesis and feed intake, etc. Candidate genes associated with various growth, carcass and reproductive traits and essential for thermoregulation were specific to Livni pigs. Livni breed belongs to the meat-and-fat type, but during development pigs could be also meat and fat types. The analysis of genetic architecture confirmed the unique structure of local breed that was bred using commercial Landrace and the Large White breeds. During formation own allelofund, the Livni breed fixed important traits, including flexibility during growing and feeding.

CONTRIBUTION

I.M. Chernukha conceived and designed the study. I.M. Chernukha, L.V. Fedulova and E.A. Kotenkova designed the methodology. L.V. Fedulova and E.A. Kotenkova analysed and described the results. I.M. Chernukha and E.A. Kotenkova wrote the manuscript. All authors contributed to data interpretation.

CONFLICT OF INTEREST

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

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Antioxidant, anti-inflammatory, antimicrobial, and anticancer properties of green broad bean pods (*Vicia faba* L.)

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Abstract:
This study featured broad/fava bean pods as by-products of food production. It assessed the chemical composition of green bean pods (*Vicia faba* L.) and their methanolic extract.
The extract was tested *in vitro* for antioxidant, anti-inflammatory, antimicrobial, and anticancer activities against prostate cancer (Pc3) and liver cancer (HepG2) cells. Broad bean pods proved to be rich in carbohydrates, fiber, protein, potassium, calcium, and magnesium. The extract contained 286 mg GAE/g total phenols and 105 mg QE/g total flavonoids. The antioxidant activity of the methanolic extract was measured by 1,1-diphenyl-2-picryl hydrazyl (DPPH) assay. The highest DPPH scavenging activity belonged to the extract concentrations of 1000 µg/mL (80.5%) and 500 µg/mL (73.7%), whereas the IC_{50} value was 87.35 µg/mL. The methanolic extract possessed the anti-inflammatory effect as it significantly reduced the hemolysis of red blood cells. The maximal inhibition percentage reached 66.7% at 1000 µg/mL. Regarding the antimicrobial activity, the broad bean pod methanolic extract inhibited *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, as well as *Candida albicans*. The extract reduced the cell viability of human hepatocarcinoma (HepG2) and prostate cancer (PC3) cells in a concentration-dependent manner. It also caused significant changes in cell shape, compared to the control.
Therefore, broad beans can be recommended for human consumption together with pods, fresh or cooked, as a potential source of bioactive substances in functional food production.

Keywords: *Vicia faba* L. pods, anticancer effect, antioxidant activity, anti-inflammatory properties, antimicrobial ability, DPPH radicals

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INTRODUCTION

Medical drugs may have side effects and are often expensive. As a result, people tend to turn to natural plant products and medicinal plants in search of nutrients and health-beneficial phytochemicals. Legumes are an important source of protein, carbohydrates, and fiber. In addition, they are low in fat [1, 2]. Legumes are introduced into human diet for numerous nutritional and health-related properties, e.g., phenolic compounds, oligosaccharides, enzyme inhibitors, phytosterols, and saponins [3, 4]. Legumes are also known to reduce the risk of cancer, cardiovascular diseases, hypertension, and diabetes [5–7].

Broad beans (*Vicia faba* L.) are a popular food of plant origin that belongs to the *Fabaceae* (*Leguminosae*) family and the *Vicia* gene cluster [8]. Broad beans are also called fava/faba beans, broad beans, Windsor beans, horse beans, tick beans, etc. In Hindi, *V. faba* is known as *kalamatar* or *bakala* [9]. *V. faba* has four subspecies that differ in the size of seeds: major (large seeds), equine (medium seeds), minor (small seeds), and paucijuga (small seeds) [10].

Broad beans are cultivated in many regions of the world, including Egypt, India, the Netherlands, Spain, Sudan, Saudi Arabia, and China. The seed coat can be white, buff (or beige), purple, green, or red. However, buff beans are the most accepted for human consumption.

Phenolic compounds are micro components that receive a lot of scientific attention due to their health-improving qualities, e.g., antioxidant activity. Procyanidins, catechins, flavanols, isoflavones, phenolic acids, and tannins are natural antioxidants, and broad beans contain them all [11–14]. Phenolic chemicals of plant origin impede the digestion of lipids and carbohydrates, thus inhibiting their absorption. They may reduce postprandial hyperglycemia in diabetic patients and facilitate weight loss in patients with obesity [15].

The high content of flavonoids and phenolic acids renders *V. faba* coat antioxidant and anticancer properties [16]. The acetone extract of its seed coat revealed antioxidant, antibacterial, anti-inflammatory, and anticancer properties [17]. Mejri *et al.* reported that the methanolic extract of broad bean pods decreased the high levels of serum alanine aminotransferase, aspartate aminotransferase, creatinine, and uric acid in the serum of diabetic rats [18]. The methanol extract of broad bean pods also reduced oxidative stress by activating such antioxidant enzymes as catalase, glutathione peroxidase, and superoxide dismutase [18, 19]. Broad beans lowered blood sugar and total cholesterol as well as prevented heart conditions, eye diseases, various cancers, and dysfunction of kidney and liver [16, 20–25].

Egypt is one of the leading consumers of broad beans. There, they are known as *ful*. Stewed (*ful medames*) or fried broad beans (*falafel*) are considered the main dish of a typical Egyptian breakfast. Broad bean pods are usually cast off as wastes. However, young broad bean pods are traditionally consumed together with beans in Egyptian village cuisine.

This research tested *in vitro* the methanolic extract of *V. faba* pods for their antioxidant, antimicrobial, anti-inflammatory, and anticancer properties.

STUDY OBJECTS AND METHODS

Materials. Immature broad bean *Vicia faba* L. pods were purchased on a local market in Mansoura, Egypt.

Chemicals. All chemicals were obtained from Al-Gomhoria Company (Mansoura, Egypt), which produces medicines and medical supplies.

Permission to conduct the experiment was granted by the Scientific Research Ethics Committee of the Faculty of Specific Education, Mansoura University (No, 12-3/11/22).

Methods. ***Preparing pod powder.*** Broad beans were cleaned and thoroughly washed in water. Afterwards, the beans were separated, and the green pods were oven-dried at 40°C until constant weight, ground to a fine powder, and stored at −20°C.

Preparing methanolic extract. We soaked 250 g of pod powder in 1 L methanol, mixed, left it overnight, and filtered through filter paper. The filtrate was kept in a dark-glass bottle. After that, we took another portion of methanol, added it to the residue, shook thoroughly, left it overnight, and filtered. The new filtrate joined the previous one. Finally, the residue was resoaked in methanol overnight and filtered. The three filtrates were collected to make the methanolic extract solution. We removed the solvent by evaporating it in a rotary evaporator. The obtained extract was collected and dried in a desiccator to a constant weight, then kept in dark-glass bottles for further use.

Chemical analysis. The methods recommended by the Association of Official Analytical Chemists provided experimental data on ash, fat, fiber, protein, and moisture contents [26]. Carbohydrates were calculated as 100 – (ash + fiber + protein + water). We employed the method of inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Horiba Jobin-Yvon Ultima 2 CE) to determine the mineral composition under optimal experimental conditions [27].

Phytochemical screening. The pod extract underwent phytochemical tests for the qualitative profile of glycosides, phenolics, tannins, alkaloids, flavonoids, and saponins. This part of the research followed the methods described by Trease & Evans and Harborne [28, 29].

Total phenolics and total flavonoids. We applied the Folin-Ciocalteu colorimetric method as recommended by Singleton & Rossi to define the total phenolic content at 765 nm [30]. The results were expressed as 1 mg gallic acid equivalent per 1 g pod extract (mg GAE/g). The total flavonoid content was calculated using the method described by Dehpour *et al*., i.e., colorimetericall-ly at 415 nm [31]. The results were represented as 1 mg quercetin equivalent per 1 g extract (mg QE/g).

Antioxidant activity. *DPPH radical scavenging assay.* The methanolic extract of broad bean pods was tested for its capacity to scavenge free radicals with the help of 1,1-diphenyl-2-picryl hydrazyl (DPPH). Initially, 1 mL of DPPH methanol solution (0.1 mM) was mixed with 3 mL of the pod extract at various concentrations: 3.9, 7.8, 15.62, 31.25, 62.5, 125, 250, 500, and 1000 g/mL. The mix was briskly shaken before being left to stand at room temperature for 30 min. After that, we used a UV-visible spectrophotometer to detect absorbance at 517 nm [32]. The log dosage inhibition curve made it possible to determine the IC_{50} value, i.e., the concentration the sample needed to block 50% of the DPPH free radical. If the absorbance was low, the free radical activity was high [33]. The percentage of the DPPH scavenging effect, %, was calculated by the following Eq. (1)

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

where A_0 was the absorbance of the control reaction; A_1 was the absorbance of the extracted samples.

Ferric reducing power assay. We evaluated the antioxidant capacity of the sample extract using the reducing power as described by Debnath *et al*. [34]. In line with the procedure, we combined 1 mL solution with 2.5 mL of sodium phosphate buffer (0.2 mM, 6.6 pH) and 2.5 mL of 1% $K_3[Fe(CN)_6]$. The resulting mix incubated at 50°C for 20 min. To halt the reaction, we added aliquots of 10% CCl_3 COOH (2.5 mL). Finally, 2.5 mL reaction mix, 2.5 mL distilled water, and 1 mL fresh 0.1% $FeCl_3$ solution reacted at room temperature for 10 min. The absor-

bance was measured at 700 nm. High absorbance corresponded with high reducing power.

In vitro anti-inflammatory assay. *Preparing erythrocyte suspension.* Three healthy volunteers provided blood, 3 mL each, which was collected into heparinized tubes and centrifuged at 3000 rpm for 10 min. The red blood pellets were dissolved in a volume of normal saline equal to the supernatant. Dissolved red blood pellets were measured in volume and reconstituted in an isotonic buffer solution (10 mM sodium phosphate buffer, pH 7.4) as a 40% v/v suspension to be used later as the erythrocyte suspension.

Hypotonicity-prompted hemolysis. In centrifuge tubes, we dissolved the pod extract samples in a hypotonic solution (distilled water) at concentrations of 100, 200, 400, 600, 800, and 1000 µg/mL. Isotonic solutions (5 mL) were also prepared in centrifuge tubes with 100–1000 µg/mL of pod extracts. In addition, the vehicle control tube contained 5 mL of distilled water. Each sample received 0.1 mL erythrocyte suspension and was mixed lightly. The tubes were incubated at 37°C for 1 h and then centrifuged at 1300 g for 3 min. To determine the hemoglobin content in the supernatant, we measured the absorbance, or optical density (*OD*), at 540 nm.

The inhibition percentage of hemolysis, %, was calculated as follows:

$$\text{Inhibition of hemolysis} = \frac{OD_2 - OD_1}{OD_3 - OD_1} \times 100$$

where OD_1 was the absorbance of the extracted sample in the isotonic solution; OD_2 designated the absorbance of the extracted sample in the hypotonic solution; OD_3 stood for the absorbance of control sample in the hypotonic solution.

Antimicrobial activity of broad bean pod methanolic extract. Agar well diffusion method: the agar well diffusion made it possible to assess the antibacterial activity of the pod extract. We covered the entire agar surface with microbial inoculum and diluted the extract solution to the necessary concentration. A well with a diameter of 6 to 8 mm was drilled aseptically with a sterile drill. The agar plates were incubated in the proper environment for each type of microbe. The widths of the acquired inhibition zone around the wells (mm) were measured after 16–24 h (*Mucoraceae*), 24 h (*Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*), and 48 h (other microbial species) of incubation. Gentamicin was used as a reference standard at a concentration of 4 µg/mL. The abovementioned microbial strains could not develop in agar media due to the potent antimicrobial properties of the extract, which diffused into the medium [35].

Effect of pod methanolic extract on Pc3 and HepG2 cells. Cell viability and proliferation assay (MTT): the MTT test assessed the cytotoxic activity of the methanolic extract of broad bean pods against HepG2 and PC3 cells. The method involved a 96-well culture plate as recommended in [36].

A full monolayer sheet formed after 24 h of incubation at 37°C with 1×10^5 cells/mL (100 µL/well) in the 96-well tissue culture plate. After the confluent sheet of cells had developed, we decanted the growth medium from 96-well microtiter plates and washed the cell monolayer twice with wash media. Then, we prepared twofold dilutions of the extract in RPMI medium with 2% serum as a maintenance medium. Three wells served as controls and received only the maintenance medium after 0.1 mL of each dilution was poured in various wells. The plate was tested after incubation at 37°C. Cells were evaluated for any indications of toxicity, such as shrinkage, granulation, or a partial/total loss of monolayer. After adding 20 µL of MTT solution (5 mg/mL) to each well, the medium was mixed with the MTT using a shaking table at 150 rpm for 5 min. The MTT then metabolized during 1–5 h of incubation at 37°C and 5% CO_2. After that, the medium was discarded, and the plate was dried with a paper towel to remove residue, if necessary. Then, we resuspended metabolic by-product of MTT, formazan, in 200 µL of dimethyl sulfoxide and agitated it at 150 rpm for 5 min to combine formazan with the solvent. The optical density was measured at 560 nm, the subtract background was determined at 620 nm. The cell count and optical density were directly connected.

Statistical analysis. The data were presented as the mean ± SD. All tests were processed using the SPSS statistical analysis program (Version 24), as described by McCormick & Salcedo [37].

RESULTS AND DISCUSSION

Proximate chemical analysis of broad bean (*Vicia faba* L.) pods. Table 1 shows the chemical composition of the broad bean pods in their green state after oven-drying at 50°C. The dried pods contained 9.27% moisture, 8.38% protein, 0.38% fat, 7.22% ash, 14.59% fiber, and 60.16% carbohydrates. In our research, the content of carbohydrates and dietary fiber appeared to be quite high. However, Mateos-Aparicio *et al.* reported different data, especially for fiber and protein: 40.1% dietary fiber, 13.6% protein, 6.3% ash, and 1.3% fat on a dry weight basis [38]. Our results also differed from those published by Mejri *et al.*, who detected a high moisture content of 79.26% on a wet weight basis, with 13.81% proteins, 18.93% carbohydrates, 0.92% lipids, and 57.46% dietary

Table 1 Proximate chemical composition of broad bean pods, g/100g dry weight

Components	Moisture	Protein	Fat	Ash	Fibers	Carbohydrates
Proximate composition	9.27 ± 0.08	8.38 ± 0.06	0.38 ± 0.03	7.22 ± 0.10	14.59 ± 0.03	60.16 ± 0.16

Each value is the mean ± SD

Table 2 Mineral contents of broad bean pods

Minerals	Concentration, mg/100 g dry weight
Potassium	3.483
Calcium	937.2
Magnesium	340.4
Phosphorus	340.1
Iron	32.09
Copper	4.442
Manganese	2.189

Table 3 Phytochemical screening of broad bean pods methanolic extract

Glycosides	Phenols	Tannins	Alkaloids	Flavonoids	Saponins
+++	+++	+	+	++	+

Table 4 Total phenols and flavonoids in broad bean pod methanolic extract

Total phenols	286 mg GAE/g
Total flavonoids	105 mg QE/g

fiber [18]. In a study reported by Vernaleo *et al*., fava beans proved rich in dietary fiber and phytonutrients, e.g., isoflavone and plant sterols [39]. The differences in the chemical composition of broad bean pods obtained by different research teams could be attributed to the geographical location, handling, processing, or variety.

Mineral contents of broad bean pods. Table 2 demonstrates the mineral profile of broad bean pods per 100 g. Obviously, broad bean pods proved to be a good source of potassium, calcium, magnesium, and iron. Our results were in line with those by Vernaleo *et al*., who also revealed that broad beans were rich in phosphorus, iron, copper, manganese, calcium, magnesium, and potassium [39]. Similarly, Mateos *et al*., who studied broad bean pods as by-products, reported that they contained a lot of potassium, calcium, and iron [38].

Phytochemical screening of broad bean pod methanolic extract. Table 3 illustrates the results of a phytochemical screening, which revealed phenolic compounds, flavonoids, glycosides, tannins, alkaloids, and saponins in the methanolic extract of broad bean pods.

The ethanolic extract of *V. faba* L. was found to contain all phytochemicals except for anthracenosides, sterols, and triterpenes (*Fabaceae*). The aqueous extract contained less tannins, alkaloids, glycosides, sterol, triterpenes, and saponins than the ethanolic extract. Reducing sugars were present in the ethanolic extract exclusively [40]. Broad beans are known to contain polyphenols in leaves, roots, and seeds [41]. The content of cotyledons in beans was reported to exceed that in hulls. According to recent studies, broad beans and their derivatives may be included in diets against hypertension, diabetes, and cardiovascular diseases [42].

Total phenols and total flavonoids in broad bean pod methanolic extract. Table 4 shows the total phenols and flavonoids in the methanolic extract of broad bean pods. The phenol content was 286 mg GAE/g, while the total flavonoid content was 105 mg QE/g. The data obtained were higher than those reported by Mejri *et al*., where the total phenolic compounds in the methanol extract of broad bean pods were 115.21 mg GAE/g extract and the total flavonoids were 47.34 mg QE/g extract [18]. According to Valente *et al*., the total free phenols in dried pods depended on the variety and ranged from 10.87 to 26.34 mg/100 g, while the total esterified phenolics ranged from 8.76 to 26.72 mg/100 g dry weight [43]. Chan *et al.* reported that the methanolic extract of broad bean pods was rich in total phenolics and flavonoids, including numerous polar aglycones and flavonoid glycosides [44]. The phenolic content issue still requires more scientific attention. Chaieb *et al.* studied 13 genotypes of broad bean pods grown in the same area and under the same conditions [45]. Their phenol content ranged from 56.97 to 149.21 mg GAE/g whereas the total flavonoids ranged from 10.23 to 45.92 mg RE/g, depending on the genotype.

Antioxidant activity of broad bean pod methanolic extract. ***DPPH assay.*** The antioxidant activity of the broad bean pod extract was measured by 1,1-diphenyl-2-picryl hydrazyl (DPPH) assay. Table 5 and Fig. 1 show that the DPPH scavenging percentage increased together with the extract concentration. The highest value of DPPH scavenging activity reached 80.5% at the extract concentrations of 1000 µg/mL. The concentrations of 500 and 250 µg/mL also showed high levels of DPPH scavenging activity, which reached 73.7 and 65.7%, respectively.

IC_{50} is the concentration of the antioxidant substance needed to reduce the initial DPPH concentration by 50%. Low IC_{50} indicates high antioxidant activity. In our research, IC_{50} was quite low and equaled 87.35 µg/mL, which means that the broad bean pods had high antioxidant activity. Mateos-Aparicio *et al.* also reported high reducing power and free-radical scavenging activity of polyphenols extracted from broad bean pods [19]. The antioxidant activity of broad bean pods probably came from their high phenolic content [46].

Some plants are known to contain natural substances with good anticancer potential. Broad bean pods are rich in fiber, phenolic acids, and flavonoids, which can prevent the oxidation of cell membranes and protect the cells from free radicals and toxic substances. In addition, tannins in broad beans could provide hydroxyl radical scavenging activity [47]. Hypothetically, broad bean pod extract prevents the reaction of hydroxyl radicals with the hydrogen atoms of the sugar moiety of DNA and hence protects DNA from damage [48].

Antioxidant activity of broad bean pod methanolic extract: reducing power. Table 6 shows that the reducing power of the broad bean pod methanolic extract increased together with its concentration. The IC_{50} values reached 177.32 mg/mL.

Any substance with a reducing power combines with potassium ferricyanide (Fe^{3+}) to generate potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride

Table 5 Antioxidant activity of broad bean pod methanolic extract: DPPH assay

Extract concentration, µg/mL	Optical density	DPPH scavenging %
1.000	0.295	80.5
500	0.397	73.7
250	0.518	65.7
125	0.687	54.5
62.50	0.879	41.8
31.25	0.978	35.3
15.625	1.158	23.4
7.8125	1.224	19.0
3.90	1.305	13.6
1.95	1.356	10.3
IC_{50} (87.35 µg/mL)		

Table 6 Antioxidant activity of broad bean pod methanolic extract: reducing power

Concentration, mg/mL	100	200	400
Inhibition, %	33.9	61.04	79.1
IC_{50} (177.32 mg/mL)			

Table 7 Anti-inflammatory activity of broad bean pod methanolic extract

Concentration, µg/mL	Absorbance		Hemolysis inhibition, %
	Hypotonic solution	Isotonic solution	
Control	0.759		0
1000	0.291	0.057	66.7
800	0.341	0.051	59.0
600	0.367	0.045	54.9
400	0.394	0.041	50.8
200	0.429	0.039	45.8
100	0.503	0.036	35.4

to form a ferric-ferrous complex, or Perls Prussian blue, which is absorbed at 700 nm [49]. Reductive action and antioxidant activity are connected [50]. As mentioned before, the BBP methanol extract demonstrated high antioxidant activity (60.72%). The lowest IC_{50} for the DPPH and ABTS assays corresponded with the highest free radical scavenging activity [18].

Anti-inflammatory activity of broad bean pod methanolic extract. We appealed to the HRBC (human red blood cells) method in vitro to study the anti-inflammatory effect of the broad bean pod extract. According to the procedure, the erythrocyte membrane and the lysosomal membrane are comparable; therefore, by stabilizing the erythrocyte membrane, the extract from broad bean pods may stabilize the lysosomal membrane.

Table 7 shows that all the extract concentrations exhibited a significant reduction in the hemolysis of red blood cells: the maximal inhibition percentage reached 66.7% at 1.000 µg/mL. The inhibition percentage decreased with lowering the extract concentration. Therefore,

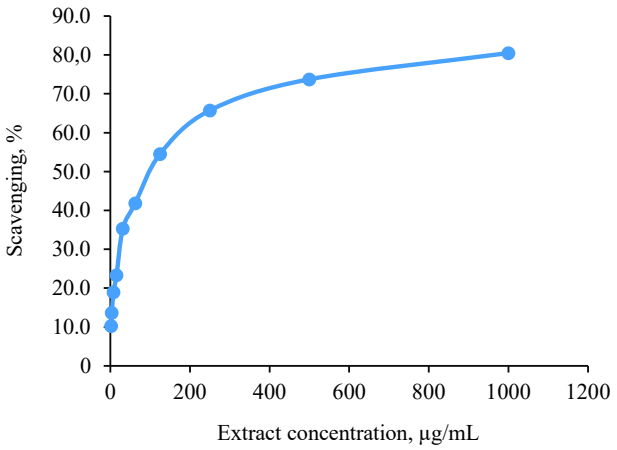

Figure 1 Scavenging activity percentage of DPPH by methanolic extract of broad bean pods

the pod extract indeed possessed anti-inflammatory properties in the studied models.

The hypotonic solution causes hemolysis of red blood cells because fluid accumulates in the cells, thus rupturing their membranes. The damaged red blood cells become more susceptible to lipid oxidation via free radicals. As a result, some components, e.g., protein and fluids, start entering the tissues, which is similar to inflammation [51].

The extract of broad bean pods proved able to preserve the red blood cell membranes by preventing the oxidation of lipids in them. In addition, it stabilizes the red blood cell membrane by preventing the production of lytic enzymes and active inflammatory mediators.

In this research, the broad bean pod extract proved to contain flavonoids, alkaloids, and saponin, which are known for their anti-inflammatory properties. Many studies reported the antioxidant and anti-inflammatory effects of plant flavonoids [52–54].

Plant flavonoids may owe their anti-inflammatory properties due to their ability to inhibit the enzymes of arachidonic acid metabolism, as well as the enzymes that contribute to the production of inflammatory mediators [55, 56].

Antimicrobial activity of broad bean pod methanolic extract. The antimicrobial activity of the methanolic extract isolated from broad bean pods was assessed *in vitro* by the agar well diffusion method against four pathogenic bacteria strains and two kinds of fungi. The bacteria strains included two Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and two Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) samples, while the two fungi were represented by *Candida albicans* and *Aspergillus fumigatus*. Antimicrobial activity was determined by agar diffusion (100 µL), 6.0 mm disc diameter. All samples were dissolved in normal saline (0.9% NaCl), which had no antimicrobial activity against all the tested pathogenic strains.

Table 8 shows that the pod extract prevented the bacterial growth of *B. subtilis*, *S. aureus*, *E. coli*, and

Table 8 Antimicrobial activity of broad bean pod methanolic extract

Pathogenic microorganism	Inhibition zone diameter, mm	
	Sample	Reference (gentamicin)
Bacillus subtilis (ATCC 6633)	16	[25]
Staphylococcus aureus (ATCC 6538)	17	[15]
Escherichia coli (ATCC 8739)	15	[17]
Pseudomonas aeruginosa (ATCC 90274)	28	[22]
Candida albicans (ATCC 10221)	23	[21]
Aspergillus fumigatus	n.d.	[15]

n.d. – not detected

Table 9 Effect of broad bean pod methanolic extract on liver and prostate cancer cells *in vitro*

Pod extract Concentration, µg/mL	Liver cancer cells (HepG2)		Prostate cancer cells (PC3)	
	Viability	Toxicity	Viability	Toxicity
Control	100.0^{a}	0.00^{f}	100.00^{a}	0.00^{f}
1.000	$4.07^{f} \pm 0.12$	$96.02^{a} \pm 0.26$	$3.43^{f} \pm 0.78$	$96.44^{a} \pm 0.59$
500	$18.46^{e} \pm 2.79$	$81.54^{b} \pm 2.79$	$11.59^{e} \pm 0.98$	$88.41^{b} \pm 0.98$
250	$35.52^{d} \pm 7.21$	$64.48^{c} \pm 7.21$	$21.32^{d} \pm 2.83$	$78.68^{c} \pm 2.83$
125	$49.54^{c} \pm 2.81$	$50.40^{d} \pm 2.91$	$48.63^{c} \pm 3.58$	$51.38^{d} \pm 3.58$
62.5	$89.99^{b} \pm 1.49$	$10.01^{e} \pm 1.49$	$89.52^{b} \pm 2.62$	$10.48^{e} \pm 2.62$
31.25	$99.83^{a} \pm 11.90$	$0.17^{f} \pm 11.90$	$99.17^{a} \pm 2.52$	$0.83^{f} \pm 2.52$
IC_{50} dil.	126.97 µg/mL		125.12 µg/mL	

P. aeruginosa. It also inhibited fungus *C. albicans*. The corresponding inhibition zones were 16, 17, 15, 28, and 23 mm, respectively. However, the *A. fumigatus* fungus appeared resistant to the broad bean pod extract. The antimicrobial effect was more effective against *S. aureus*, *P. aeruginosa*, and *C. albicans* than gentamycin, which served as reference control. To some extent, these results agreed those reported by Peyvast & Khorsandi, who also registered the antimicrobial activity of broad bean seed hull ethanolic extract against *E. coli*, *B. subtilis*, and *S. aureus* [57].

Anticancer activity of broad bean pod methanolic extract. Liver cancer is the fourth most common cause of death in the world [58]. This type of cancer has high mortality and morbidity because hepatitis C virus infection has become wide-spread in the last decades. Hepatitis C virus is the leading cause of cirrhosis, which is one of the risk factors for liver cancer [59, 60]. Since anticancer medications have so many side effects, natural products have good prospects as a novel anticancer remedy.

In this study, we tested the effect of methanolic extract of broad bean pods on human hepatocellular carcinoma (HepG2) and prostate cancer (PC3) cells. Table 9 and Figs. 2 and 3 show that the methanolic extract of broad bean pods reduced cell viability and increased cell toxicity of both HepG2 and PC3 in a concentration dependent manner. The low extract concentration of 31.25 had no significant effect on cell viability. However, all other concentrations caused significant decreases in the viability of the two kinds of cells, increasing their cell toxicity. In HepG2 cells, the viability for the extracts with concentrations of 125, 250, 500, and 1000 µg/mL was 49.54, 35.52, 18.46, and 4.07%, respectively; in PC3 cells, it was 48.63, 21.32, 11.59, and 3.43%, respectively. The broad bean pod methanolic extract exhibited high toxicity to HepG2 and PC3 cells: the toxicity percentage exceeded 96% for 1000 µg/mL. The IC_{50} values of the pod extract were observed at concentrations of 126.97 µg/mL for HepG2 cells and 125.12 µg/mL for PC3 cells, which are good results for an anticancer agent.

Figures 2 and 3 demonstrate that the methanolic extract of broad bean pods caused remarkable alterations in the cell shape, compared to the control. The changes in the cellular morphology increased together with the extract concentration. A large amount of dead and detached cells indicated a toxic effect of the pod extract on the proliferation of tumor cells after 24 h of incubation. The low concentration of 31.25 µg/mL caused no significant alterations. In contrast, high concentrations triggered substantial changes in the morphology of the tumor cells, and these changes increased together with the extract concentration.

Plant by-products burden the environment, and their utilization attracts a lot of scientific attention. For example, some fruit and vegetable wastes can be used as feed for cattle and sheep; others can be used in soil fertilization [61]. Bioactive components found in plant and vegetable wastes can become a source of antioxidant and anticancer nutraceuticals [62]. In addition, polyphenols and micronutrients found in legumes possess important biological values [63, 64]. Polyphenols are known to protect the human organism from chronic diseases, such as cardiovascular conditions, diabetes, asthma, cancer, and inflammation [65]. The anticancer

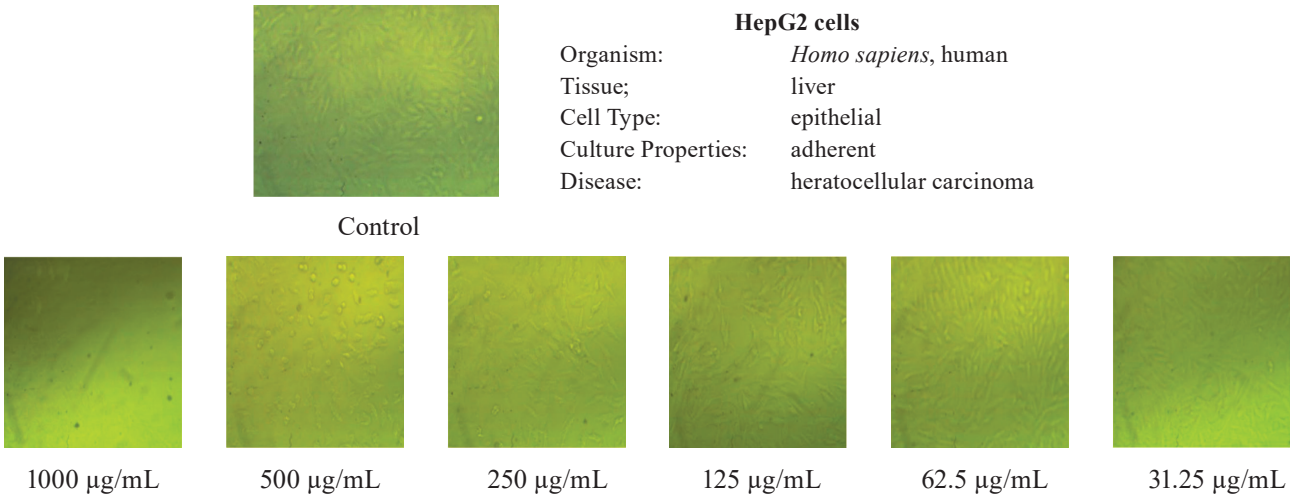

Figure 2 Effect of broad bean pod extract on HepG2 cells at different concentrations

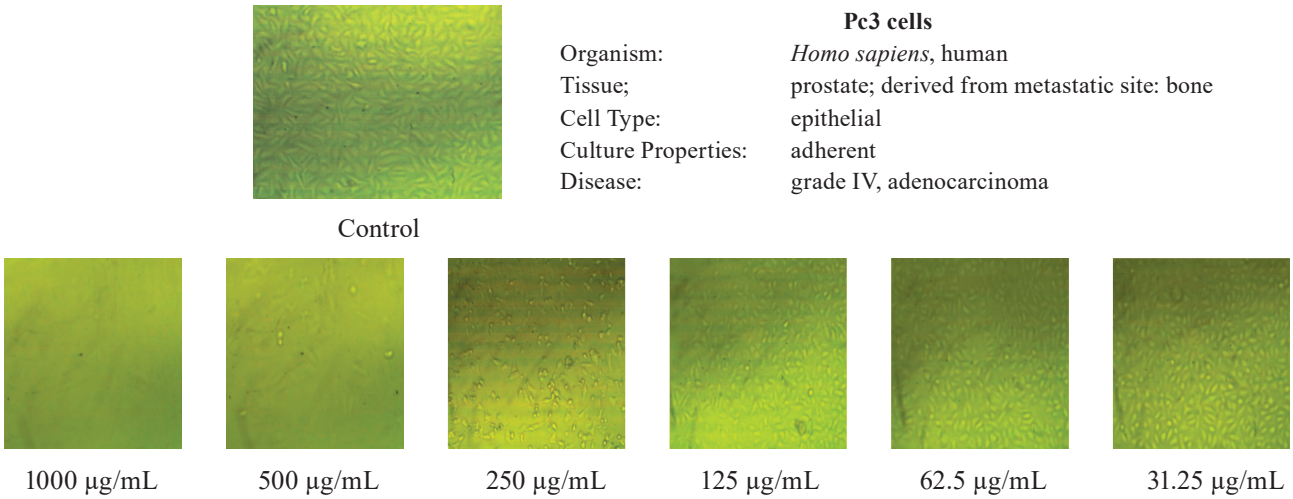

Figure 3 Effect of broad bean pod extract on Pc3 cells at different concentrations

activity of the broad bean pod extract is attributed to its high content of phenolic compounds

p-Coumaric and ferulic acids are present in the phenolic acid profile of broad bean pods. In other studies, they displayed anticancer activity against different types of cell lines [66].

Ceramella *et al*. performed DPPH and ABTS assays on extracts of broad bean pods in acetone, methanol, and 70% ethanol [67]. All three extracts demonstrated an excellent antioxidant activity, as well as a satisfactory anticancer activity against melanoma SK-Mel-28 cells.

Polyphenols were found extremely important in preventing and treating chronic inflammation-related illnesses, such as cardiovascular diseases, obesity, neurodegeneration, cancers, and diabetes [68, 69]. Polyphenols can suppress toll-like receptors and pro-inflammatory genes. The antioxidant activity of polyphenols is attributed to their ability to inhibit enzymes that contribute to the production of eicosanoids and their anti-inflammation properties. For example, they inhibit certain enzymes that produce reactive oxygen species, e.g., xanthine oxidase and NADPH oxidase. On the one hand, they boost other endogenous antioxidant enzymes, e.g., superoxide dismutase, catalase, and glutathione peroxidase. On the other hand, they inhibit phospholipase A2, cyclooxygenase, and lipoxygenase, thus reducing the production of prostaglandins and leukotrienes, as well as inflammation antagonism. These effects that polyphenols have on the immune system mitigate the syndromes of various chronic inflammatory diseases [69].

CONCLUSION

Pods of broad beans (*Vicia faba* L.) proved to contain such bioactive substances as phenolic compounds, flavonoids, tannins, and alkaloids, not to mention dietary fiber. The methanolic extract of dried fresh green pods demonstrated a potent antioxidant activity towards DPPH radicals, as well as good anti-inflammatory properties. The pod extract also showed antimicrobial activity against some food-born pathogenic microorganisms. In

addition, it possessed anticancer activity against HepG2 and PC3 cell lines. These properties belonged to phytochemicals and soluble fibers in the methanolic extract. Therefore, fresh immature broad bean pods can be recommended for human consumption, raw or cooked. Dried ripened pods can be solvent-extracted to obtain various bioactive components that may serve as additives in functional food production.

CONTRIBUTION

All the authors were equally involved in the research analysis and manuscript writing.

CONFLICT OF INTEREST

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

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Application of fat replacers in dairy products: A review

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Abstract:
The consumption of fat raises the risk of coronary heart disease and a number of chronic diseases such as obesity. However, removing fat or reducing its level in the final product may give it undesirable properties since fat plays an important role in the quality of food, mainly its texture and flavor. Therefore, natural or artificial fat replacers are utilized in food formulations instead of natural fat. Fat mimics are based on carbohydrates, proteins, and/or lipids, with energy values of 0–38 kJ/g (0–9 kcal/g). They mimic physical properties and sensory attributes of fat but have less energy and calories. Fat substitutes have physical and functional characteristics of conventional fat molecules which are directly replaced with synthetic molecules that provide no calories or structured lipid molecules. Dairy products represent a principal part of consumer diet all over the world.
Therefore, this review aimed to expound how fat replacers can be used to overcome the defects of fat absence or reduction in dairy products. It was reviewed different types and sources of fat replacers, both micro- and nanoparticulated, and highlighted their application in cheese, ice cream, frozen yogurt, fermented milk, and fatty dairy products. Some of the currently applied micro-particulated proteins include Simplesse® (whey protein), APV LeanCreme™, and Dairy-Lo® (micro-particulate protein + micro-particulate cellulose).
While whey protein has a great role in the dairy sector today, there is a need for further research in this field.

Keywords: Nano particles, dairy products, fat replacers, fat mimetic, ice cream, frozen yogurt, hard and soft cheese

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INTRODUCTION

One of the challenges of modern food products is that consumers expect them to be simultaneously delicious and healthy, while fat, or lipid, is the most important energy source for the human body. Fat is vital for normal growth, supplying the body with fatty acids and fat-soluble-vitamins. However, the immoderate consumption of saturated and hydrogenated fat has resulted in the opposite impact on human health. High fat consumption may correlate to chronic diseases like obesity, type-2 diabetes, cancer, and cardiovascular diseases. On the other hand, fat is required in food for its palatability, acceptability, as well as textural and emulsion properties [1, 2].

In view of consumers' health consciousness, food manufacturers need to produce low-fat products to lower cholesterol and the risk of coronary heart disease in humans [3–5]. The American Dietetic Association (ADA) described several terms such as reduced, light, low-fat, and non-fat, or no-fat, dairy products [6]. It is currently recommended that the fat intake should be reduced to < 30% of calories and that saturated fats should account for < 10% of total energy intake. The American Heart Association also recommends that people with elevated levels of low-density lipoprotein cholesterol or cardiovascular disease should restrict saturated fats to < 7% of calories. To achieve a healthier dietary pattern, people should increase the intake of vegetables, fruits, and grains, as well as modify the type and amount of fat consumed [7–10].

However, consumers are often discouraged from buying low-fat or non-fat food products since their texture and sensory properties are not as good as those of full-fat products. This gave rise to fat replacers which simulate the most abundant features of full-fat food [11].

Fat replacers are classified into fat substitutes and fat mimetics. Fat substitutes are the most novel molecules used to minimize fat ratios in food. They supply dairy products with sensory and functional characteristics of fat but are low in calories or have no calories at all. There is a variety of fat substitutes, including carbohydrate-derived fat substitutes, which can hold water

and have a creamy texture close to that of fat (such as starches and gums), protein-derived fat substitutes (such as egg white, milk, and whey), synthetic fat substitutes, and fat compound substitutes. Fat mimetics are ingredients which partially mimic the organoleptic features of animal fat. They are comprised of food hydrocolloids such as gums, cellulose microfibrils, and pectin. They consist of protein, protein aggregates, protein-polsaccycharide composites, and emulsion gels, which have received more attention lately. Decreasing calorie intake by consuming low- or non-fat food is one of the most serious strategies to overcome many common diseases [11–13]. A high-protein diet could reduce the risk of sarcopenia and other types of muscle loss [14].

The high reactive features of protein-based fat replacers towards pH, temperature, ions, and enzymes can be tuned with distinct physiochemical properties for expanded food and dairy applications, e.g., in yogurt, cream cheese, salad dressings, as well as cheese and frozen desserts. Animal proteins have a greater quality due to their well-balanced amino acid profiles and high digestibility and bioavailability compared to plant proteins, which are low in cysteine and methionine [15, 16].

There has been plenty of research into types of fat replacers, as well as their action, properties, and food applications. However, only few studies have discussed the development of protein-based fat substitutes and their effects depending on the source of protein, its partial weight and solubility. We aimed to present several types of fat replacers and describe their action and applications in some dairy products.

STUDY OBJECTS AND METHODS

The objective of this review was gathering the academic papers concerning with methods of application of various fat replacers in dairy products. For this, we used pertinent keywords, namely "fat mimetic", "nanoparticles" and "dairy products". We focused for English-language articles at most published in 1999–2023 because 1999 was the year when the issue of fat replacers was first raised, boosting scientific research. All papers have a citation index of more than 1000 and at least 50 citations in digital databases, namely Web of Science, eLIBRARY.RU, and Scopus. The list of publication was limited to high-quality peer-reviewed journals and all references were screened for relevant researches. There was no need to include books or non-academic materials into the review.

RESULTS AND DISCUSSION

Definition, classification, and action of fat replacers. Solanke *et al.* defined a fat replacer as a material that replaces all or some of the fat properties in food and gives it a taste, texture, or mouthfeel identical to those of full-fat food [17]. Fat replacers serve two purposes: they reduce the amount of fat and lower the caloric value of a food product. They have energy values of 0–9 kcal/g, as mentioned by Syan *et al.* [18]. Nourmohammadi *et al.* classified fat replacers into two groups according to their properties and processing method [11]. The first group includes fat substitutes that function similarly to fat and have few or no calories. There are three main types of fat substitutes based on their source, namely 1 carbohydrate-based fat substitutes, which can hold water and have a creamy texture close to that of fat (such as starches and gums); 2 protein-based fat substitutes (such as egg white, milk, and whey); and 3 fat-based fat substitutes, which are too large to be digested with little contribution to calories (such as Caprenin, a cocoa butter fat substitute and Olestra). The second group of fat replacers covers fat mimetics – constituents that partly imitate the sensory qualities of animal fat, such as hydrocolloids food (gums, cellulose microfibrils, pectin), proteins, protein aggregates, protein – polysaccharide composites, and emulsion gels. Fat mimetics are generally polar, water-soluble compounds which cannot replace some of the non-polar functional characteristics of fats. Their polar nature facilitates water binding, which helps generate a sense of creaminess in food similar to that in full-fat products [19, 20].

In 2006, Jones and Jonnalagadda mentioned that fat replacers facilitate the development of low-fat and fat-free food that have the taste and texture of high-fat food [21]. Urgu *et al.* pointed out that fat replacers or mimetics alter the casein matrix in cheese [22]. When added to milk, they do not interact with the casein network but do fill spaces. They are commonly used for a fat reduction of ≥ 50%. These materials are usually starches or denatured whey protein aggregates. Thomas *et al.* reported that fat mimetics tended to have a higher moisture-holding capacity than casein, so they promoted a higher moisture level in cheese [20]. Fat replacers and fat mimetics boosted the mellowness and softened the body. However, their exaggerated use could increase stickiness and reduce shreddability. Fat replacers increased the flow ability of cheese when heated. In addition, more whey released into the cheese matrix made the texture softer.

Fat replacers or mimetics cannot be used in cheese where the standard of identity does not allow for their use. They are typically synthetic molecules which provide no calories or structured lipid molecules. Fat substitutes can successfully maintain the palatability of food as they can imitate the texture and mouthfeel of fat. They are generally heat-stable. Thus, fat replacers exchange fat molecules in food for components with comparable properties and supply a minimal calorific value [23].

Fat substitutes available as artificial materials. In 2016, Ognean *et al.* listed the following fat replacers:

– olestra, a mixture of hexa-, hepta-, and octa-esters of sucrose;
– caprenin (capro-capryl-obehenic-triacyl-glyceride) manufactured from glycerol by esterification with caprylic, capric, and behenic fatty acids;
– salatrim, which is the generic name for a family of structured triglycerides comprised of a mixture containing at least one short-chain fatty acid;

– emulsifiers (such as sucrose fatty acid esters, mono- and di-glycerides, lecithin, sodium stearoyl-2-lactylate, and poly-glycerol esters) containing both hydrophilic and lipophilic properties that enable them to act as emulsifiers;
– orbestrin, a mixture of tri-, tetra-, and penta-esters of sorbitol and sorbitol anhydrides with fatty acids;
– dialkyldihexadecylmalonate, a fatty alcohol dicarboxylic acid ester of malonic acid and alkylmalonic acid;
– esterified propoxylatedglycerols, a family of derivatives of propylene oxide prepared by reacting glycerol with propylene oxide; and
– tri-alkoxy-tri-carballylate, trialkoxycitrate, and trialkoxyglyceryl ether, polycarboxylic acids with two to four carboxylic acid groups esterified with saturated or unsaturated alcohols [9].

Other commercial fat replacers. Etenia™: an E-number-free fat substitute classified as starch and maltodextrin in Europe and malto-dextrin in the rest of the world. The product is easy to add when processing dairy and bakery products or emulsified low-fat products. It has unique thermo-reversible gelling characteristics. Etenia™ is used in indulgent products and saves the main cost in fresh and cream cheese, quark, and yogurt [24]. Etenia™ enhances creaminess and reduces fat in ice cream. It can also be used in fat-reduced cake mixes and dough. This thermo-reversible amylopectin hydrocolloid builds texture during 16–24 h only when the product is cooled (1–4°C). Small amounts of this fat replacer enhance the perception of creaminess in low-fat yogurt (1.5%) to that of full-fat yogurt (5%), as reported by Alting *et al.* [25].

Inulin: a non-digestible prebiotic fiber and carbohydrate polymer consisting of three to sixty units of D-fructose known as fructans. Fructans are linked together with beta(2-1) glucosidic bonds and usually have a D-glucose unit at one end. This fat replacer can be safely used without specific limitations in a wide variety of dairy products [26, 27]. Inulin, which is low in calories, possesses many health benefits, especially due to prebiotics that stimulate the growth of beneficial intestinal bacteria [28, 29]. In addition, inulin has been effectively used to modify textural and organoleptic enhancement in food products [29, 30]. Meyer *et al.* concluded that the effect of inulin on the rheological behavior and texture of dairy products depends not only on its concentration but also on the degree of its polymerization [31]. The ability of inulin as fat replacer is not only related to the modification thickness or hardness of the product but also to changes of other mouth feel. Junyusen *et al.*, who studied the usage of inulin as a partial fat replacer in reduced-fat cheese, reported that it improved the product's textural, thermal, and microstructural characteristics, making them comparable to those of full-fat cheese [5].

Micro- and nanoparticles as fat replacers in dairy products. Recently, policy-makers and consumers have sought reduced calorie intake, particularly through low-fat dairy products. However, low-fat products can have some defects. This motivated food scientists to design fat mimetics that could echo the functional and sensual features of fat [32]. For example, Peng and Yao differentiated between macromolecules and microparticles [33]. The former had similar properties to those of full fat, while the latter had shapes and sizes similar to those of fat globules, thus allowing them to behave like fat.

Fat mimetic substances imitate the organoleptic, physicochemical, and microstructural characteristics of fat by utilizing biocompatible and biodegradable proteins and/or carbohydrates in a native form, in an aggregated state, or in the form of bio-polymeric particles [34]. Figure 1 illustrates the reported bibliographic data for fat replacers, including protein-based fat replacers. It presents scientific literature and citations, with the highest number of publications produced in 2017, high-lighting the topical nature and importance of this field in the period from 1998 to 2018. A substantial yearly increase can be observed from 2012 for both total and protein-based fat replacers, with the latter contributing to nearly one-third of the total publications to-date. The data characterize this filed as a priority area in the food science, as mentioned by Kew *et al.* [34].

Protein-based microparticles as fat replacers. Microparticulate proteins have a smaller particle size (0.1–20 μm diameter) compared to concentrate/isolate protein. They are created using thermal treatments and high shear processes at low pH. Protein microparticles are known under such common names as Simplesse® (whey protein), APV LeanCreme™ (SPX Technology), or Dairy-Lo® (micro-particulate protein + micro-particulate cellulose) [34].

Torres *et al.* reported that reducing fat in yogurt contributed to higher syneresis, weaker body, and unsatisfying texture [35]. Milk whey protein might behave as "active fillers", further increasing the viscosity of low-fat yogurt [36]. Furthermore, where milk whey protein is larger than the protein of fat globules, it creates a higher level of serum separation, increased graininess, and lower firmness compared to whey protein concentrate.

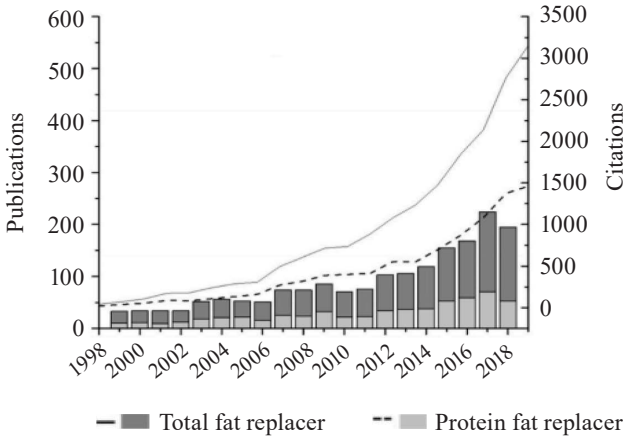

Figure 1 The publication numbers (bars) and citations (lines) for fat replacers (black bar, solid line) and protein-based fat replacers (white bar, dashed line)

According to Li *et al.*, the interaction of gel emulsion with yogurt proteins enhanced the stability of the network structure, resulting in a higher water-holding capacity and better texture or storage stability of emulsion gel yogurts [37]. However, the structure of the yogurt made with whey protein emulsion gel microparticles prepared from vegetable oil was more easily destroyed than that of the yogurt made with whey protein emulsion gel microparticles prepared from milk fat.

El-Aidie *et al.* used three types of commercial microparticulate whey protein at 0.3–0.9% in low-fat Edam cheese and found that it reduced firmness, restored proteolysis and opaqueness, as well as improved sensory acceptability [38]. Milk whey protein acted as "inactive fillers" in yogurt [35]. The microstructure of low-fat pickled cheese significantly opened up after supplementation with Simplesse® at the 0.9% concentration, resulting in the lowest firmness. This effect was also seen in a study by Akin and Kirmaci, who used Simplesse® at 1% [39]. However, higher concentrations of this milk whey protein (3–7% w/w) increased the product's hardness and firmness, which limits its effective use in cheese to small concentrations [40]. As for sensory properties, it was established that milk whey protein at 0.6% contributed to optimum body and texture and the cheese with DairyLo® and Protelo® had a better flavor than that with Simplesse® [38].

Many studies have explained how to design dairy products with little or no fat by using proteins and micro-particulate proteins to simulate full-fat products. Whey protein appears to dominate the dairy sector. Figure 2 shows some types of protein-based fat replacers [18]. Although there has been a recent interest in the use of plant proteins, their characterization, understanding, and application are still highly limited. In addition to rheology, particle size, and sensory evaluation, tribology is currently applied to understand the lubrication properties of fat replacers. Combining tribology with muco-adhesion techniques can provide powerful screening tools for identifying fat mimetics with just-right mouthfeel properties. Besides proteins and commercial micro-particulate proteins, micro-gels have recently demonstrated superior rheological and lubrication performance as potential fat replacers. However, literature lacks information on the sensory perception of micro-gels and the challenges of their commercial production appear to be a bottleneck that delays their application in food [34].

Application of fat replacers in dairy products. Low- or reduced-fat dairy products are generally in high demand. Consequently, many fat substitutes are used to mitigate the textural and sensory disorders in the final products caused by minimizing fat content. Common fat replacers used in dairy products are oligo-fructose, inulin, maltodextrin, poly-dextrose, milk proteins, soy proteins, dietary fibers, and starches.

Fat replacers in cheese. Cheese is a favorite dairy product for people of all ages. Today, consumers are increasingly looking for low-fat cheese with favorable qualitative properties, which raises the importance of using fat replacers in cheese manufacture.

Processed cheese spread. Mounsey and O'Riordan reported that rice starch appears to have the greatest potential as a partial casein substitute in imitation cheese [41, 42]. The action of starch on processed cheese properties was referred to phase attitudes between protein and starch, as shown in confocal laser scanning micrographs by Diamantino *et al.* [43]. A study by Mehanna *et al.* also presented low-fat processed cheese spreads based on starch [44].

In 1999, Mounsey and O'Riordan manufactured processed cheese with various levels of pre-gelatinized maize starch and found that the product's melt-ability decreased with increasing levels of starch [41]. In their study in 2001, the same authors used mixtures of Ras cheese and acid or rennet curd of skimmed milk with various fat replacers such rice powder, whey protein concentrate powder, Jerusalem artichoke, oat, and Simplesse®100 at a concentration of 0.5% [42]. Different microstructures observed in the low-fat spreads and

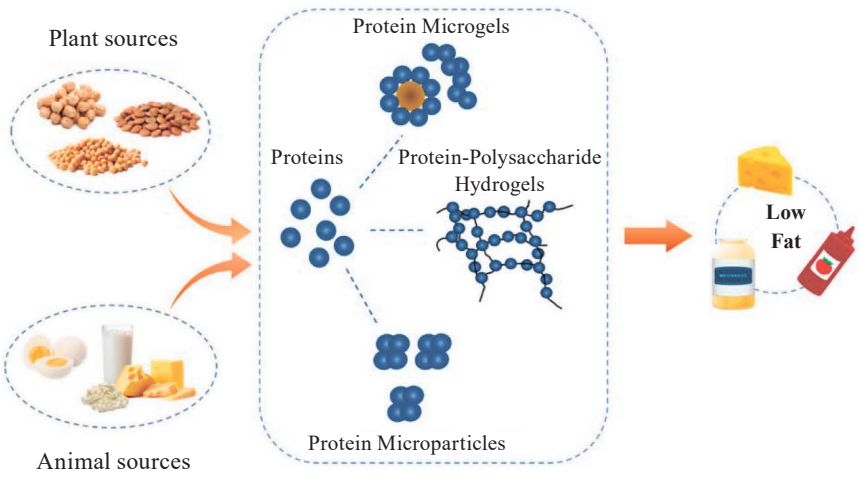

Figure 2 Protein-based fat replacers

the control reflected the differences in their fat contents and the contents of fat replacers. However, Wahed *et al.* replaced milk protein concentrate in low-fat spreadable cheese with a synergized filler branded as "Gervizol", which contained soy proteins, soy fibers, maltodextrin, and modified starch [45]. El-Assar *et al.* revealed the impact of inulin on the physicochemical, rheological, and sensory characteristics of low-fat processed cheese spread and evaluated the stability or changes in these characteristics under cold storage conditions [46]. According to their results, replacing milk protein in low-fat processed cheese spread with 5% of inulin lowered the hardness and increased the adhesiveness of the product compared to low-fat cheese free of inulin.

In 2020, Schädle *et al.* studied the influence of inulin, corn dextrin, polydextrose, and micro whey protein combined with rennet-casein on the melting behavior, dynamic rheological properties, and hardness of reduced-fat processed cheese [40]. They found that increasing concentrations of inulin and corn dextrin diminished the flow ability of cheese.

In recent study, Sołowiej *et al.* examined the effect of fat replacement with whey protein micro-coagulates on the physicochemical properties and microstructure of acid casein model processed cheese [47]. According to their results, partial replacement of anhydrous milk fat with whey protein micro-coagulates (3–8%) increased the hardness and viscosity of acid casein processed cheese. Whey protein micro-coagulates acted as a fat substitute without causing negative changes to the structure of the final product. Physiochemical properties showed higher viscosity or good melting properties.

Mozzarella cheese. There has not been much research into Mozzarella cheese. Daia *et al.* added konjac glucomannan to fat-reduced Mozzarella cheese, which mainly affected its texture, color, and browning, as well as slightly increased its moisture and A_w [48]. The authors recommended konjac glucomannan as a potential fat replacer to be used in fat-reduced Mozzarella cheese. Low-fat Mozzarella cheese was also prepared by Chatli *et al.* by incorporating 0.3% sodium alginate as a fat replacer with comparable and acceptable physiochemical, textural, color, and sensory attributes [49]. The cheese was almost by 44% lower in calories than full-fat Mozzarella cheese.

Esena and Güzeler indicated that the reduction of fat and the use of whey protein as a fat replacer affected the composition, proteolysis, texture, and melt-ability, as well as the microstructural and sensory properties of reduced-fat Boru-type Künefe cheese during storage [50]. Increased amounts of whey protein concentrate (especially in Boru-type Künefe cheese) allowed the authors to obtain low-fat cheese with similar characteristics to those of full-fat cheese.

Soft and semi-soft cheese. Abd El-Gawad mentioned that 1% fat Ricotta cheese supplemented with 2% Dairy-Lo® was superior in quality and sensory characteristics, having a whiter color and a smooth creamy texture [51]. Dairy-Lo® replaced fat globules with a gel structure, which decreased the junction in the casein matrix and increased the whiteness of the Ricotta cheese. In a study performed by Rahimi *et al.*, indicated that the fat content of gum tragacanth and the ripening time had serious effects on the cheese-making yield, as well as chemical, rheological, and microstructural characteristics of Iranian white cheese [52]. As the fat content in cheese decreased, the instrumental hardness parameters increased and the microstructure became more compact. Adding gum tragacanth to the low-fat cheese increased its moisture content and improved its sensory properties. Gum tragacanth improved the rheological properties of texture due to its water-binding ability. The instrumental hardness parameters decreased during ripening, and the interactions with gum tragacanth caused visible undesirable effects on the cheese's characteristics after 42 days of ripening.

However, Alnemr *et al.* recommended adding 2 and 4% inulin to Karish cheese to promote its sensory properties and nutritional value [53]. In 2016, the authors produced high-quality low-fat Domiati cheese (Gbnah-Beeda) by using hydrocolloids as a fat mimetic. As a result, low-fat cheese showed a significant augmentation in the physiochemical characteristics, yield, and moisture. Furthermore, its sensory properties were both highly acceptable and comparable to those of full-fat cheese during 75 days of ripening.

In a study by Alnemr *et al*., the authors improved the quality of low-fat Domiatti cheese by using Simplesse or Z-trim as fat replacers [54]. The composition, proteolysis, and voluptuous properties of low-fat cheese (70% fat reduction) were examined throughout aging for 60 days. All the control low-fat products demonstrated decreased yield and significantly increased moisture and protein content. However, the low-fat cheeses made with the fat replacers showed a significantly increased extent of proteolysis and lipolysis compared to the control cheese.

Rashidi *et al.* used fat replacers to produce low-fat UF Feta cheese with good textural and sensory characteristics [55]. The response surface methodology showed improving effects of WPC80 (0–20 g/kg), lecithin (0–2 g/kg), and a mixture of xanthan and guar (0–1 g/kg) on the sensory and instrumental texture characteristics of low-fat UF-Feta cheese made with 6% fat retentate. Lecithin and xanthan-guar had positive effects, while WPC80 had negative effects, on the product's hardness, chewiness, and gumminess. Furthermore, lecithin had positive effects on taste, acceptance, and appearance, as well as a positive effect on sensory texture. Whey protein concentrate improved the taste, while xanthan-guar enhanced the sensory texture and acceptance scores.

Basiony *et al.* manufactured low-fat Munster cheese as a semi-soft French type from whole cow's milk [56]. They concluded that adding fat replacers such as Slendid®200 and Simplesse®100 increased the moisture and ripening indices. Also, these fat replacers decreased the product's hardness, adhesiveness, cohesiveness, gumminess, and chewiness, as well as increased its springiness.

All the sensory properties of low-fat Munster-like cheese were improved by adding fat replacers to the cheese milk.

Saraiva *et al.* added inulin to reduced-fat cheese, which induced fat and protein recovery [57]. Furthermore, the use of inulin markedly reduced the hardness of the cheese, particularly after 7 days. Kadpe *et al.* produced low-fat Quarg cheese by using such fat replacers as carrageenan and Simplesse-100@ [58]. The cheese was tested for its sensory, physiochemical, and microbial characteristics. The ultimate score was awarded to the Quarg cheese prepared with 0.5% Simplesse-100.

In 2019, NurAinie *et al.* described the formulation of analogue cheese produced with sweet corn extract used as a fat replacer [59]. This analogue cheese satisfied the people's need for alternative cheese prepared from cow's milk. Its sensory characteristics were similar to those of cheese from cow's milk. In particular, the product had a yellowish-white color, distinctive aroma of cheese, soft texture, good spreadability, and no sour taste.

Recently, Giha *et al.* summarized the effects of milk fat replacement with vegetable oils on the rheological, textural, and microstructural properties of cheese analogues [60]. Their findings suggest that the primary effects of modifying fat in cheese analogues are associated with an alteration in the interactions between the components of the protein matrix caused by milk fat extraction. Overall, changes in the functional properties of analogue cheese depend on the sort of oil, the percentage of fat modification, and the type of cheese microstructure and fat globule size. Processing causes structural bonds to break, thus creating different size particles.

In 2020, Sharaf *et al.* optimized the textural hardness of ultra-filtrated, low-fat cheese by using galactomannan and novagel (0.1–0.5%) [61]. AI-Bedrani *et al.* manufactured low-fat, low-calorie soft cheese from reconstituted skim milk by adding whey protein concentrate as a fat substitute [62]. This improved the product's rheological properties such as compressibility and springiness. In addition, whey protein concentrate increased the percentage of total solids (especially protein content) and the cheese yield, reduced total energy, and improved the product's sensory properties.

The consumer's desire for plant-based cheese analogues is expanding due to the ease and versatility of their use. However, the products available on the market are nutritionally poor. They are low in protein, high in saturated fat and sodium, and are often composed from a long list of ingredients. Mefleh *et al.* applied dry-fractioned pea protein concentrates combined with emulsion gel with inulin and emulsion volatile olive oil to develop, on a laboratory scale, a clean label and nutritious spreadable plant-based cheese analogue [63]. The authors masked the typical bean flavor offered by the dry-fractionated pea by adding spices without causing any adverse sensory characteristics. They concluded that expanding the range of plant-based products with alternatives characterized by a clean label and a higher nutritional value will better accommodate a broader audience of "healthy" consumers aware of the importance of a plant-based diet.

Basiony and Hassabo studied the effect of modified maize starch used as a fat replacer on low-fat Halloumi cheese [64]. The addition of modified starch to cow's milk decreased the time of rennet coagulation and curd syneresis but increased curd tension. The overall results showed a possibility of producing low-fat Halloumi cheese with attractive appearance, good body, and acceptable flavor by adding modified starch. Table 1 presents some of the fat replacers that have been used in cheese production in the last two decades.

Table 1 Fat replacers used in cheese varieties in the last two decades

Cheese type	Fat replacer type	Ratio of additives	Reference
Ricotta cheese	Dairy- Lo@	2%	[51]
Iranian white cheese	Gum tragacanth	0.75 g/kg	[52]
Karish cheese	Inulin	2 or 4%	[53]
Low-fat spreadable processed cheese	Soy proteins, soy fibers, maltodextrin, and modified starch	Gervisol 218	[45]
UF-Feta cheese	WPC80, lecithin, and a mixture of xanthan and guar	0–20, 0–2 and 0–1 g/kg	[55]
Domiatti cheese	Kappa carrageenan:locust bean:xanthan gums	60:20:20 g/kg	[54]
Munster-like cheese	Slendid®200 and Simplesse®100	0.2%	[56]
Low-fat cheese	Inulin	4%	[57]
Low-fat Mozzarella cheese	Carboxymethyl cellulose and sodium alginate	0.4–0.3%	[49]
Quarg cheese	Carrageenan and Simplesse®100	0.5%	[58]
Low-fat processed cheese	Inulin	5%	[46]
Cheese analogue	Citric acid, papain, and maltodextrin	0.20, 0.029, and 20%	[59]
Processed cheese	Inulin, corn dextrin, polydextrose, and micro-particulated whey protein	3.0, 4.0, 5.0, 6.0, and 7.0%	[40]
Ultra-filtrated, low-fat cheese	Novagel and galactomannan	0.32 and 0.5%	[61]
Low-fat soft cheese	Whey protein concentrate	1.0, 1.5, 2.0, and 2.5%	[62]
Processed cheese	Whey protein micro-coagulates	3–8%	[47]
Low-fat Halloumi cheese	Modified starch	1, 2, and 3%	[64]
Boru-type Künefe cheese	Whey protein	0.5%	[50]

Fat replacers in ice cream or frozen yogurt. Ice cream is one of the most popular dairy products that has a distinctive taste and contains a high percentage of fat. Replacing milk fat in ice cream with either carbohydrates or protein-based fat substitutes is one of the new strategies to reduce the fat content and produce a safe and healthy product. Mahdian and Karazhian produced low-fat ice cream (5% fat) using milk protein concentrate (65%) and multiple contents of inulin as a fat replacer (0, 2, and 4%), as well as two sorts of commercial stabilizer-emulsifier blends (Stab-IC80 and Stab-6924) at 0.3 and 0.4% concentrations [65]. The product's hardness was not affected by the type or amount of fat replacers or stabilizers. However, the overrun values decreased with increasing concentrations of milk protein concentrate, inulin, and stabilizers in the formulation.

In another study, Junyusen *et al.* manufactured ice cream (6% fat) with 2 and 4% of inulin and compared it with regular ice cream (10% fat) and inulin-free fat-reduced ice cream [5]. Their results confirmed that the lower fat content (6%) significantly affected the physicochemical characteristics of the ice cream products. The addition of inulin motivated the clustering of fat globules, resulting in the growth of larger-sized particles (1–10 μm), but it significantly decreased the hardness of the reduced-fat ice cream. Furthermore, 4% of inulin lowered the melting average of the reduced-fat ice cream and enhanced the physicochemical characteristics. In a study by Salem *et al*., the fat content in ice cream was partially replaced with whey protein, dietary fibers, oat, wheat germs, and modified starch. The use of oat and wheat germs increased the fiber content of the product. Additionally, all ice cream treatments had a significant free radical scavenging activity similarly to the control [66].

Babu and Parimalavalli used modified sweet potato starch (2%) in ice cream [67]. Although it did not significantly affect the protein content, it decreased the overrun. The product's hardness increased with 1% replacement but decreased with 2% starch. According to Warren and Hartel, the overrun can affect the size of air cells in ice cream products during the freezing and storage processes [68]. The use of modified sweet potato starch as a fat substitute reduced the melting rate in ice cream. This can be affected by the weakening of ice cream's structural texture due to the lower amount of fat.

In 2020, Hatipoğlu and Türkoğlu studied the quality properties of fat-reduced ice creams produced with some fat substitutes [69]. The authors found that 10% fat ice cream behaved similarly to the control group. Then, they added 6% Simplesse®100 or 2% Maltrin040 as fat substitutes to 7.5, 5, and 2.5% fat ice cream. According to the results, the viscosity of the 7.5% fat ice cream with Maltrin040 or Simplesse®100 was higher than that in the control group. Furthermore, decreasing the fat content in ice cream had the opposite effect on the sensory properties and viscosity. Thus, the quality defect caused by the lack of fat can be remedied by protein and starch fat replacers. These fat substitutes can also help solve obesity problems by offering calorie-reduced ice creams. The findings showed that the 7.5% fat ice cream with 6% Simplesse®100 can be recommended as fat-reduced ice cream.

El-Shafei manufactured three types of probiotic frozen goat's yogurt using full-fat (4%) goat's milk (control), low-fat (2%) and non-fat (0.5%) goat's milk with probiotic bacteria and chia flour (1, 2, and 3%) as a fat replacer, as well as stabilizers [70]. The study showed that increased levels of chia flour decreased the specific gravity and melting resistance of low-fat and non-fat probiotic frozen goat's yogurt.

Abdou *et al.* prepared low-fat ice cream by partially replacing buffalo milk fat with various fat substitutes (inulin, maltodextrin, modified starch, whey protein concentrate powder, and oat) [71]. Milk fat was standardized to 2% to meet the consumer's demand for low-energy foods (control), while full-fat ice cream was standardized to 6% fat. The best treatment was the low-fat ice cream with maltodextrin as it achieved the same panelist scores as the full-fat ice cream, followed by the sample with inulin.

Silantjeva *et al.* used pumpkin puree, sugar, and whey concentrate in ice cream [72]. They found that larger amounts of pumpkin puree and gelatin decreased the product's hardness and increased its overrun and viscosity. In 2022, Hamad *et al.* produced low-fat and low-calorie frozen yogurt using Etenia 457 as a fat replacer and Sativoside as a sweetener [73]. The control and four treatments were prepared with 0, 25, 50, 75, and 100% of the fat replacer and sweetener. The treated samples were lower in fat and calories compared to the control. According to the results, the product's specific gravity and viscosity increased with larger amounts of the additives, while its overrun decreased. The sensory evaluation showed that the sample with 25% of the fat replacer and sweetener had the best properties, as well as the highest total scores among all the treatments.

Shahein *et al.* evaluated the potential benefits of adding Jerusalem artichoke tuber powder (0, 5, 10, 15, and 20% w/w) as a fat and sugar replacer for the physicochemical properties and survival of probiotics in frozen yogurt [74]. The sample with 10% of this fat replacer showed the highest viability of probiotics. The results also revealed that the frozen yogurt with Jerusalem artichoke tuber powder had higher acidity, melting resistance, overrun, viscosity, and sensory attributes compared to the control. The authors concluded that enriching frozen yogurt with 20% of this fat replacer will provide it with functional properties to benefit consumers' health.

Fat replacers in fermented milk. The use of skim milk is a strategy to increase milk yogurt acceptability. However, it can negatively influence yogurt's rheology because fat plays a crucial role in dairy structural integrity. Salem *et al.* used inulin as a fat replacer to produce low-fat Labneh [66]. The findings showed that inulin stimulated the growth of *Lactobacillus reuteri*,

Lactobacillus johnsonii, and *Lactobacillus salivarius*, resulting in ameliorated viability of the organisms.

Atallaha *et al.* produced low-fat yogurt fortified with whey protein concentrates, Ca-caseinates (Ca-Cns), and spirulina (*Spirulina platensis*) powders [75]. They found that the yogurt with spirulina powder had a high total phenolic content, while the yogurt containing whey protein concentrates had higher ($p < 0.05$) viscosity and water-holding capacity than the other samples. In the whey protein concentrates-fortified low-fat yogurts, the gel exhibited a various structure with a fine network containing numbers with very small pores. The gel from the Ca-Cns-fortified yogurt had a dense and finely perforated microstructure, similar to that of the whey protein concentrates-enriched yogurt. Generally, the low-fat yogurt fortified with whey protein concentrates performed best in the sensory evaluation, followed by the sample with Ca-Cns.

Abd El-Galeel *et al.* studied the effect of adding different levels (0.5 and 1%) of fat replacers (Dairy-Lo or Maltrin) on the quality of non-fat yogurt [76]. They found that the fat replacers had no significant effect on the chemical composition of the resultant yogurt but increased the soluble nitrogenous compounds, as well as the formation of acetaldehyde, diacetyle, and total volatile fatty acids (flavor compounds). Also, there was an improvement in the yogurt's syneresis and viscosity. Overall, the non-fat yogurt containing 0.5 and 1% of Maltrin was similar in quality characteristics to the full-fat control yogurt.

Carob bean gum is widely used in food systems as a carbohydrate-based fat replacer to modify quality attributes and shelf-life as a thickening and gelling agent. Sonmez and Ozcan prepared reduced-fat (12%) and low-fat (6%) yogurt using Carob bean gum an found that it increased the textural and sensory attributes of the product [77]. The low-fat yogurts with Carob bean gum showed significant firmness and stickiness, as well as a higher yellowness index. Thus, the addition of Carob bean gum to reduced-fat and low-fat yogurts improved their sensory properties and acceptance index.

Zbikowska *et al.* explored the influence of various concentrations of inulin (3, 6, 9, 12, and 15% w/w) added to natural yogurt on its physicochemical properties [78]. They found that these concentrations of inulin significantly enhanced the product's physical stability. Godoy-garcia *et al.* prepared fat-reduced Greek-style yogurt by using glycomacropeptide powder as a fat replacer [79]. Adding 0.75% of glycomacropeptide reduced the granular consistency and syneresis of the yogurt by 50 and 10%, respectively. The flow curves and firmness analysis showed that this fat replacer decreased the elastic and viscous modulus, as well as the yoghurt's firmness. The consumer acceptance of low-fat Greek-style yogurt containing 0.75% of glycomacropeptide decreased only after 30 days of storage. The resulting product showed lower syneresis and graininess.

Costa *et al.* prepared goat milk yogurts with inulin [80]. The authors reported a decrease in firmness and consistency during storage. On the other hand, the viscosity index significantly increased during refrigerated storage. Furthermore, all the treatments exhibited viscoelastic behavior [81]. Table 2 summarizes some of the fat replacers used in ice cream and yogurt (frozen yogurt) production in the last decade.

Fat replacers in fatty products. Consumers often associate reduced-fat food products with poor taste, despite the potential benefits they can offer for managing diet-related health problems. Therefore, it is important to use high-quality fat substitutes to create appealing reduced-fat options. In a study by Míčková *et al.*, polydextrose was used as a low-calorie fat replacer and bulking agent in butter [82]. The presence of polydextrose was confirmed through the use of petroleum ether extraction to remove fats from the butter. Solid fractions of both regular butter and butter with polydextrose were prepared and analyzed using FT-IR spectroscopy. The IR marker bands specific to poly-dextrose,

Table 2 Fat replaces used in ice cream and (frozen) yogurt in the last decade

Product type	Fat replacer type	Ratio of additives	Reference
Reduced-fat ice cream	Inulin	0, 2, and 4%	[5, 65]
Ice cream	Whey protein, dietary fibers (oat, wheat germs), and modified starch	1 and 2%	[66]
	Modified sweet potato starch	2%	[67, 68]
Reduced-fat ice cream	Simplesse®100 and Maltrin040	6 and 2%	[69]
Non-fat ice cream	Inulin, maltodextrin modified starch, whey protein concentrate powder, oat	2%	[71]
	Pumpkin puree, gelatin whey concentrate		[72]
Low-fat frozen yogurt	Etenia 457	0, 25, 50, 75, and 100%	[73]
	Jerusalem artichoke tuber powder	0, 5, 10, 15, and 20%	[74]
Low-fat goat milk yogurt	Inulin, maltodextrin, whey protein	1, 2, and 6%	[73, 77]
Low-fat yogurt	Whey protein concentrate, caseinates (Ca-CNS), Spirulina powders		[75]
	Carob bean gum	1%	
Non-fat yogurt	Dairy-Lo or Maltrin	0.5 and 1%	[76]
Natural yogurt	Inulin	3, 6, 9, 12, and 15%	[78]
Greek-style yogurt	Glycomacropeptide	0.75%	[79]

which appear at 1150, 1076, and 1040 cm^{-1}, were only observed in the butter sample containing poly-dextrose.

In 2020, Iftikhar and Dutta conducted a study using raw, retrograded, and retrograded-annealed starches from three rice varieties with varying amylose contents (22.7, 9.8, and 0.3%) to replace some of the fat in fresh cream [83]. The goal was to create a whipped cream consistency with around 15% of fat. The study found that the fat replacement reduced the whipping time to 60 s and improved the stability of the whipped cream foam, resulting in a significant overrun of up to 44%. The use of starch also improved water retention and structural stability, reducing the amount of liquid that weeps out upon freezing and thawing. When using modified waxy starch, the resulting creamy texture was closest to that of the commercial cream standard, indicating that efficient fat replacement had been achieved. Overall, it was concluded that whipped cream with the fat content of more than by 62% less than in the commercial variants could be produced by incorporating starch.

Ahmadi *et al.* optimized the thermal, functional, and rheological properties of ethyl cellulose-based oleogel by using different concentrations of behenic acid and assessing the stability of water in oleogel (w/og) emulsions [84]. Similarly, Palla *et al.* investigated the formulation and characterization of filling creams for sandwich cookies using mono-glyceride oleogel as a fat material [85]. The study revealed that the filling creams formulated with 260 g/kg oleogel had viscoelastic moduli values similar to those in a filling cream of commercial sandwich cookies used as a reference. However, the oil-binding capacity of the filling creams decreased with an increase in the oleogel content. Furthermore, larger amounts of oleogel in the formulation resulted in decreased hardness, but increased adhesiveness and cohesiveness. The replacement of animal fat with vegetable fat offers an additional advantage, since it agrees with the increasing demand for animal-free products by consumers.

Schädle *et al.* examined the impact of fat replacers on reduced-fat emulsions, including their rheological, tribiological, and aroma release properties [86]. The authors emphasized the importance of high-quality fat replacers in developing appealing reduced-fat products. They reduced the fat content of a full-fat emulsion by replacing it with water, lactose, corn dextrin, inulin, poly-dextrose, or micro-particulated whey protein. Corn dextrin exhibited similar values to the full-fat emulsion in terms of Kokini oral shear stress and viscosity, while micro-particulated whey protein strongly increased these properties. All the four fat replacers improved the lubricity of the reduced-fat samples. None of the formulations had a significant effect on the droplet size distribution. The aroma of the emulsions comprising micro-particulated whey protein and corn dextrin were most similar to those of the full-fat emulsion. Thus, the authors found corn dextrin a promising fat replacer for reduced-fat emulsions. Butanoic acid, heptan-2-one, ethyl butanoate, and nonan-2-one were more affected by changes in the formulation than butane-2, 3-dione, and 3-methylbutanoic acid.

Schädle *et al.* also conducted another study in which they used varying concentrations of corn dextrin as a replacement for fat in mayonnaise [87]. The findings indicated that tri-biological measurements could replace sensory analysis in determining the stickiness attribute. Furthermore, the use of Stevens' power law demonstrated a strong correlation between Kokini oral shear stress and the creaminess sensory attribute. Additionally, the instrumental texture properties (firmness, stickiness) were also related to sensory perception. These correlations obtained by comparing different methods could aid in predicting the potential applications of new fat replacers and assist in innovative product development.

Bayat *et al.* decreased the fat content of whipped creams by using two types of gums derived from chia and mero seeds [88]. Their study evaluated various physicochemical characteristics such as acidity, pH, viscosity, humidity, volume, water content, and sensory attributes of the treatments on days 1, 5, and 10. The findings indicated that the use of gums did not affect the acidity or pH of the treatments. However, the samples containing chia seed gum alone and chia seed gum with mero showed a reduction in moisture and an increase in viscosity and overrun. In conclusion, the study demonstrated that chia and mero seed gums can be effectively used to produce whipped creams with reduced fat content.

Additionally, Cui *et al.* noted that animal fats and shortenings are commonly used in various food preparations [89]. However, these types of fats are high in saturated fatty acids, and partially hydrogenated shortenings contain trans fats, which have been linked to an elevated risk of coronary heart disease. Consequently, nutrition experts suggest reducing the consumption of saturated and trans fats in people diets. Hence, food technologists and scientists are collaborating to create healthy substitutes for these types of fats. One promising solution is to use oleogels, which are solid-like structures formed by structuring edible oils. While several types of oleogels have been developed and shown potential, some technological hurdles limit their widespread use in the food industry.

Kim *et al.* created canola oil oleogels for filling creams by combining candelilla wax and glycerol monostearate in different ratios, resulting in a firmer texture and lower melting point than the traditional shortening substitutes [90]. The use of binary blends of oleo-gelators in oleogels could be a promising approach to improving the quality and healthfulness of food products by mitigating the drawbacks and limitations associated with using a single oleo-gelator.

In a study conducted by Onsri *et al.* in 2022, the use of inulin and whey protein concentrate in the crust and cream puff filling was investigated at varying percentages [91]. The addition of inulin and whey protein concentrate resulted in a significant decrease in viscosity and specific volume. However, using 20% of inulin in

Table 3 Fat replacers used in fatty products in the last decade

Product type	Fat replacer type	Ratio of additives	Reference
Butter	Polydextrose	1%	[82]
Whipping cream	Annealed starches from rice	10%	[83]
Emulsions based oleogels	Ethyl cellulose and behenic acid	2:4 and 1:5 wt %	[84]
Filling creams	Monoglyceride oleogel	22–26%	[85]
Emulsions	Water, lactose, corn dextrin, inulin, polydextrose, or micro-particulated whey protein	15%	[86]
Mayonnaise	Corn dextrin	0.5–8%	[87]
Whipped cream	Chia and mero seeds	0.15–0.3%	[88]
Shortening	Oleogels		[89]
Filling creams	Candelilla wax and glycerol monostearateoleogels	60:40%	[90]
Crust and cream puffs filling	Inulin Whey protein concentrate	20, 35, and 50% 5, 10, and 15%	[91]
Whipping cream analogues	Lactic acid bacteria Hydroxyl-propyl methylcellulose and casein sodium	38 %, w/w 0–0.9%	[92]
Shortening	Beeswax and palm oil oleogels	0.5–5%	[93]
Chocolate	Gum Arabic-based water-in-oil emulsions	2:8, 3:7, and 4:6 v/v	[94]

crust puff filling helped maintain the structure. In sensory evaluations, the formula containing 20% of inulin and 10% of whey protein concentrate received the highest scores for appearance and taste. For cream puff filling, the treatments with 35 and 50% of inulin resulted in high viscosity. The panelists preferred the 20% inulin and 5% whey protein concentrate formulas for texture, taste, odor, and overall acceptability. These formulas helped reduce total energy from fat and increase dietary fiber and protein contents. Thus, inulin and whey protein concentrate proved to be successful fat replacers, extending shelf life from 9 to 13 days while meeting microbiological standards.

Recently, in a 2023 publication by Jiang *et al.*, lactic acid bacteria were combined with hydroxyl-propyl methylcellulose and casein sodium salt to replace the saturated fat content in whipping cream analogues [92]. By using both hydrophobic and hydrophilic strains, the whipping cream had similar overrun (107%) and drainage stability (drainage area 1.4 mm^2) compared to those of commercial dairy whipping cream (30% and 2.7 mm^2, respectively). The Pickering capability and aggregating properties of the strains affected the foam stability of the whipped cream. The addition of the components and the whipping process did not have any negative effects on bacterial viability. Therefore, the use of edible lactic acid bacteria as fat replacers could offer viable alternatives to using naturally derived components as active structural building blocks for colloidal food systems such as whipping cream.

Suriaini *et al.* conducted a study to examine how beeswax and palm oil concentrations affect the properties of oleogels, which were then compared to a commercial shortening to determine their potential as shortening substitutes [93]. Crude palm oil and palm oil-based cooking oil both contain high levels of palmitic acid (C16:0), oleic acid (C18:1), and linoleic acid (C18:2), but the former has more total saturated fatty acids than commercial cooking oil. The researchers used binary blends of palm oil (crude palm oil and cooking oil) and beeswax in different concentrations to produce palm oil-based oleogel. The results showed that the oleogels with higher beeswax concentrations had a greater oil-binding capacity and produced needle-like and spherical crystals. The crude palm oil-based oleogel had higher heat resistance and was more similar in profile to shortening than the cooking oil-based oil. The palm oil-based oleogel products had properties comparable to commercial shortening and can thus be used as substitutes.

A recent study by You *et al.* created 3D printed chocolate with a reduced fat content by substituting cocoa butter with water-in-oil emulsions made from gum Arabic [94]. The optimal formulations for 3D printability contained cocoa butter, icing sugar, and cocoa powder in a ratio of 2:1:2.5. Various levels of cocoa butter (25, 50, and 75%) were replaced with water-in-cocoa butter emulsions with different water/oil ratios (2:8, 3:7, 4:6 v/v) to produce the fat-reduced 3D printed chocolate. The results indicated that these chocolates retained the desired polymorphic form V of cocoa butter, and higher emulsion contents led to improved snap quality. The study successfully developed a functional reduced-fat chocolate using gum Arabic-based water-in-oil emulsions that could potentially incorporate both hydrophilic and lipophilic bioactives in the future. Table 3 provides examples of fat replacers that have been used in fatty products in the last decade.

CONCLUSION

Most research in the last decade has focused on designing low- or no-fat products that mimic the features of full-fat products. Fat replacers can be divided into two categories, fat substitutes and fat mimetics. They are molecules that have the physical and functional characteristics of conventional fat molecules (e.g., triglycerides). Using proteins and micro-particulated proteins has been a popular trend as nanotechnology plays a critical role in the food sector nowadays. In addition to

proteins and commercially available micro-particulated proteins, microgels designed under laboratory conditions have recently demonstrated superior rheological and lubrication performance with the ability to act as potential fat replacers. While whey protein is the most common fat replacer in the dairy field today, there is some interest in other plant proteins which have not been studied as widely yet.

The application of fat replacers in dairy products is expected to witness significant advancements in the coming years, with the growing consumer demand for healthier alternatives and the increasing focus on reducing fat content in food products. Researchers and food scientists are actively exploring novel fat replacers that can mimic the sensory and textural properties of traditional dairy products while offering improved nutritional profiles. One promising trend is the utilization of plant-based fat replacers derived from nuts, seeds, and legumes, which not only provide a creamy mouthfeel but also offer additional health benefits, including higher levels of unsaturated fats and essential nutrients. Another emerging trend is the incorporation of microencapsulation techniques to enhance the stability and release properties of fat replacers, ensuring their efficient delivery and functionality throughout the shelf life of dairy products. Additionally, advancements in biotechnology and genetic engineering may enable the development of tailored fat replacers that closely mimic the complex structure and composition of natural fats, further enhancing the overall sensory experience and consumer acceptance of low-fat dairy products. As research in this field continues to evolve, it is anticipated that these future trends will contribute to the development of innovative and nutritious reduced-fat dairy products that meet the demands of health-conscious consumers while maintaining their palatability and quality.

CONTRIBUTION

Hayam M. Abbas wrote the first section including the definition, classification, and action of fat substitutes available as artificial materials. Mona A. M. Abd El-Gawad reviewed the application of fat replacers in different types of cheese. Jihan M. Kassem described the use of fat substitutes in fermented dairy products and corresponding author. Mohamed Salama wrote the application of fat replacers in fatty products. All the authors were involved in reviewing the article.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Phenolic compounds in purple whole-wheat flour and bread: Comparative analysis

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Abstract:
Whole-wheat flour of purple wheat varieties contains anthocyanins and other phenolic compounds with high antioxidant activity, which makes it a potential raw material for functional foods. The content and composition of phenolic compounds in whole-wheat flour depends on the genotype and weather conditions; in the bread, however, they also depend on the bread-making technology. This article offers a comparative analysis of phenolic compounds in purple whole-wheat flour and bread baked from this flour.
The study featured purple soft spring wheat (*Triticum aestivum* L.) of two varieties, Nadira and line 193, which were harvested in 2016 and 2017, and corresponding bread samples. The antioxidant activity and phenolic content were measured spectrophotometrically while the qualitative analysis relied on the method of high-performance liquid chromatography (HPLC).
In the hot and dry year of 2016, the content of bound phenolic acids reached 2.0–2.4 mg/g dry weight in the flour of both genotypes. In the cool and humid year of 2017, the content of anthocyanins in the Nadira flour increased by 2.7 times and amounted to 0.65 mg/g dry weight. However, the corresponding bread sample had a much lower phenolic content: soluble phenolics were halved, anthocyanins dropped by 3–4 times, and bound phenolic acids went down by 7–17 times. The content of bound phenolic acids in the flour correlated positively with the content of free phenolic acids in the bread. The HPLC analysis revealed an increase in the content of free hydroxycinnamic acids in the bread: p-coumaric acid was the most abundant and amounted to 0.14–0.22 mg/g dry weight.
Conventional State Standard 27669-88 for bread making resulted in a total decrease in anthocyanins, bound phenolic acids, and most free phenolics. Therefore, this technology cannot be applied to functional bread. The results can help develop a bread-making technology for purple wheat varieties.

Keywords: *Triticum aestivum* L., purple wheat, whole-wheat flour, bread, phenolic compounds, anthocyanins, phenolic acids, antioxidant activity

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INTRODUCTION

Bread has always been part of the daily dietary pattern in Russia and worldwide. As a source of basic nutrients, minerals, and biologically active substances, bread provides 10–20% of calories, 20% simple and complex carbohydrates, 20% dietary fiber and 11% protein [1–3]. White-flour bread comes from debranned/refined grain, which means that each seed has been cleaned from its pericarp, seed coat, and germ. While white-flour bread remains the most popular flour product, whole-grain wheat flour is a much more balanced raw material in terms of amino acids, fibers, phenolic compounds, carotenoids, vitamins, and minerals [1–4].

Whole grains are an inherent part of almost any healthy diet [5]. Whole-grain foods reduce excess weight, normalize cholesterol, as well as prevent diabetes, cardiovascular diseases, metabolic conditions, and cancers [6, 7]. Dietary fiber improves gastrointestinal motility and intestinal microbiota while phenolic compounds produce a certain antioxidant effect [5–11]. Humans and higher animals cannot synthesize phenolic compounds and have to obtain them with plant foods. In this respect,

cereals are a daily source of not only proteins and carbohydrates, but also phenolic compounds [12]. Grain contains both free and bound phenolic compounds, e.g., in cell walls or as links between polysaccharide and/or protein molecules. Free phenolic compounds are antioxidants [10]. As a rule, symbiont intestinal microflora releases bound phenolic compounds gradually.

Phenolic acids and flavonoids are the main phenolic compounds in cereals [2, 7]. These powerful antioxidants are to be found in the pericarp, the seed coat, and the germ [7, 10, 11]. Purple wheat also contains anthocyanins in its pericarp, which give these sorts extra antioxidant properties [9, 13, 14]. Whole-wheat flours have higher antioxidant activity levels than traditional white flours, with whole-wheat flours of anthocyanin-pigmented cultivars having the highest levels of antioxidant activity [2, 11, 13]. Similarly, whole-wheat bread, especially that made of purple varieties, possesses greater antioxidant effect than white-flour bakery products [13]. In Europe and North America, purple wheats are a source of popular functional foods, e.g., bread, crackers, and even confectionery bakery products [8, 13, 15–17].

In the Russian Federation, purple-grain wheat lines adapted to the local climatic conditions have been developed [18, 19]. However, the share of therapeutic bakery products on the Russian market is as low as 2% of total production, not to mention that purple wheat has never found its way on it [20, 21]. The beneficial properties of domestic purple-wheat bread and pastry have just barely attracted scientific attention [19, 22], and no reliable production technology has been developed so far. The Nadira variety is the first Russian patented purple wheat [23]. Its white flour demonstrated baking properties very similar to those of traditional premium wheat [19]. The total baking score for white flour bread was 4.4 out of 5, while the whole wheat flour bread had a score of 4 [19]. Obviously, the Nadira flour proved suitable for baking. However, whole-wheat bread from the Nadira variety has never been tested for antioxidant properties and phenolic compounds.

Bread making includes four basic stages: kneading, fermenting, proofing, and baking. Each stage causes certain chemical changes that lead to qualitative and quantitative changes in the composition of the final product. During kneading and fermentation, gluten proteins bind water while polypeptide chains cross-link with disulfide bridges to form a single gluten matrix. The rheological properties of the dough depend on this matrix. Hydrolase activation occurs when glycosidases break down polysaccharides into simple sugars, and esterases release phenolics bound with polysaccharides [24]. Kneading, fermentation, and proofing reduce the content of anthocyanins while simultaneously increase the content of phenolic acids. The resulting content of phenolic acids depends both on the total phenolics in the flour and the duration of each stage [13]. The high temperatures triggers various processes that affect phenolic structure, i.e., isomerization and cleavage of glycosidic and/or ether bonds. As a result, during the baking stage both the formation and conversion/degradation of phenolic compounds occur. Indeed, the content and composition of anthocyanins and other phenolics in bread and other bakery products may differ from those in the grain they were made from [13, 15–17]. By changing the baking time and temperature, bakers can modify phenolic composition [13, 16, 25]. Therefore, bread making causes dynamic changes in the qualitative and quantitative phenolic content of the final product, depending on the production technology, as well as on the phenolic content and genotype of cereals [16, 17, 26].

However, genotype is not the only factor that affects phenolics. Biotic and abiotic stress factors, e.g., weather conditions, are able to increase phenolic synthesis [2]. Each genotype has its own stress-causing conditions. In a previous study, we proved that the Nadira variety experiences moderate temperatures and sufficient moisture as optimal, in contrast to line 193 [27]. Under different weather conditions, the content and composition of anthocyanin and other phenolics may vary greatly for one and the same genotype [27]. The question remains, to what extent these differences in the composition of whole-wheat flour will be passed on to the corresponding bread. Presumably, the phenolic content in bakery products from whole-wheat flour of the same genotype will be different if the harvest year is different. Numerous publications feature the effect of weather conditions on the phenolic content of cereals [2, 28, 29]. However, no studies so far attempted to identify the correlation between the genotype- and weatherrelated phenolic composition of whole-wheat flour and the phenolic composition in the bread baked from this whole-wheat flour.

This article introduces a comparative quantitative and qualitative assessment of phenolic and anthocyanin profile of bread samples baked according to State Standard 27669-88 from whole-grain flour of two varieties of purple spring soft wheat grown and harvested under contrasting weather conditions.

STUDY OBJECTS AND METHODS

This research featured purple spring soft wheats (*Triticum aestivum* L.) of two varieties, namely cultivar Nadira (hereinafter Nadira) and line Kk-193-08-1 (hereinafter line 193). The varieties were harvested by the Tatar Research Institute of Agriculture, FRC Kazan Scientific Center of RAS, in 2016 and 2017. To obtain whole-wheat flour (Fig. 1a–d), we ground the grain in a MM400 ball mill (Retsch, Germany) at 25 Hz for 3 min. The bread-making procedure followed State Standard 27669-88 under laboratory conditions. The resulting bread samples (Fig. 1e–h) were freeze-dried in an Alpha 1-4 LD-2 freeze dryer (Martin Christ, Germany) and ground in a ball mill under the above mentioned conditions.

Figure 2 illustrates the process of phenolic isolation. To extract soluble phenolics, we poured 1 mL of 60% acidified ethanol (final concentration of HCl 1%) into 100 mg of whole-wheat flour or bread. After 10 min of ultrasonication at 30 kHz and 20°C, the samples were incubated on an MR-1 shaker (BioSan, Latvia) at 4°C for 24 h.

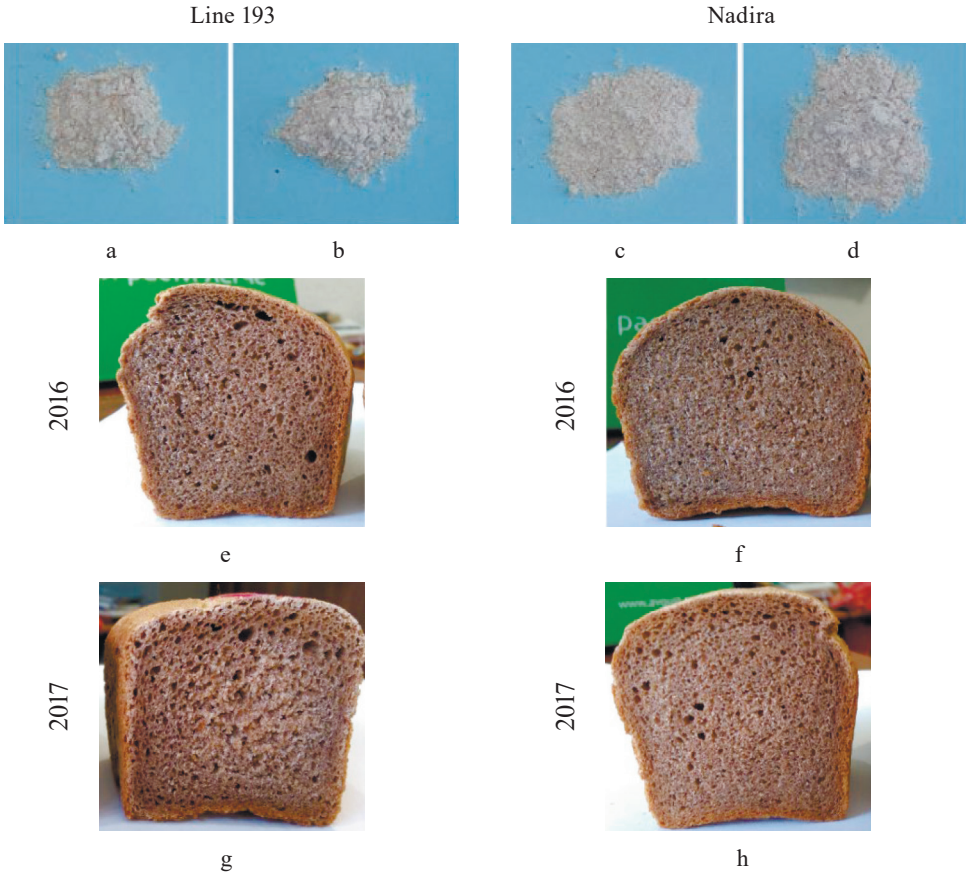


Figure 1 Whole-wheat flour (a–d) and whole-wheat bread (e–h): soft spring purple wheat of line 193 (a, b, e, g) and Nadira (c, d, f, h) varieties harvested in 2016 (a, b, e, g) and 2017 (c, d, f, h)

After that, the homogenate was centrifuged in a Mini-Spin centrifuge (Eppendorf, USA) at 10 000 g for 10 min. Upon collecting the supernatant, we washed the precipitate twice with 0.25 mL of 60% acidified ethanol and subjected it to ultrasonic treatment. The combined supernatants were used to determine the antioxidant activity as well as the content and composition of anthocyanins and other phenolics.

We used the Folin – Ciocalteu method to define the content of soluble phenolics, as well as free and bound phenolics [30]. The procedure involved a LAMBDA 25 spectrophotometer (PerkinElmer, USA). The results were calculated as milligram equivalent of gallic acid per 1 g dry weight. The anthocyanin content was determined by measuring the optical density of ethanol extracts at 525 nm. We used cyanidin-3-O-glucoside (Sigma-Aldrich, USA) to plot the calibration curve. The results were expressed as milligram equivalent of cyanidin-3-O-glucoside per 1 g dry weight. The antioxidant activity tests involved diphenylpicrylhydrazine (AlfaAesar, USA) and followed the pattern previously described [27].

To isolate phenolic acids, we modified the method developed by Kim *et al.* (Fig. 2) [31]. For free phenolic acids, we concentrated the ethanol extract at 35°C and 20 mbar on a vacuum concentrator (Concentrator Plus, Eppendorf, USA) and poured in 1% HCl to reach the final volume of 1 mL. After that, we added a mix of diethyl ether and ethyl acetate (1:1). The resulting mix was centrifuged for 5 min at 10 000 g to collect and dry the upper ether phase. The dry sample was dissolved in 80% ethanol to define the content of free phenolic acids.

For bound phenolic acids, we extracted 2 M NaOH from the sediment that remained after the extraction of soluble phenolics. The procedure lasted 4 h at 20°C on a shaker. After collecting the supernatant from the homogenate centrifuged at 10 000 g for 10 min, the pH of the extract was adjusted to 2 and added a mix of diethyl ether and ethyl acetate (1:1). To isolate bound phenolic acids from the solvent mix, we performed the same procedure as for free phenolic acids (Fig. 2). The dried samples were dissolved in 80% ethanol to determine the content of bound phenolic acids.

To compare the phenolic content in the bread samples with that in the whole-wheat flour, we used the formula below [26]:

$$\% \text{ difference} = \frac{(C_{flour} - C_{bread}) \times 1.04}{C_{flour}} \times 100$$

where C_{flour} is the content of phenolics in 1 g of flour, mg, and C_{bread} is the content of phenolics in 1 g of bread, mg. The coefficient equaled 1.04 and depended on the share of flour in the total weight of dry ingredients specified in State Standard 27669-88.

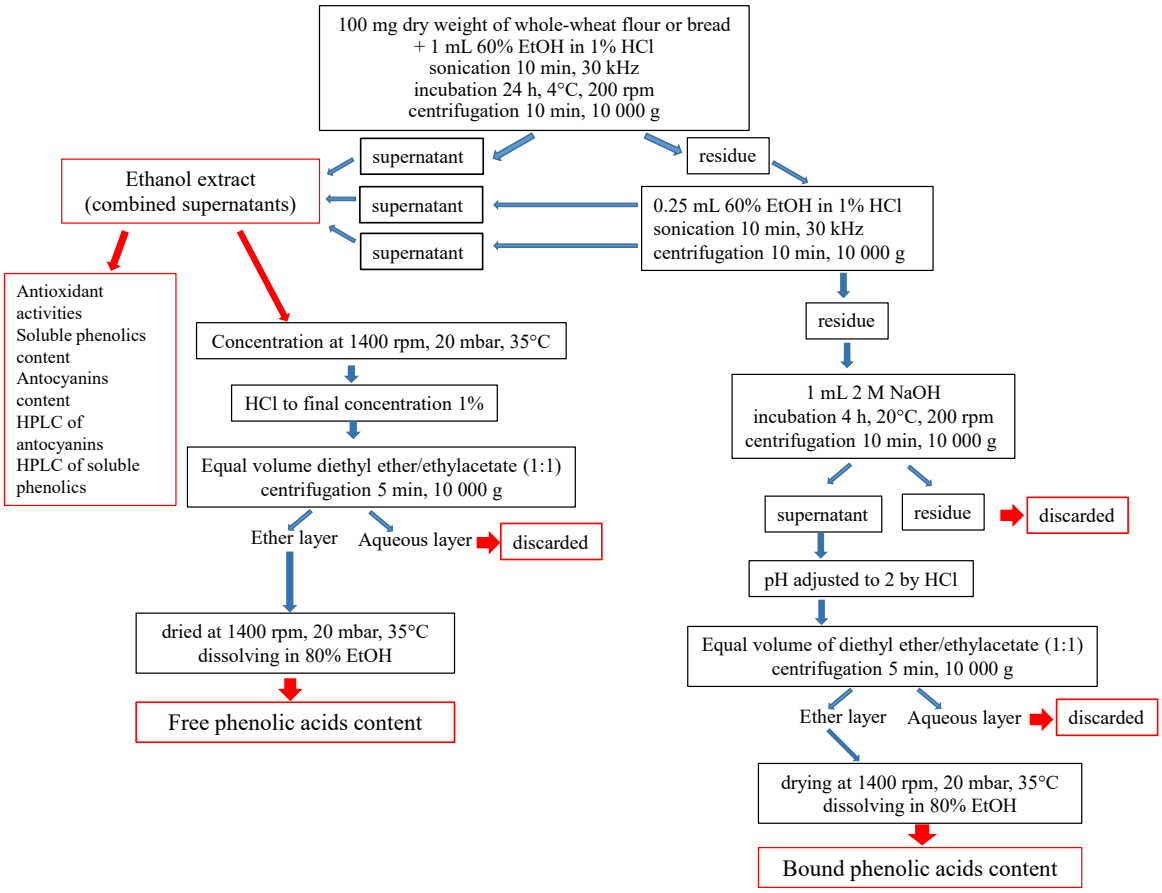


Figure 2 Scheme for isolating phenolic compounds.

A similar formula was used to calculated the difference in antioxidant activities.

The chromatographic analysis involved reverse-phase high performance liquid chromatography (RP-HPLC) on a Breeze chromatograph (Waters, USA) and a Waters 2489 UV/VIS dual-wave detector (Waters, USA). A reverse-phase Symmetry® C18 column (Waters, USA) served as the stationary phase: 3.9×150 mm column size, 5 µm particle size, 100 Å pore size.

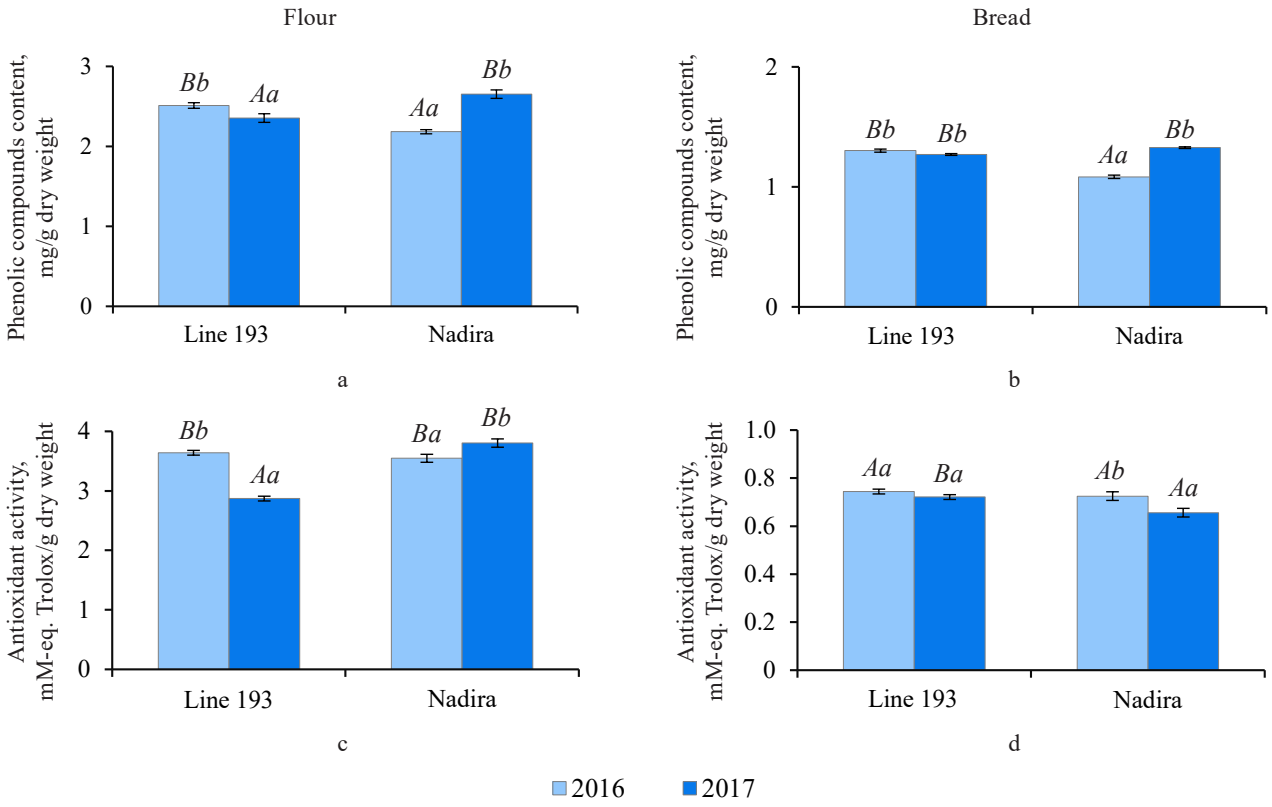
To study anthocyanins, we used two solutions as the mobile phase. Solution A consisted of 10% acetic acid while solution B involved acetonitrile, water, and glacial acetic acid (10:9:1, v/v). The separation was in line with the following pattern: 2 min – 100% solution A, 10 min – 25% solution B, 10 min – 50% solution B. The mobile phase feed rate was 0.5 mL/min. The volume of the injected sample was 20 µL. The peak detection occurred at 525 nm. Cyanidin-3-glucoside and cyanidin (Sigma-Aldrich, USA) served as standards.

For other phenolics, the mobile phase was represented by solution A (6% acetic acid) and solution B (60% methanol in 6% acetic acid). Solution B was graded as follows: 0–1 min – solution A; 1–6 min – 0–25% solution B; 6–16 min – 25–100% solution B; 16–21 min – 100% solution B; 21–25 min – 100% solution B. The mobile phase feed rate was 0.5 mL/min. The volume of the injected sample was 20 µL. The peaks were detected at 280 nm. For phenolic acids and flavonoids, the procedure relied on the reference peaks for gallic, *p*-coumaric, ferulic, sinapic, and benzoic acids and rutin, quercetin, and kaempferol (Sigma-Aldrich, USA). The contents of the chromatographically identified phenolics were determined using the calibration curves constructed from the areas of chromatographic peaks for standard phenolics.

The statistical data processing involved the SigmaPlot program and a two-factor analysis of variance (ANOVA) to be presented as mean ± standard error. The differences were declared significant based on the Tukey multiple comparison test ($p \leq 0.05$). Pearson's correlation coefficients were calculated at $p \leq 0.05$. The principal component analysis was carried out using OriginPro 2017.

RESULTS AND DISCUSSION

Defining soluble phenolics and antioxidant properties of flour and bread. The purple wheat varieties line 193 and Nadira were harvested in 2016 and 2017. Those years differed in weather conditions. The year of 2016 was hot and dry while 2017 was cool and humid. The content of soluble phenolics in the Nadira flour samples from 2016 was by 18% lower compared to the samples harvested in 2017. For line 193, the content of soluble phenolics in the 2016 samples was, on the contrary,



Note: the letters mark significant differences in mean values ($p \leq 0.05$). The capital letters indicate the difference between genotypes of the same harvest year; the lowercase letters indicate the difference between the harvest years for the same genotype

Figure 3 Soluble phenolics and antioxidant activity of ethanol extract of flour (a, c) and bread (b, d) baked from purple whole-wheat flour, harvests 2016 and 2017: a, b – soluble phenolics, mg/g dry weight; c, d – antioxidant activity of ethanol extract, mmol equivalent Trolox/g dry weight

Table 1 Antioxidant activity and soluble phenolics: bread from whole-wheat flour vs. whole-wheat flour

	Line 193		Nadira	
	2016	2017	2016	2017
Antioxidant activity	–78.74	–73.87	–78.74	–82.06
Soluble phenolics	–46.07	–43.89	–48.36	–47.98
Anthocyanins	–67.41	–59.72	–69.85	–72.19
Free phenolic acids	+4.32	–6.34	+34.11	–12.89
Bound phenolic acids	–91.16	–87.50	–94.28	–85.04

"+" is % increase, "–" is % decrease

For the coefficient, see Study Objects and Methods

by 7% higher (Fig. 3a). As for the bread, the phenolic content was by 44–48% lower than in the flour (Table 1) and amounted to 1.1–1.3 mg/g dry weight (Fig. 3b). Table 2 demonstrates that the year-related changes in the phenolic content correlated in the bread and the flour (r = +0.81).

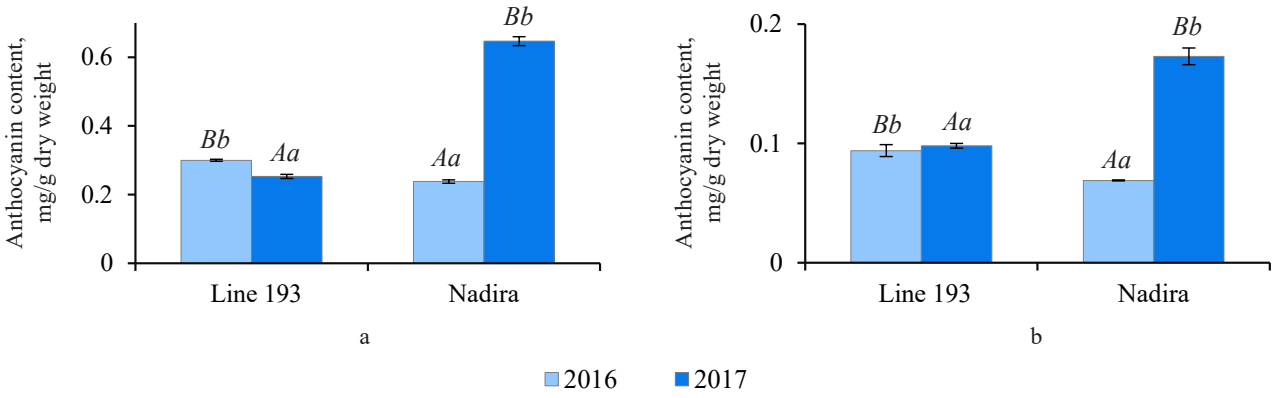
In the bread samples, the antioxidant activity was by 74–82% lower than that in the flour (Table 1). In addition, it did not depend on the phenolic content (Fig. 3c and d, Table 2). The antioxidant properties are known to depend on the phenolic structure. A particular phenolic composition may cause synergism, additivity, or antagonism [10]. Consequently, phenolic composition has a greater effect on the antioxidant activity of bread than the phenolic content in whole-wheat flour.

Analyzing the composition and content of anthocyanins in flour and bread. The anthocyanin content in the wheat of two genotypes depended on weather conditions. The dry and hot year of 2016 increased the anthocyanin content in line 193. However, it was the cool and wet year of 2017 that had the same effect on the Nadira variety (Fig. 4). After baking, the anthocyanin content dropped by 3.3–3.6 times in the Nadira variety and by 2.5–3.1 times in line 193 (Fig. 4). As a result, the bread samples maintained only 28–40% of the initial anthocyanin content (Table 1). The anthocyanin content in the flour samples correlated with their phenolic content (r = +0.76) and antioxidant activity (r = +0.57), as well as with the anthocyanin content in the corresponding bread samples (r = +0.96) (Table 2). However, we detected no positive correlation between the antioxidant activity of the bread and its anthocyanin content (Table 2). Probably, anthocyanins with high antioxidant activity were more affected by the baking process that those with poor antioxidant properties. Since anthocya-

Table 2 Correlation matrix of antioxidant activity and phenolic compounds in whole-wheat flour and bread

		Whole-wheat flour					Bread				
		Anti-oxidant activity	Soluble phenolics	Antho-cyanins	Free phenolic acids	Bound phenolic acids	Anti-oxidant activity	Soluble phenolics	Antho-cyanins	Free phenolic acids	Bound phenolic acids
Whole-wheat flour	Anti-oxidant activity	1	0.372	0.573*	–0.121	0.127	–0.681**	0.005	0.395	0.131	–0.183
	Soluble phenolics		1	0.758***	0.520*	–0.700**	–0.450	0.810***	0.810***	–0.708**	0.604*
	Antho-cyanins			1	0.542*	–0.693**	–0.788***	0.577*	0.960***	–0.604*	0.592*
	Free phenolic acids				1	–0.844***	–0.263	0.727**	0.656**	–0.820***	0.805***
	Bound phenolic acids					1	0.310	–0.850***	–0.833***	0.944***	–0.924***
Bread	Anti-oxidant activity						1	–0.153	–0.668**	0.237	–0.280
	Soluble phenolics							1	0.693**	–0.911***	0.810***
	Antho-cyanins								1	–0.745***	0.723**
	Free phenolic acids									1	–0.962***
	Bound phenolic acids										1

Bold characters represent significant correlations at p, indicated by asterisks (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$) with a correlation coefficient of $R > 0.5$. R-values for positive correlations are color-coded as follows: almond (0.51–0.70), orange (0.71–0.90), and orange-red (0.91–1.0). R-values for negative correlations are color-coded as follows: light cyan (from –0.5 to –0.7), powder blue (from –0.71 to –0.9), deep sky blue (from 0.91 to 1.0)



Note: the letters mark significant differences in mean values ($p \leq 0.05$). The capital letters indicate the difference between genotypes of the same harvest year; the lowercase letters indicate the difference between the harvest years for the same genotype

Figure 4 Anthocyanin content, mEq of cyanidin-3-O-glucoside/g dry weight

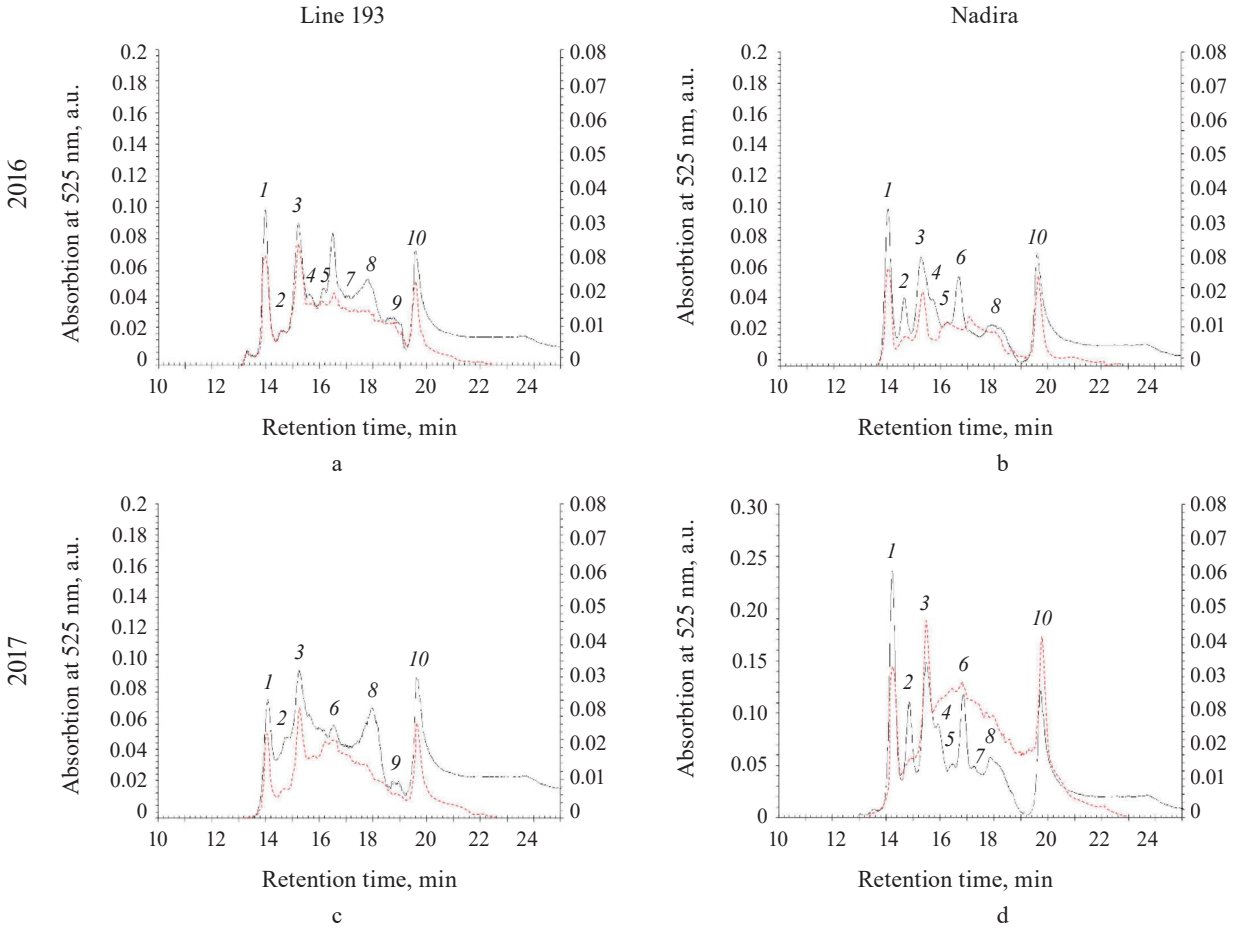
nins vary in antioxidant properties and thermal stability, some of them can be strong antioxidants and thermolabile compounds at the same time [9, 16, 17].

To assess the baking qualities of the whole-wheat flour, we carried out baking under laboratory conditions in line with State Standard 27669-88. It presupposes a straight dough procedure with the total kneading, fermentation, and proofing time of ≥ 250 min at 28°C. The baking time was 55 min at 200–210°C. The processing time exceeded the standard time for other bread formulations: the fermentation and proofing were 2.1–6.4 times as long and the baking was 2.2–3.9 times as long [13, 15–17].

Under the effect of peroxidases, anthocyanins may start degrading as early as during kneading [24]. Beta *et al.* used the Canadian Short Process Bread Baking to register a significant decrease in anthocyanins (1.1–1.2 times) and qualitative changes in the phenolic composition during the first three stages [13]. However, it was the baking stage that produced the most significant effect and reduced the anthocyanin content by 2.2 times [13]. Bartl *et al.* reported that a shorter baking time preserved more anthocyanins [16]. After 30 min at 180°C, 27% of anthocyanins remained in the flour; however, 21 min at even higher temperatures (240°C) spared 39% of anthocyanins [16]. Gamel *et al.* reported similar results for the bread-making standards published by the American Association of Cereal Chemists (Method 10.10.3) [15]. After 17 min at 215°C, the bread maintained 38% of the initial anthocyanins. Consequently, fermentation and baking time is as important as the initial anthocyanin composition and content in the flour.

According to Eliášová *et al.*, different purple wheat varieties had different anthocyanin composition, hence the different anthocyanin content in the corresponding bread [17]. The AF Jumiko variety with its peonidin-3-glucoside demonstrated the best resistance to anthocyanin degradation. The Konini variety had a lot of thermolabile delphinidin glycosides (30%) and proved to be the least resistant sample in the test. Thus, the anthocyanin content in bread depends both on the total anthocyanin content in the flour, the qualitative composition of anthocyanin in the flour, and the baking mode.

Figure 5 illustrates the HPLC analysis of anthocyanins in the flour and in the corresponding bread samples. Since the anthocyanins obtained from the flour and the bread demonstrated very different peak absorption intensity, the chromatograms contained two ordinate axes: the left axe illustrated the data obtained for the flour samples and the right axe was reserved for the bread samples. The HPLC revealed ten compounds (peaks 1–10): nine belonged to both lines while the compound that peaked 9 was specific to line 193. Cyanidin-3-glucoside (peak 1) and its aglycone, cyanidin (peak 10), covered the largest area (Fig. 5). In 2017, the content of cyanidin-3-glucoside in the Nadira flour was 2.7 times higher than in 2016. It exceeded the data for line 193 by 2.2 and 2.6 times in 2016 and 2017, respectively. Most publications report cyanidin-3-glucoside as the most frequent



1 – cyanidin-3-glucoside, *2-9* – unidentified compounds, *10* – cyanidin

Note: axis *x* represents retention time, min; axis *y* represents absorbance at 525 nm; left axis marks flour samples; right axis marks bread samples

Figure 5 Chromatograms of extracts of anthocyanin pigments in whole-wheat flour (black solid line) and bread (red dotted line): line 193 (a, c) and Nadira variety (b, d) of purple soft spring wheat, harvests 2016 (a, b) and 2017 (c, d)

anthocyanin identified for purple wheats [2, 32]. Other studies may report other cyanidin glycosides, as well as glycosides of pelargonidin, peonidin, malvinidin, petunidin, and sometimes delphinidin [16, 32].

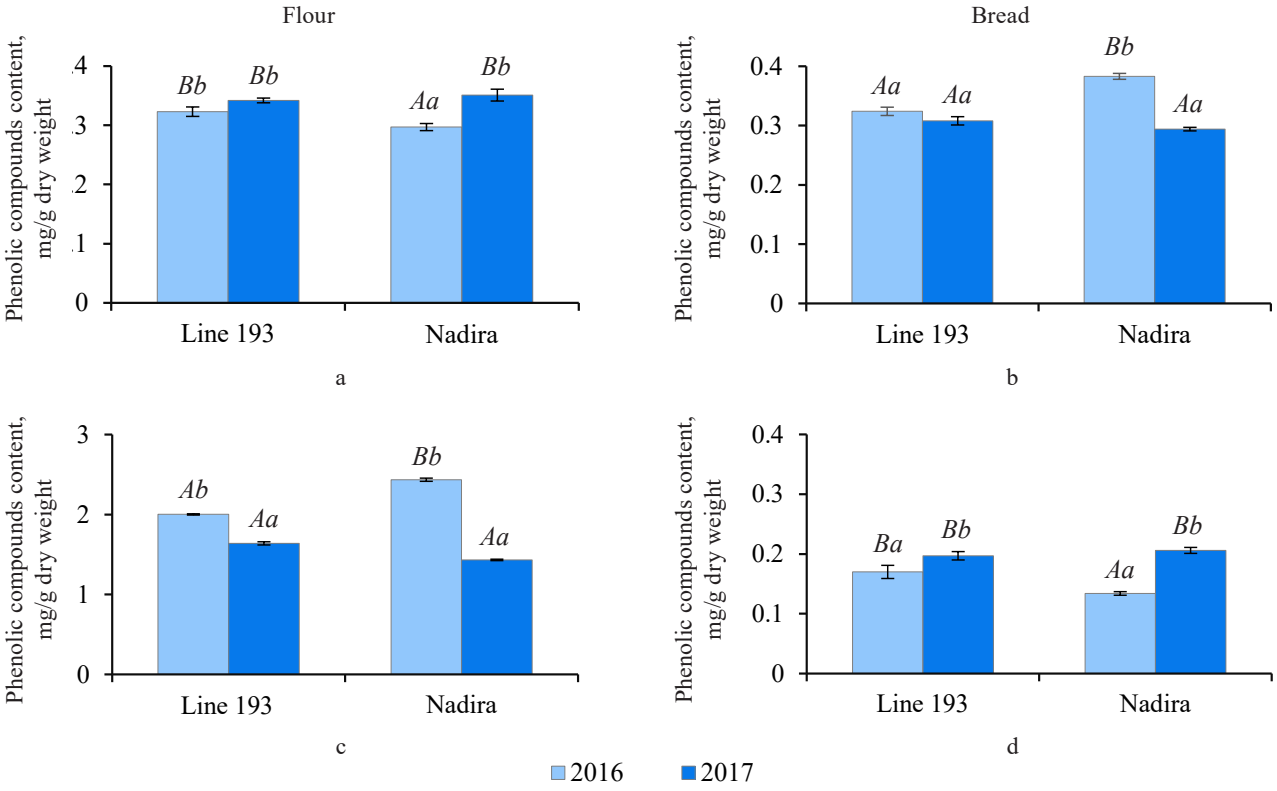
The HPLC-analysis also revealed genotype differences. The height of peak 8 in the line 193 samples exceeded that in the Nadira samples, while peak 2 showed an opposite trend (Fig. 5). The whole-wheat flour obtained from line 193 in 2016 (peak 8) had less anthocyanin than the samples obtained from the harvest of 2017; peaks 3, 6, and 9 were more intensive. Compared to 2016, the content of all anthocyanins of Nadira increased in 2017 and the changes in the proportions of anthocyanins were minor.

The bread samples demonstrated a different HPLC anthocyanin spectrum. The total area of all peaks combined dropped by 4.5–5.4. Peaks 1, 3, and 10 were the most common, their share in the total area of all anthocyanin peaks occupied 87% for line 193 and 91% for the Nadira variety. Other peaks became minor (Fig. 5): apparently, they corresponded with thermolabile anthocyanins. Cyanidin-3-glucoside, peak 3, and cyanidin appeared to be the most variable peaks for the bread samples. The changes were quantitative and mirrored the variations in the corresponding flour samples.

It takes some time for heat to destroy anthocyanins. First, they are deacylated and deglycosylated, releasing sugars. Acylated anthocyanins release phenolic acids. Acylated anthocyanins are the most numerous anthocyanins in purple wheats [16, 32]. Long thermal exposure destroys anthocyanidins, releasing phloroglucinaldehyde and hydroxybenzoic phenolic acids, corresponding to the structure of the B-ring of the anthocyanin. For example, protocatechuic acid comes from cyanidin, while gallic acid comes from delphinidin [33]. Since phloroglucinaldehyde is thermolabile and hydroxybenzoic acids are more stable, these phenolic acids usually end up in the fraction of free phenolic acids of bread [33].

Phenolic acid fractions in flour and bread. Table 2 clearly demonstrates a high degree of negative correlation between the fractions of free and bound phenolic acids in the flour samples (r = –0.844). In the dry year of 2016, the Nadira variety had a 1.2-fold decrease in soluble phenolic acids and a 1.7-fold increase in bound phenolic acids, compared to 2017. The line 193 samples also demonstrated an increase in bound phenolic acids, although to a lesser extent (1.22 times), compared to the Nadira variety (Fig. 6). During droughts, more phenolics enter cell walls to strengthen their structure [34]. However, this process is different in different genotypes.

In bread from the wheat harvested in 2017, the content of soluble phenolic compounds decreased by 1.1–1.2 times, compared to the corresponding flour samples. As for the harvest of 2016, the situation was different:



Note. The letters mark significant differences in mean values ($p \leq 0.05$). The capital letters indicate the difference between genotypes of the same harvest year; the lowercase letters indicate the difference between the harvest years for the same genotype

Figure 6 Free (a, b) and bound (c, d) phenolic acids in flour (a, c) and bread (b, d) baked from whole-wheat flour obtained from line 193 and Nadira varieties of spring soft purple wheat, harvests 2016 and 2017

in the line 193 samples, the content of soluble phenolic compounds remained the same; in the Nadira variety, it increased by 1.3 times (Fig. 6a and b). The content of bound phenolic acids in the bread dropped down to 5–15% of their initial content in the flour, i.e., from 1.4–2.4 mg to 0.13–0.21 mg (Fig. 6, Table 1). The data obtained for bound phenolic acids in the flour and in the bread demonstrated a high degree of negative correlation ($r = –0.924$, $p \leq 0.001$) (Table 2). A similarly high degree of negative correlation occurred between bound and free phenolic acids in the bread samples ($r = –0.962$, $p \leq 0.001$) (Table 2). However, a high positive correlation was detected between bound phenolic acids in the flour and free phenolic acids in the bread ($r = +0.944$, $p \leq 0.001$) (Table 2).

The complex biochemical processes that take place at the dough fermentation stage release bound phenolic acids [24]. For instance, most muffins and cookies require no fermentation stage. As a result, they maintain the initial content of bound phenolic acids while bread, which cannot be baked without fermentation, contains less phenolic acid [26]. High-temperature baking destroys phenolic acids [35, 36]. In this research, the bread had less phenolic acid compared its initial content in the flour because its production time was quite long and presupposed conditions for a more active release of bound phenolic acids during proofing and fermentation, and then free phenolic acids were destroyed by baking. State Standard 27669-88 establishes 55 min of baking time at 200–210°C, which destroys most phenolic compounds. Studies that involved other formulations reported an increase in the content of free phenolic acids in bread [13, 26]. In our study, only one bread sample demonstrated this trend: it was the bread baked from the Nadira grain harvested in 2016 (Fig. 6b). Apparently, this increase corresponded with the higher content of bound phenolic acids in this flour, which was 2.44 mg while other flour samples had it as low as 1.4–2.0 mg (Fig. 6c). This sample seemed to release more bound phenolic acids, part of which do not completely degrade during the baking stage.

HPLC analysis of soluble phenolics. Ferulic, vanillic, sinapic, and hydroxybenzoic acids are the most abundant free phenolic acids in wheat. Their proportion depends on the genotype and weather conditions [2, 28, 29]. Ferulic and vanillic acids were usually reported as domineering, with some exceptions [28, 31, 37, 38]. The HPLC analysis of soluble phenolics (Fig. 7) revealed peaks of varying intensity, which corresponded with hydroxycinnamic acids, i.e., ferulic, sinapic, and *p*-coumaric. Due to their high antioxidant activities, hydroxycinnamic acids exhibit various therapeutic and preventive effects on people and animals [10, 39]. Ferulic and *p*-coumaric acids are the most bioavailable compounds from whole-wheat flour. In addition, they are believed to prevent cancer and cardiovascular diseases [39, 40]. In the Nadira flour, the content of ferulic and sinapic acids exceeded that in the line 193 flour samples (Table 3). In line 193, the content of coumaric acid depended on the year: it amounted to 30.1 and 40.2 μg/g dry weight in 2016 and 2017, respectively. Unlike line 193, the Nadira flour maintained almost the same content of coumaric acid, which stayed 39–40 μg/g dry weight under different weather conditions.

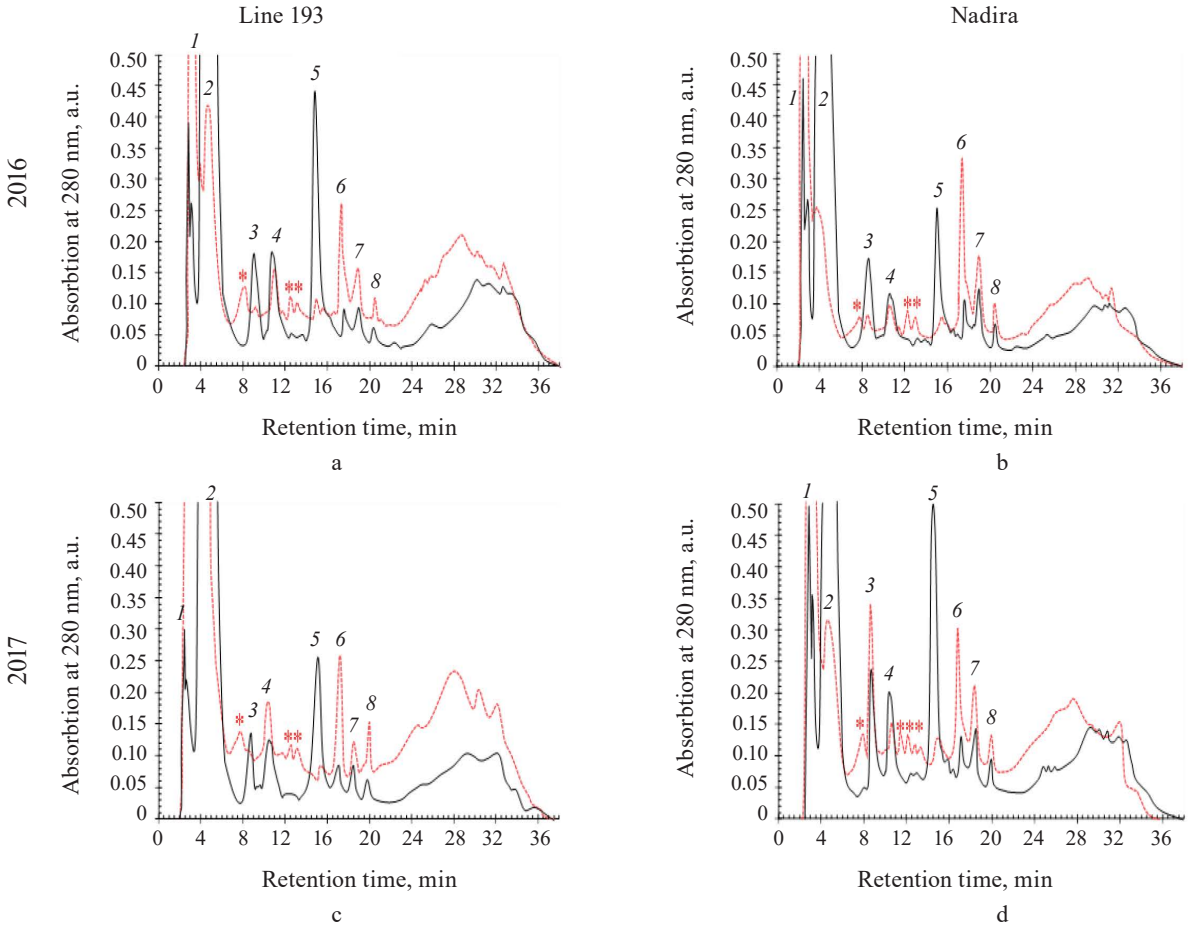
The Nadira bread samples contained more ferulic acid than the line 193 bread samples. The bread samples made from the grain harvested in 2016 demonstrated the most significant difference (2.7 times) (Table 3) because ferulic acid accumulated in the bound phenolic acids of flour to be released. Ferulic acid dominates the fraction of bound phenolic acids of wheat grain, regardless of its variety [28, 31, 37]. Some phenolic acids are thermostable and some are thermolabile. Ferulic acid was reported to be a thermolabile compound while gallic, caffeic, protocatechuic, and coumaric acids proved more resistant to high temperatures [35, 36, 41].

In our study, however, the content of coumaric acid appeared to be much higher in bread than in the corresponding flour samples (Table 3). Other hydroxycinnamic acids also had more intensive peaks in the bread samples than in the flour although the difference was quite unremarkable (Fig. 7). The long baking time apparently contributed to the destruction of ferulic acid and the accumulation of more thermostable coumaric acid. The fermentation stage releases phenolic acids associated with oligo- and polysaccharides, as well as flavonoids, including anthocyanins. Acylated anthocyanins were reported to contain *p*-coumaric acid [16]. For instance, Beta *et al.* showed that the content of coumaric acid in bread made from purple whole-wheat flour was seven times as high as in bread baked from white-wheat whole-grain flour [13].

Peaks 1, 2, and 5 had the largest areas among the unidentified peaks of flour phenolic compounds (Fig. 7). In the flour samples of both genotypes, the area of peak 1 was smaller than in the bread while peak 2, on the cont-

Table 3 Hydroxycinnamic acids in whole-wheat flour and bread

Phenolic compounds	Hydroxycinnamic acids content, μg/g dry weight							
	Line193				Nadira			
	Flour		Bread		Flour		Bread	
	2016	2017	2016	2017	2016	2017	2016	2017
p-coumaric acid	30.1	40.2	140.3	210.3	39.8	38.6	217.1	174.6
Ferulic acid	36.2	40.4	30.2	46.4	61.7	51.1	82.7	65.2
Sinapic acid	18.5	21.2	30.4	26.3	25.3	29.7	31.8	30.9



1–5 – unidentified compounds in flour and bread; *6* – coumaric acid; *7* – ferulic acid; *8* – sinapic acid, * – unidentified compounds in bread samples

Figure 7 Chromatograms of phenolic compounds extracted from whole-grain flour (black line) and bread (red dots) from line 193 (a, c) and the Nadira variety (b, d) of purple soft spring wheat, harvests 2016 (a, b) and 2017 (c, d)

rary, was significantly larger. The total area of peaks 2–5 was much smaller in the bread samples. The only exception was peak 3 in the bread made from the Nadira wheat harvested in 2017 (Fig. 7). The response to weather conditions depended on the wheat variety. In the Nadira flour, peaks 4 and 5 were lower in 2016 than in 2017; in line 193, the picture was quite opposite (Fig. 7).

The unidentified peaks may relate to flavonoids or some phenolics that are typical of whole-wheat flour, e.g., hydroxybenzoic, vanillic, gallic, and syringic acids [28, 31, 38]. Other studies reported thermostable protocatechuic acid in grain and bread obtained from purple wheats [13, 38, 42]. Protocatechuic acid can be released from the fraction of bound phenolic acids; it may also appear during the destruction of cyanidin and/or ferulic acid [43]. Some minor phenolic peaks that we registered in the bread but not in the flour could also be attributed to the destruction of thermolabile phenolics, primarily anthocyanins. In Fig. 7, they are marked with an asterisk.

To sum up, the content of soluble phenolics in the bread was halved compared to their initial content in the flour while the content of hydroxycinnamic acids increased. Most likely, it happened after the release of bound phenolics or after the destruction of complex phenolics, e.g., anthocyanins, which, as a result, were underrepresented in the bread.

The principal component analysis made it possible to identify the most variable factors and the differences between the samples for all factors. According to the biplot (Fig. 8), principal component 1 depended on most parameters, i.e., antioxidant activity, anthocyanin content, phenolic substances, and bound phenolic acids. It covered 69.54% of the differences between the samples. Principal component 2 was associated primarily with the content of free phenolic acids and was responsible for 21.2% cases. The samples made up several clusters (Fig. 8). The flour samples grouped on the positive side of principal component 1, which means that they had higher values for these parameters compared to the bread samples, which occupied the negative side of the graph along axis *x*. The Nadira flour samples from 2016 and 2017 demonstrated more differences than the flour obtained from line 193. The Nadira bread from the harvest of 2016 formed a separate cluster while the other bread samples seemed to be rather similar in most characteristics. In most cases, the weather-induced

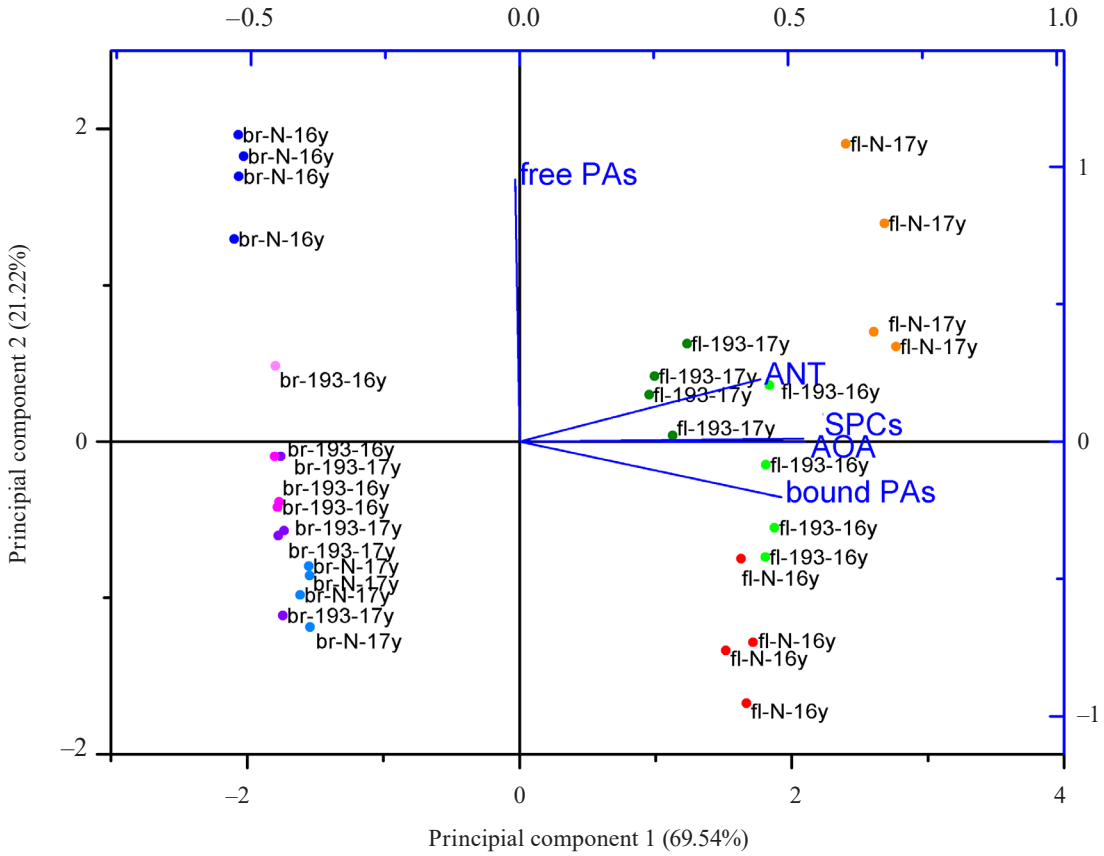


Figure 8 Principal component analysis scores plot. AOA – antioxidant activity, SPCs – soluble phenolic compounds, ANT – anthocyanins, PAs – phenolic acids; samples: fl-N-16y (red dots) – Nadira flour, 2016; fl-N-17y (orange dots) – Nadira flour, 2017; fl-193-16y (light green dots) – line 193 flour, 2016; fl-193-17y (dark green dots) – line 193 flour, 2017; br-N-16y (blue dots) – Nadira bread, 2016; br-N-17y (blue dots) – Nadira bread, 2017; br-193-16y (pink dots) – line 193 bread, 2016; br-193-17y (purple dots) – line 193 bread, 2017

phenolic changes in the flour were leveled out after the baking. However, the abundance of bound phenolic acids in the flour may have been that single beneficial weather-related change which resulted in the abundance of free phenolic acids in the bread after baking.

CONCLUSION

New functional food technologies require an integrated approach. The phenolic content of wheat depends on the combination of weather conditions and genotype while it is the bread-making technology that preserves biologically active substances in whole-wheat flour. The high anthocyanin content in the purple wheat of the Nadira variety harvested in 2017 seemed a valuable property to be used in functional foods. However, the long bread-making process in line with State Standard 27669-88 destroyed most anthocyanins and other soluble phenolics. As a result, the bread had much poorer antioxidant properties than the flour it was made from.

The bread baked from the Nadira wheat harvested in 2016 was the only sample to increase the content of free phenolic acids because the grain accumulated a lot of bound phenolic acids in response to the dry and hot weather conditions in 2016.

As a result of the bread-making procedures stipulated by State Standard 27669-88, the content of bound phenolic acids in the bread dropped by 85–95%, compared to the flour. However, the content of free hydroxycinnamic phenolic acids increased, especially that of *p*-coumaric acid. The amount of ferulic and sinapic acids also increased but to a lesser extent because the long baking destroyed them.

Therefore, bread-makers need to develop new technology to produce functional bread from purple wheat if they want to preserve the original amount of anthocyanin, phenolic acids, and other phenolics. Apparently, this new technology should have a shorter fermentation and baking time.

CONTRIBUTION

A.I. Valieva developed the research concept, provided the data validation and curation, performed the formal analysis, wrote the draft, designed the figures and tables, and proofread the manuscript. A.N. Akulov designed the methodology, validated and curated the data, performed the empirical research, and proofread the manuscript. N.I. Rumyantseva supervised the research, obtained funding, validated the data, obtained the resources, and proofread the manuscript. All

the authors have read and agreed to the final version of the manuscript.

CONFLICT OF INTEREST

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

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Extremophilic bacteria as biofertilizer for agricultural wheat

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Abstract:
Wheat (*Triticum L.*) is a strategically important agricultural crop because its quality and yield provide food security for the population. Biological fertilizers improve the growth and development of agricultural crops. Unlike chemical ones, they have no toxic effect on people and the environment. This research assessed the positive effect of extremophilic microorganisms isolated from coal dump soils of the Kemerovo Region (Russia) on the growth and development of wheat.

The study featured bacterial isolates of *Achromobacter denitrificans*, *Klebsiella oxytoca*, and *Rhizobium radiobacter*, as well as their consortia in four different ratios: 1:1:1 (Consortium A), 2:1:1 (Consortium B), 1:2:1 (Consortium C), 1:1:2 (Consortium D), respectively. The beneficial effect was assessed by determining such factors as nitrogen fixation, solubilization of phosphates, potassium, and zinc, and production of gibberellic acid, siderophores, and hydrogen cyanide. The wheat samples were checked for germination, root length, and stem length.

R. radiobacter demonstrated the best nitrogen fixation properties. Consortium D, with two shares of *R. radiobacter*, yielded the best results for zinc solubilization. *R. radiobacter* proved to be the most efficient potassium solubilizer while the isolate of *A. denitrificans* was the best phosphate solubilizer. The largest amount of gibberellic acid belonged to *K. oxytoca*. Consortium C, which included two shares of this isolate, appeared to be the most effective siderophore producer. All samples but *A. denitrificans* were able to produce hydrogen cyanide. The best seed germination rate (84%) belonged to Consortium C, which contained a double share of *K. oxytoca*. Consortia C and B (two shares of *A. denitrificans*) had the greatest positive effect on the root length. Treatment with Consortium B resulted in the longest average stem length.

Extremophilic microorganisms isolated from coal dump soils of the Kemerovo Region (Russia) had a good potential as biofertilizers that could improve wheat quality and local food security.

Keywords: Food safety, wheat, biofertilizers, extremophilic microorganisms, seed germination

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INTRODUCTION

The current world population of 7.9 billion people is expected to reach 10 billion people by 2050 [1]. Such rapid growth rate challenges the agricultural sector as the demand for food resources keeps growing. According to the Food and Agriculture Organization of the United Nations, the global production will have to increase by 60% over the next two decades to feed the growing population [2]. Almost 90% of all food comes from 12 crops and 14 animal species [3]. In particular, wheat, rice, and corn cover more than half of the world's food demand.

The agricultural importance of wheat can hardly be overestimated. Wheat is the main source of plant proteins in human and animal diets in more than 80 countries [4, 5]. In fact, one third of global population obtain 13–57% of their caloric intake from wheat, which makes wheat their main source of energy. Wheat is the second main source of energy in 26 countries, including China and India, and the third main source of energy in

another 16 countries. In total, about 85% of the world's population depend on wheat as their main source of energy. Therefore, increasing wheat production volumes is one of the most urgent tasks that the food industry has to tackle in the nearest future.

Traditionally, crop farming relies on chemical methods, which means severe man-induced environmental load and poor phytosanitary condition of agricultural lands [6–8]. Bacterial plant growth promotion, known in Russian agriculture as biologization, is a promising direction in wheat cultivation as it harnesses the potential of plant growth-stimulating bacteria [9–11]. These bacteria and their metabolites provide biofertilizers that boost the rhizospheric biogenicity, thus improving the ecological condition of the entire agrocenosis. Under proper conditions, microorganisms produce metabolites of agricultural importance [12]. Microorganisms and their metabolites break down complex soil minerals, turning them into growth-promoters for a particular crop.

Nitrogen is vital for plant growth. Soil contains two main forms of nitrogen, i.e., inorganic, or mineral, nitrogen (2%) and organic nitrogen (98%) [13–16]. Inorganic nitrogen includes ammonia (NH_3), ammonium (NH_4^+), nitrite (NO_2^-), and nitrates (NO_3^-) [17]. Organic nitrogen is to be found in organic nature, e.g., soil biota, fresh remains of animals and plants, etc., as well as in inorganic nature, e.g., as humified or non-humified compounds [18]. Mineral nitrogen is available to plants either as ammonium nitrogen (NH_4^+-N) or as nitrate nitrogen (NO_3-N) [19]. Organic nitrogen becomes available to plants only after mineralization into ammonium or nitrate [20]. Biological nitrogen fixation is another way of soil nitrification for plant nutrition. It converts dinitrogen (N_2) into a form suitable for plant uptake, e.g., NH_4^+. Rhizospheric microorganisms provide the biological fixation of atmospheric nitrogen.

Phosphorus is second to nitrogen in terms of plant growth and development. It is an essential macronutrient for plant metabolism, i.e., cell division, energy production, macromolecule biosynthesis, membrane integrity, signal transduction, and photosynthesis [21]. Phosphorus is inherent to plant respiration. Unfortunately, most phosphorus compounds are insoluble and non-bioavailable [22].

The total phosphorus content in soil approximates 0.05% (w/w). Plants are able to absorb as little as 0.1% of bioavailable phosphorus because of such processes as cation precipitation in the soil, immobilization, adsorption, and interconversion to organic form [23]. As a result, phosphate fertilizers are an extremely popular means of continuous supply of phosphorus to plants. However, they possess a significant disadvantage: they tend to prepitate in the soil in great quantities, which is associciated with such adverse effects as accumulation of heavy metals, soil depletion, etc. Thus, crop farming needs a green approach that could provide the same effect as chemical fertilizers without negative consequences for the environment.

Microorganisms consume phosphorus in several ways, depending on the inaccessible forms of its compounds in the soil. They can solubilize inorganic phosphates by acidification, protonation, or chelation. They can also mineralize organic phosphates biochemically, e.g., via such enzymes as phosphatase, phytase, phosphonatase, and C-P-lyase [22].

Potassium, the third plant nutrient element, is abundant in agricultural soil. Potassium is important for photosynthesis: it produces adenosine triphosphate, transports sugar, water, and nutrients, and synthesizes starch, as well as participates in legume- and enzyme-based nitrogen fixation and protein synthesis. However, barely 5% of all potassium in soil is available for plant intake since 95% of potassium is bound with various minerals [24]. Potassium is present in soil in mineral (unavailable), soluble (available), non-exchangeable (fixed), and exchangeable forms. Fixed potassium remains a reserve source, whereas exchangeable potassium is easily absorbed by plant roots.

While potassium is not the most important element for plants, young plants need it even more than nitrogen and phosphorus [25]. Potassium improves their growth and development, as well as increases their resistance to diseases and stresses, e.g., drought, frost, pests, etc. In addition, it improves the quality of the crop and extends the shelf life of agricultural products. Potassium was found able to promote photosynthesis, which means it affects the formation of carbohydrates, fats, and proteins, regulates water absorption by plant roots, and helps shape a healthy root system [26]. Irrational and excessive use of potassium fertilizers reduces the yield, disrupts the microbial soil community, and causes groundwater pollution [27, 28]. Potassium solubilizing bacteria are a safe alternative to chemical fertilizers. Microbial potassium mobilizes and solubilizes insoluble potassium-containing minerals, e.g., mica, muscovite, feldspar, biotite, illite, orthoclase, etc. [29]. It also releases potassium compounds by producing oxalic, citric, tartaric, succinic, or acetic organic acids [30]. Figure 1 shows which organic acids appear as a result of the activity of potassium-solubilizing microorganisms.

The abovementioned acids of microbial production release potassium ion from potassium-containing minerals by chelating ions of Al^{3+}, Si^{4+}, Ca^{2+}, and Fe^{2+}. Some microorganisms form biofilms on the surface of minerals or stones. This film creates a controlled optimal microenvironment around the cells, which facilitates solubilization by organic acids and secondary metabolites. This mechanism sometimes lowers the pH of the rhizosphere: as a result, the potassium-containing mineral dissolves better and becomes more available for plant uptake.

Zinc facilitates carbohydrate and auxin metabolisms. In addition, it possesses antioxidant properties [31]. Zinc deficiency slows down shoot growth, as well as causes chlorosis, leaf-size reduction, withering, and fungal infections. Zinc also affects grain yield, pollen production, root development, absorption and transport of water, etc. Plants absorb zinc in the form of a divalent cation, which is present in soil in very small quantities. Other forms of zinc include insoluble complexes and minerals. Zinc

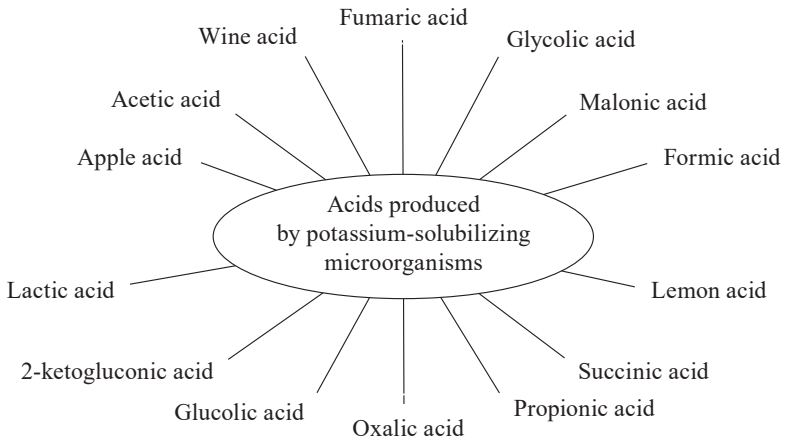

Figure 1 Organic acids involved in the release of potassium from potassium-containing minerals

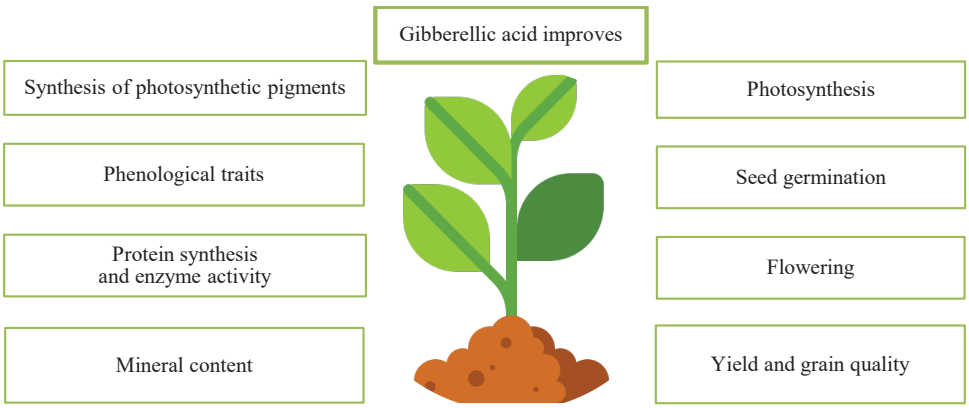

Figure 2 Effect of gibberellic acid on crops

is part of many fertilizers because zinc-poor agricultural products contribute to the development of zinc deficiency in people.

Zinc fertilizers have a long history. They include zinc sulfate, regular crop rotation, intercropping, cross-breeding, transgenic methods, and genetic engineering. However, all these methods are expensive, labor-intensive, and time-consuming, which makes zinc solubilizing microorganisms a prospective alternative. Zinc solubilizing microorganisms use a variety of strategies to convert zinc into a soluble form. For instance, they produce organic acids which bind zinc cations and reduce the pH in the immediate soil environment [32]. During acidifying, anions can also chelate zinc and increase its solubility. Other means of zinc solubilization include siderophores.

Siderophores are secondary low-molecular-weight metabolites with iron chelating properties. Iron is a vital element that participates in many biological processes, e.g., electron transport, oxygen metabolism, nitrogen xation, DNA and RNA synthesis, etc. [33–36]. Agricultural crops always experience serious iron deficiency because the content of available iron is negligible (10^{-9}–10^{-4} mol/L) and insufficient for plant growth and development. Most soils, especially alkaline ones, are extremely low in soluble iron, which is picomolar (10^{-9}–10^{-18} mol/L) [37]. Severe iron deficiency can cause plant death as early as at the seedling stage, thus reducing the yield. Siderophores use the transport mechanism of their cell membrane to transport iron ions. Metal ions combine with the siderophores produced by microorganisms in the soil around plant roots. While some complex compounds enter the cell membrane, metal ions remain in the periplasm and siderophores are released for cyclic use. Other complex compounds enter the cytoplasm through the cell membrane via the TonB mechanism [38]. Together with producing iron from insoluble hydroxide forms, siderophores also facilitate its release from iron citrate, iron phosphate, iron transferrin, iron in flavone pigment, sugars, and glycosides [39].

Growth regulators are important for plant development and protection. Gibberellic acid is an example of effective plant growth promoter. It is a phytohormone that affects the growth of roots and stems, as well as seed germination [40]. Figure 2 illustrates the role of gibberellic acid in plant growth and development.

Seed germination is an important stage controlled by environmental variables, e.g., light, humidity, temperature, etc., as well as by endogenous phytohormones, e.g.,

abscisic acid, gibberellic acid, etc. Gibberellic acid proved to inhibit the action of abscisic acid, a hormone that reduces the growth and development of seeds [41]. Endosperm cells cannot rapture without gibberellic acid, the level of which increases during swelling, which means that gibberellic acid is vital for root development.

Hydrogen cyanide is also important due to its toxic effect on plant pathogens. Hydrogen cyanide chelates metal ions and improves phosphate availability [41, 42]. As a product of bacterial synthesis, hydrogen cyanide is applied in the production of indolylacetic acid, antibiotics, and fluorescent insecticidal toxins, as well as in utilization of 1-aminocyclopropane-1-carboxylate deaminase [43].

The Kemerovo Region aka Kuzbass is an industrial region with numerous enterprises related to fuel and energy production, metallurgy, chemistry, coal mining, etc. Their anthropogenic effect threatens the quality of life of the population, not to mention the local biodiversity and biological soil capability [44, 45]. Industrial pollution leads to severe heavy-metal soil contamination. Heavy metals reduce the growth and productivity of agricultural plants and lower or even eliminate the effect of biological preparations. As a result, the local agricultural sector strives to develop safe methods of bioremediation of contaminated soil. At present, the local agricultural sector focuses on the concept of soil-protective and resource-saving agriculture to boost production volumes and improve the quality of agricultural products [46]. Some biofertilizers are based on extremophilic microorganisms isolated from polluted and disturbed soils [47]. These microorganisms have unique properties. In particular, some are able to accelerate plant growth and development. In addition, they are resistant to adverse environmental factors, e.g., heavy metals.

This research focused on extremophilic microorganisms isolated from coal dump soil to be used in biofertilizers that increase wheat yields.

STUDY OBJECTS AND METHODS

The study involved such extremophilic bacteria as *Klebsiella oxytoca*, *Rhizobium radiobacter*, and *Pseudomonas fluorescens*. They had been isolated from coal dump soil (53°26′ N; 87°25′ E) (Fig. 3) [48]. Microorganisms were isolated on a medium that contained salts of heavy metals, namely $CuSO_4$, $ZnSO_4$, $FeSO_4$, $CdCl_2$, $MgSO_4$, and $MnSO_4$.

The biocompatibility tests performed at the previous stage revealed several variants of bioconsortia (Table 1), which, together with individual extremophilic strains, became the objects of the current study.

Nitrogen fixation. The degree of nitrogen fixation was determined by spectrophotometry in Nfb nutrient medium at pH 6.5 [49]. The nutrient medium was sterilized at 121°C for 15 min.

To construct a calibration curve, we used 23 tubes with 2 mL sterile Nfb medium in each. Next, we added 0.93 N NH_4OH solution at the following quantities: 0.5, 1.0, 1.5, 2, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 11.0, and 12.0 µL. We used

Figure 3 Sampling coal dump

Table 1 Bioconsortia

Bioconsortium	*Achromobacter denitrificans*:*Klebsiella oxytoca*:*Rhizobium radiobacter* ratio
A	1:1:1
B	2:1:1
C	1:2:1
D	1:1:2

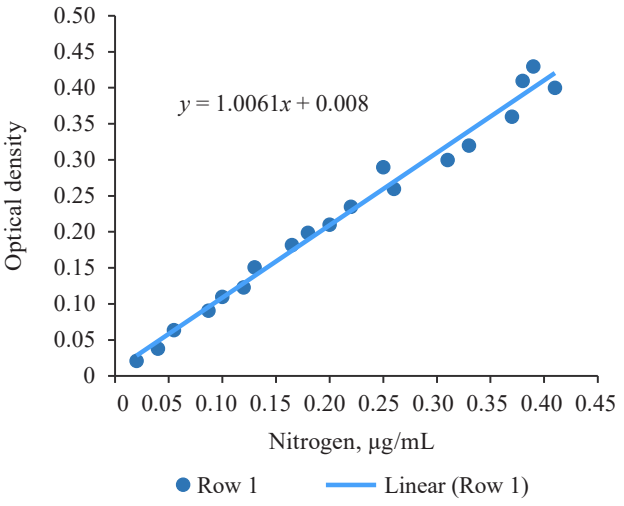

Figure 4 Standard absorbance curve

1.42 N HCl as a secondary standard. The total volume of each tube was adjusted to 3 mL with Nfb medium. We calculated the nitrogen concentration in each test tube based on the total volume and the volume of the NH_4OH solution we added. The optical density tests occurred at 610 nm using a UV 1800 spectrophotometer (Shimadzu, Japan). The measurements were carried out in triplicates to obtain a standard curve of how absorption depended on nitrogen concentration (Fig. 4).

We added 100 µL of inoculated bacterial strains and consortia to 4.9 mL of sterile Nfb medium and incubated at 25 ± 2°C for 48 h with constant stirring on an

LSI-3016A/LSI-3016R incubator shaker (Daihan Labtech, South Korea). Then, the tubes underwent centrifuging at 5000 rpm for 15 min. The optical density was measured at 610 nm. The sterile Nfb nutrient medium served as a control. The amount of nitrogen fixed by extremophilic microorganisms was obtained graphically using a curve that showed the dependence of nitrogen concentration in the nutrient medium on the optical density of standard solutions.

Measuring zinc solubilizing properties. This experiment involved spot inoculation of a daily bacterial culture/consortia onto Petri dishes with the following media: 1.00% of glucose, 0.10% $(NH_4)_2SO_4$, 0.02% KCl, 0.01% K_2HPO_4, 0.02% $MgSO_4$, 1.50% agar, 0.10% ZnO. The Petri dishes were incubated at 28 ± 2°C for 7 days. Clear inhibition zones around colony dots indicated that the extremophilic bacteria had some solubilizing ability. The efficiency of zinc solubilization was calculated as follows:

$$E_Z = \frac{D_{c+a}}{D_c} \times 100 \quad (1)$$

where E_Z is the efficiency of zinc solubilization, %; D_{c+a} is the diameter of the colony together with the inhibition zone, cm; D_c is the diameter of the colony, cm.

Determining potassium solubilizing properties. This experiment involved spot inoculation of a daily bacterial culture/consortia on Petri dishes with the following media: 1.000% of glucose, 0.500% $MgSO_4$, 0.005% $FeCl_3$, 0.100% $CaCO_3$, 2.000% $Ca_3(PO_4)_2$, 1.500% agar, and 5000% zeolite [30]. The Petri dishes were incubated at 28 ± 2°C overnight. Inhibition zones around colony dots indicated the ability of the culture to solubilize potassium. The efficiency of potassium solubilization was calculated as in Eq. (2):

$$E_K = \frac{D_{c+a}}{D_c} \times 100 \quad (2)$$

where E_K is the efficiency of potassium solubilization, %; D_{c+a} denotes the diameter of the colony together with the inhibition zone, cm; D_c stands for the colony diameter, cm.

Determining phosphate solubilizing properties. This experiment involved spot inoculation of a daily bacterial culture/consortia on Petri dishes with the following media: 5.00 g of $Ca_3(PO_4)_2$, 20.00 g glucose, 0.20 g NaCl, 0.10 g $MgSO_4$, 0.01 g $MnSO_4$, 0.01 g $Fe_2(SO_4)_3$, 15.00 g agar, and 1.0 L distilled water [50]. The Petri dishes were incubated for 4 days at 28 ± 2°C to form inhibition zones around colony dots. The efficiency of phosphate solubilization was determined as follows:

$$E_{ph} = \frac{D_{c+a}}{D_c} \times 100 \quad (3)$$

where E_{ph} is the efficiency of phosphate solubilization, %; D_{c+a} denotes the colony together with the inhibition zone, cm; D_c stands for the colony diameter, cm.

Producing gibberellic acid. We added 280 µL of 1 M $(CH_3COO)_2Zn$ and 10.6% $K_3[Fe(CN)_6]$ solution to 2 mL of culture liquid. After stirring, the culture liquid was centrifuged at 4500 rpm for 10 min. The resulting supernatant was mixed with 30% HCl at a ratio of 1:1. The solution was left to settle for 75 min. A similarly prepared nutrient medium served as control. The optical density was measured relative to 5% HCl using a spectrophotometer at a 254 nm wavelength [51]. The optical density of the sample was determined using the follow Eq. (4):

$$OD = OD_s - OD_c \quad (4)$$

where OD is the optical density; OD_s is the indicated optical density; OD_c denotes the optical density of the control sample.

The amount of synthesized gibberellic acid was determined using a calibration graph of a standard gibberellic acid solution between 10 and 200 µg/mL.

Obtaining siderophores. We added 100 µL of culture fluid to 100 µL of fresh Chrome Azurol S (CAS) reagent. The resulting solution was left to settle for 20 min. After that, the optical density was measured at 630 nm. A similarly prepared nutrient medium served as control [52]. The amount of siderophores synthesized was calculated as follows:

$$N_s = \frac{OD_c - OD_s}{OD_c} \quad (5)$$

where N_s is the amount of siderophores, %; OD_s stands for the optical density of the experimental sample; OD_c denotes the optical density of the control sample.

Producing hydrogen cyanide. This test involved a modified 4% nutrient agar medium with 4.4 g/L of amino acid L-glycine. We soaked filter paper in 0.5% picric acid in 1% Na_2CO_3 solution and applied it to the inner surface of the Petri dish lid. To synthesize hydrogen cyanide, we transferred bacterial colonies to plates with the modified 4% nutrient agar medium and uninoculated control. The Petri dishes were sealed with paraffin and incubated at 28 ± 1 °C until browning, which indicated hydrogen cyanide synthesis [53].

Effect of bacterial isolates and consortia on wheat growth. A suspension of the isolate in 2 mL of sterile distilled water was brought up to McFarland standard of 0.8–1.0 using a Densichek plus densitometer at 1.5×10^{-8} CFU/mL. Next, we added 1 mL of suspension to 10 mL of Luria Bertani nutrient medium and cultivated it on an incubator shaker at 28 ± 2°C and 110 rpm for 72 h.

Before seed inoculation, wheat seeds were sterilized with 2.5% NaClO for 3 min and washed three times with distilled water. After being planted in soil and watered with a suspension of bacterial isolates and consortia, the seeds were germinated for 10 days at 25°C and 50–60% humidity. Sterile control seeds were germinated for 10 days and watered with sterile distilled water.

Equation (6) made it possible to assess the germination rate of wheat seeds:

$$G = \frac{N_{gs}}{N_{ts}} \times 100 \quad (6)$$

where G means the germination rate, %; N_{gs} is the number of germinated seeds; N_{ts} stands for the total of seeds planted

The lengths of the roots and aerial parts of wheat were measured on graph paper with an accuracy of 0.5 mm.

All studies were triplicated. The obtained data values were expressed as the mean of three measurements with standard deviation. The statistical analysis involved Microsoft Office Excel 2007 and a one-sample paired Student's t-test for each pair. Differences were statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Nitrogen fixation. Table 2 shows the nitrogen-fixing capacity of extremophilic bacteria and bioconsortia A, B, C, and D.

Nitrogen fixation by extremophilic microorganisms ranged from 16.45 to 40.42 μg/mL nutrient medium. The data confirmed the results obtained for nitrogen-fixing properties of diazotrophic bacteria *Acinetobacter pitti*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Kosakonia oryzae* isolated from the rhizosphere of *Agave angustifolia* [49]. Their nitrogen-fixing capability was 18.34–42.06 μg/mL nutrient medium.

In this research, the best nitrogen fixation belonged to *Rhizobium radiobacter* at a nitrogen concentration of 30.86 μg/mL Nfb. As for the three-strain consortia, the best nitrogen fixation belonged to Consortium D with two shares of *R. radiobacter* at 40.42 μg/mL Nfb. It owed its nitrogen-fixing properties to the nitrogenase enzyme typical of *Rhizobium* sp. cultivated on NH_4^+ [54]. Other microorganisms in Consortium D also possessed certain nitrogen-fixing properties, which apparently enhanced the efficiency of nitrogen fixation. The Nfb medium changed color from green to blue as an indicator of bacteria with nitrogen-fixing ability. The color change was caused by bromothymol blue with its pH-dependent structure and color.

After the biological nitrogen fixation, ammonium ions accumulated in the nutrient medium and affected the pH. Two chemical forms can explain the effect of pH on the color of bromothymol blue. The quinoid form with one negative charge predominates in an alkaline environment, and it is responsible for yellow. A quinoid-phenolate structure with two negative charges predominates in an acidic environment and is associated with the blue color.

On average, consortia of extremophilic microorganisms showed better nitrogen fixation compared to individual bacterial strains. We recorded the worst result for *Klebsiella oxytoca*, which demonstrated a nitrogen-fixing capacity of 16.45 mg/mL Nfb. Apparently, the low result is connected with the poor ability of this microorganism to produce nitrogenase.

Determining zinc solubilizing properties. Table 3 illustrates the zinc-solubilizing potential of extremophilic isolates and bioconsortia.

The efficiency of zinc solubilization by soil bacteria and their consortia was 135–182%. *Bacillus megaterium* AN24, *Bacillus aryabhattai* AN30, *B. megaterium* AN31 and AN35 are routinely used in agriculture as growth promoters. Their zinc solubilization efficiency was reported as 120–258% [55]. Extremophilic microorganisms demonstrated zinc solubilization properties similar to those of growth-stimulating microorganisms.

Consortium D with a double share of *R. radiobacter* demonstrated the best zinc solubilization potential of 182.34% while Consortium B with two shares of *Achromobacter denitrificans* had the lowest result for the consortia samples (163.61%). As for individual extremophilic isolates, *R. radiobacter* proved to be the best zinc-solubilizer (154.36%). However, this achievement was far below the lowest result in the consortia group. Probably, *R. radiobacter* produced a lot of anionic organic acids, e.g., gluconic and *α*-ketogluconic, which could chelate zinc through carboxyl and hydroxyl groups, thus increasing its solubility and improving the mineral uptake by the plant [56].

Determining potassium solubilization properties. Table 4 demonstrates the potassium solubilizing capability for the soil extremophilic microorganisms and their consortia.

Table 2 Nitrogen-fixing ability of extremophilic bacteria and their consortia based

Sample	Nitrogen, μg/mL Nfb
Achromobacter denitrificans	20.32 ± 0.02
Klebsiella oxytoca	16.45 ± 0.06
Rhizobium radiobacter	30.86 ± 0.10
Consortium A (1:1:1)	24.84 ± 0.01
Consortium B (2:1:1)	38.41 ± 0.03
Consortium C (1:2:1)	35.81 ± 0.05
Consortium D (1:1:2)	**40.42 ± 0.06**

Table 3 Zinc solubilization properties of extremophilic bacteria and their consortia

Sample	Colony diameter, mm	Colony diameter + inhibition zone, mm	Zink solubilization, %
Achromobacter enitrificans	6.13 ± 0.05	8.32 ± 0.09	135.73 ± 0.14
Klebsiella oxytoca	7.36 ± 0.12	10.87 ± 0.04	147.69 ± 0.04
Rhizobium radiobacter	7.23 ± 0.11	11.16 ± 0.06	154.36 ± 0.07
Consortium A (1:1:1)	5.32 ± 0.03	9.52 ± 0.02	178.95 ± 0.05
Consortium B (2:1:1)	6.32 ± 0.04	10.34 ± 0.11	163.61 ± 0.14
Consortium C (1:2:1)	6.89 ± 0.11	11.83 ± 0.15	171.70 ± 0.12
Consortium D (1:1:2)	**6.23 ± 0.05**	**11.36 ± 0.02**	**182.34 ± 0.11**

Table 4 Potassium solubilization properties of extremophilic bacteria and their consortia

Sample	Colony diameter, mm	Colony diameter + inhibition zone, mm	Potassium solubilization, %
Achromobacter denitrificans	5.36 ± 0.10	8.63 ± 0.11	161.01 ± 0.01
Klebsiella oxytoca	6.43 ± 0.03	8.26 ± 0.02	128.46 ± 0.03
Rhizobium radiobacter	**6.52 ± 0.04**	**12.58 ± 0.03**	**192.94 ± 0.05**
Consortium A (1:1:1)	6.12 ± 0.11	10.78 ± 0.04	176.14 ± 0.05
Consortium B (2:1:1)	7.10 ± 0.13	11.59 ± 0.04	163.24 ± 0.07
Consortium C (1:2:1)	7.30 ± 0.06	13.65 ± 0.09	186.99 ± 0.03
Consortium D (1:1:2)	7.10 ± 0.08	13.10 ± 0.07	184.51 ± 0.04

Table 5 Phosphate solubilization properties of extremophilic bacteria and their consortia

Sample	Colony diameter, mm	Colony diameter + inhibition zone, mm	Phosphate solubilization, %
Achromobacter denitrificans	**3.21 ± 0.04**	**6.32 ± 0.05**	**196.88 ± 0.01**
Klebsiella oxytoca	2.62 ± 0.02	5.12 ± 0.02	195.42 ± 0.02
Rhizobium radiobacter	2.68 ± 0.14	3.65 ± 0.06	136.19 ± 0.09
Consortium A (1:1:1)	3.67 ± 0.01	4.56 ± 0.07	124.25 ± 0.05
Consortium B (2:1:1)	2.37 ± 0.03	2.87 ± 0.01	121.10 ± 0.04
Consortium C (1:2:1)	1.61 ± 0.08	1.80 ± 0.09	111.80 ± 0.03
Consortium D (1:1:2)	3.11 ± 0.01	4.32 ± 0.06	138.91 ± 0.05

Efficiency of potassium solubilization ranged from 128 to 193%. These data were slightly lower than those published by Jabin *et al.*, who obtained 185.00–257.32% for isolates of *Bacillus*, *Pseudomonas*, and *Sinorhizobium* [57].

R. radiobacter demonstrated the best potential for potassium solubilization (192.94%). Presumably, this strain produced organic acids, e.g., gluconic, oxalic, *α*-ketogluconic, succinic, or citric, which dissolved the mineral potassium by protonation and acidification [56]. *K. oxytoca* had the lowest results of 128.46%, which also proved to be the lowest among all the study objects due to its failure to produce organic acids.

Determining phosphate solubilization properties. Table 5 illustrates the potential of soil bacteria and their consortia for phosphate solubilization.

Efficiency of phosphate solubilization ranged from 111.80 to 196.88%. Blanco-Vargas *et al.* studied a consortium of *Pseudomonas* sp. and *Serratia* sp., isolated from Colombia's soil and achieved phosphate solubilization indices of 210 and 200%, respectively [59].

In this research, *A. denitrificans* proved to be the best phosphate-solubilizer with 196.88%. It produced a lot of organic acid, e.g., oxalic, gluconic, acetic, malic, etc., which acidified the environment and dissolved phosphorus [60]. Unlike individual isolates, all consortia showed poor phosphate solubilization. Obviously, extremophilic symbioses are inefficient as phosphate solubilizers. Consortium D with a double share of *R. radiobacter* had the highest solubilization efficiency (138.91%), which was as high as the lowest solubilization efficiency for individual isolates, i.e., *R. radiobacter* with its 136.19%. Consortium C had the lowest phosphate solubilization of 111.80%.

Producing gibberellic acid. Table 6 illustrates the potential of soil bacteria and their consortia for gibberellic acid production.

All the samples demonstrated gibberellic acid production potential between 475.00 and 611.50 μg/mL.

Table 6 Gibberellic acid production potential of extremophilic bacteria and their consortia

Sample	Gibberellic acid, μg/mL
Achromobacter denitrificans	475.00 ± 0.50
Klebsiella oxytoca	**611.50 ± 0.31**
Rhizobium radiobacter	601.50 ± 0.06
Consortium A (1:1:1)	551.50 ± 0.12
Consortium B (2:1:1)	479.00 ± 0.13
Consortium C (1:2:1)	589.00 ± 0.24
Consortium D (1:1:2)	581.50 ± 0.38

Table 7 Siderophore production by extremophilic soil microorganisms and their consortia

Sample	Siderophores, %
Achromobacter denitrificans	60.25 ± 0.03
Klebsiella oxytoca	28.69 ± 0.05
Rhizobium radiobacter	53.36 ± 0.08
Consortium A (1:1:1)	67.82 ± 0.07
Consortium B (2:1:1)	57.43 ± 0.03
Consortium C (1:2:1)	**82.61 ± 0.03**
Consortium D (1:1:2)	71.32 ± 0.03

Kaur *et al.*, who isolated and tested bacteria from natural sources in India, reported 550 μg/mL [61]. In another study, *Microbacterium laevaniformans* RS0111 produced 67.23 μg/mL [62].

The largest amount of gibberellic acid belonged to *K. oxytoca* and reached 611.50 μg/mL. *A. denitrificans* had the lowest result of 475 μg/mL. As for the consortia group, Consortium C with a double share of *K. oxytoca* appeared to be the most efficient gibberellic acid producer, yielding 589 μg/mL.

Producing siderophores. Table 7 sums up the percentage of siderophores produced by soil bacteria and their consortia.

Table 8 Hydrogen cyanide production by extremophilic soil microorganisms and their consortia

Sample	Hydrogen cyanide
Achromobacter denitrificans	–
Klebsiella oxytoca	+
Rhizobium radiobacter	+
Consortium A (1:1:1)	++
Consortium B (2:1:1)	+
Consortium C (1:2:1)	++
Consortium D (1:1:2)	+

"+" Moderate hydrogen cyanide production; "++" active hydrogen cyanide production; "–" no hydrogen cyanide production

The samples demonstrated siderophore production properties in the range from 28.69 to 82.61%. The rhizobacterial strain of *Pantoea dispersa* was reported to produce 70.54% siderophores [50]. Li *et al.* studied inoculants of *Paenibacillus tundrae*, *Bacillus mycoides*, and *Brevibacterium frigoritolerans* isolated from the soil of the Qinghai-Tibet Plateau, and they proved to be efficient siderophore producers (89.58–94.74%) [63].

In this research, Consortium C with two shares of *K. oxytoca* showed the best potential for siderophore production (82.61%). The medium turned from blue to yellow-pink as siderophores chelated. Individual isolates of extremophilic microorganisms performed poorly, compared to bacterial consortia. *K. oxytoca* demonstrated the lowest potential for siderophore production (28.69%). Solutions with Hg^{2+} and Ag^{2+} demonstrated a subtle color change from blue to sunset yellow, which resulted in weak siderophore binding to Hg^{2+} and Ag^{2+} [64]. As for the isolates, *A. denitrificans* demonstrated the best indicator of 60.25%.

Producing hydrogen cyanide. Table 8 displays the ability of bacterial isolates and consortia to produce hydrogen cyanide.

All the samples but *A. denitrificans* were able to produce hydrogen cyanide. Consortia A (with all isolates in equal shares) and C (with two shares of *K. oxytoca*) were quite efficient in this respect while *R. radiobacter*, *K. oxytoca*, Consortium B (with a double share of *A. denitrificans*), and Consortium D (with a double share of *R. radiobacter*) demonstrated moderate results. Therefore, isolates *K. oxytoca* and *R. radiobacter*, as well as all the consortia, could protect agricultural plants from diseases caused by phytopathogenic fungi [53]. These findings corresponded with those published by Mowafy *et al.*, who reported the ability of microorganisms of the *Rhizobium* genus to produce hydrogen cyanide [65]. Similarly, Walpola *et al.* found *K. oxytoca* capable of producing hydrogen cyanide [66].

Effect of bacterial isolates and consortia on wheat growth. Figure 5 illustrates the wheat germination results.

On experiment day 10, all extremophilic bacteria and consortia increased the germination rate of wheat seeds compared to the control samples. The germination of control wheat samples, which received distilled water, stayed below 75%. The best seed germination rates belonged to Consortium C (with a double share of *K. oxytoca*), which showed the best results for siderophores and hydrogen cyanide. Consortium-treated samples demonstrated 84% germination. *K. oxytoca* had the lowest effect on germination rate (76%).

Figure 6 shows the average length of wheat roots.

All the bacterial isolates and consortia were able to increase the length of wheat roots. The average root length of control wheat samples was 80.3 mm. Consortia C (with a double share of *K. oxytoca*) and B (with a

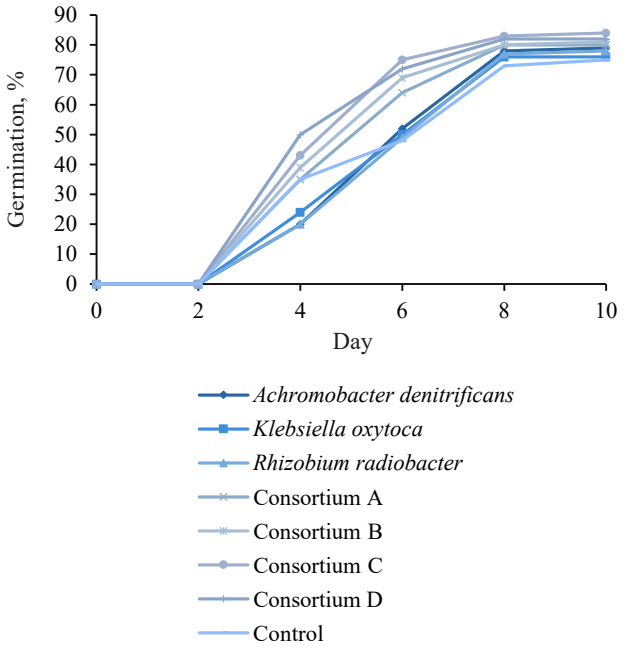

Figure 5 Wheat germination

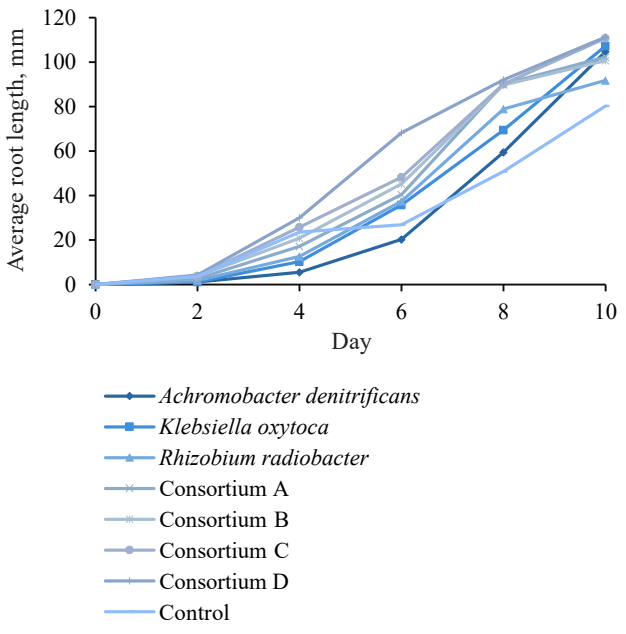

Figure 6 Effect of extremophilic bacteria and consortia on average root length

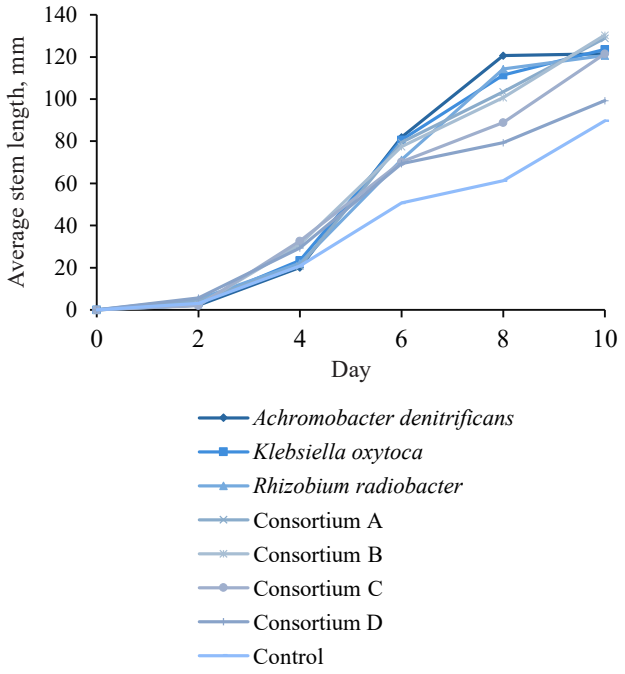

Figure 7 Effect of extremophilic bacteria and consortia on average stem length

double share of *A. denitrificans*) had the greatest positive effect on root length: 110.8 and 111.3 mm, respectively. *R. radiobacter* proved least effective in stimulating wheat roots (91.8 mm).

Figure 7 shows the average length of wheat stems.

The extremophilic soil bacterial isolates and consortia were able to stimulate stem growth in wheat. The average stem length in the control wheat samples was 89.7 mm. Consortium B (with a double share of *A. denitrificans*) produced the longest average stem (130.4 mm). A similar result of 128.9 mm belonged to Consortium A, where all the isolates were represented in equal shares. As for the bacterial isolates, *K. oxytoca* proved to be the most effective strain with 123.6 mm of average stem length. Consortium D (two shares of *R. radiobacter*) with its 99.3 mm proved to be the least effective sample.

In this research, experimental microorganisms and their consortia demonstrated their efficiency in nitrogen fixation, solubilization of zinc, potassium, and phosphates, as well as proved to be descent hydrogen cyanide and gibberellic acid producers. These properties had a positive effect on the growth and development of wheat seeds, as evidenced by germination rate and stem and root lengths.

Rhizobacteria with plant-growth promoting properties increase the growth and development of agricultural crops. They improve the overall health of plants by promoting nutrient uptake, protecting against phytopathogenic microbes, and increasing resistance to various abiotic stresses [67]. Rhizobacteria are capable of producing phytohormones, e.g., gibberellic acid, as well as siderophores. They solubilize phosphates, zinc, and potassium. In addition, they fix nitrogen, which improves plant growth and fertility. Some extremophilic microorganisms demonstrate rhizobacterial potential due to their ability to produce secondary metabolites and enzymes that are of great commercial interest to many industries, e.g., agriculture [68]. Finally, extremophiles are able to survive under aggressive environmental conditions. As a result, products that contain extremophiles have a better storage capacity. Extremophilic microorganisms maintain their effectiveness in polluted areas.

In this study, soil microorganisms isolated in the Kemerovo Region demonstrated plant growth-promoting properties and potential for agricultural use.

Due to these properties, extremophilic microorganisms isolated from disturbed areas and their consortia were able to increase seed germination from 76 to 84%. The stem length in the experimental wheat increased by 24–45%, while the average root length grew by 14–39%.

Research prospects include the enzyme complex of extremophilic isolates and their consortia. We plan a qualitative and quantitative analysis of metabolites that render microorganisms their growth-stimulating properties. A set of chromatographic methods will make it possible to develop biofertilizers that will improve the quality of agricultural crops and ensure food security.

CONCLUSION

Bacterial isolates of *Achromobacter denitrificans*, *Klebsiella oxytoca*, and *Rhizobium radiobacter*, as well as their consortia were able to improve the growth and development of wheat seeds. They proved to be efficient nitrogen fixators, solubilizers of phosphates, zinc, and potassium, and producers of siderophores, hydrogen cyanide, and gibberellic acid. The best nitrogen fixation properties belonged to *R. radiobacter* and reached a nitrogen concentration of 30.86 μg/mL Nfb. The best ability to solubilize zinc solubilization efficiency of 182.34% was observed in Consortium D with a double share of the same isolate. *R. radiobacter* also was the most efficient sample in potassium solubilization (192.94%) while *A. denitrificans* was the most efficient phosphate solubilizer (196.88%). *K. oxytoca* produced the largest amount of gibberellic acid (611.50 μg/mL). Consortium C with a double share of *K. oxytoca* was the most efficient siderophore producer (82.61%). All the samples were good at hydrogen cyanide production, with the exception of *A. denitrificans*. The best seed germination rate of 84% belonged to Consortium C. Consortium C (with a double share of *K. oxytoca*) and Consortium B (with a double share of *A. denitrificans*) had the greatest positive effect on root length: 110.8 and 111.3 mm, respectively. Consortium B was also responsible for the longest average stem length (130.4 mm).

Extremophilic microorganisms isolated from disturbed soils of the coal-mining Kemerovo Region and their consortia improved the growth and development of wheat. They proved to be a promising source of biofertilizers that improve food security and quality of agricultural crops.

CONTRIBUTION

The authors contributed to the manuscript equally and are equally responsible for any potential plagiarism.

CONFLICT OF INTEREST

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

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Seasonal determination of proximate composition and essential elements in commercial fishes from Pakistan and human health risk assessment

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Abstract:
The seasonal variability in proximate composition and essential elements demonstrates that the habitat and feeding habits of fish species play a vital role in energy transfer.
We aimed to ascertain seasonal variability in the biochemical composition (protein, lipids, carbohydrates, ash, and moisture) and the amounts of Na, K, Ca, Mg, Mn, and Zn in the species *Nemipterus japonicus*, *Epinephelus erythrurus*, *Nematalosa nasus*, and *Ilisha striatula* inhabiting pelagic and demersal zones. We compared the nutritional profile of these fish species and their seasonal importance. The essential elements were detected by flame atomic absorption spectrometry and found in the following order: K > Na > Ca > Mg > Mn > Zn. To determine the proximate composition, we employed a number of methods: the Lowry method for protein analysis, the acid hydrolysis method for fat/lipid analysis, a formula for carbohydrates and moisture, and the incineration method for ash content.
The spring inter-monsoon season showed the highest values for the essential elements in both pelagic and demersal species. However, the pelagic species had the highest biochemical composition levels during the southwest monsoon. The autumn inter-monsoon had the lowest bio-profile for the fishes of both regimes.
The summer season, which is not thought to be good for fish consumption, showed the highest biochemical composition levels in the pelagic fish. The nutritional profile of fish flesh can be affected by feeding habits, seasonal variation, and habitat.

Keywords: Seasonal determination, proximate/biochemical composition, essential elements, health risk assessment, distinct marine regimes

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INTRODUCTION

Feeding habits, habitat characteristics, and the inner physiological rhythm are the key factors that maintain the composition of macro- and micronutrients in organisms. The marine ecosystem is generally segregated into pelagic and demersal zones where physiochemical properties and behavioral adaptations of an organism are of great concern [1]. Sustainable utilization of marine resources can fulfill global seafood needs [2, 3]. According to the FAO, the total fisheries production in 2018 worldwide was 96.4 million tons, to which China contributed 15% and remained on the top, while Pakistan contributed merely 1.037% [4].

The analysis of fish habitats reveals a great abundance of biological resources and species distribution [5]. Feeding habits also vary according to the habitat. Pelagic fishes show different trends in feeding habits compared to demersal ones because they are found on the upper level of waters and hence depend largely on microorganisms like plankton and small fishes. These microorganisms make the largest biomass in the marine environment and are an important fundamental link

within the food chain [6]. On the other hand, demersal fishes dwell near the bottom and mainly feed on fishes, benthic organisms, and zooplankton [7]. Fishes of both pelagic and demersal zones are of great importance in nutritional values, but these values depend exclusively on the specific feeding habit of a fish and its habitat characteristics. *Nemipterus japonicus* belong to the carnivorous species of the demersal zone, feeding on mollusks, annelids, fish, and fish larvae. Their gut contains crabs, shrimp, and fish juveniles [8]. *Epinephelus erythrurus* is another group of species with a carnivorous feeding behavior found mostly between 10 and 200 m. Corals are an ideal habitat for these species, while their ideal food includes large invertebrates including crustaceans and fishes close to the substrate [9].

Nematalosa nasus belong to omnivorous species [10–12]. Interestingly, mud and sand account for the bulk of their gut content, and their stomach is modified like a gizzard. Both traits may be due to the bottom-feeding habit of this species [13]. *Ilisha striatula*, a species with a carnivorous feeding behavior, also has a gizzard-like stomach [14].

Significance of proximate composition and essential elements. Longwe and Kapute stated that essential elements and other nutrients (protein and fats) help to increase a healthy and nutritious food supply [15]. Fish contains vitamins A, D, and B, as well as vital elements. Seafood consumption facilitates the overall nutritional quality of a mixed diet [16]. Nutritional components of fish have many functional benefits for humans, with fish oil proven to be the most essential source of polyunsaturated fatty acid [17, 18]. About 60% of food demand is fulfilled by fish in developing countries [19].

Fish is primarily composed of water (72%), protein (19%), and fats (8%) [20]. Seafood in general and fish in particular contain a large number of metals as they can accumulate these from their environment [21]. A blue economy concept has been initiated in a few countries to utilize marine resources in a better way and to promote sustainable fishing. According to Sari and Muslimah, the blue economy will also ensure food security, environmental sustainability, and economic growth [22]. Gram and Dalgaard reported that along with fulfilling the food demand, preserving fish with all its nutritional values is a global challenge that accounts for 25% spoilage of total production [23]. Although studies on the nutritional values and proximate composition of fish have been done throughout the world, including Pakistan, there is a lack of comparative analysis of fish species with different feeding regimes [24–26]. This analysis could help consumers choose a more nutritional fish as part of their diet.

Protein. Seafood consumption plays an immense role in meeting the protein requirement of the human body. Among seafood, fish attain the top ranking in the aquatic food chain and high-quality protein [27]. Fish contain such macronutrients as water (63–84%), protein (14–26%), and lipids (0.1–17%), as reported by Hui *et al.* [28]. Due to different habitat and physiological characteristics, the protein content varies at 18–20% in demersal and pelagic fishes, respectively. In addition, fish's lean muscles are better protein carriers than those of red flesh [28]. The demand for high-quality animal protein, especially seafood, is steadily increasing along with the global population [29]. Protein plays a vital role in strengthening the immune system, body framework, and circulatory system. Consuming fish as a source of protein can prevent protein-calorie malnutrition. Protein also defends the human body against various microbial infections and strengthens the immune system [30, 31]. Our diet consists of various sources of protein, which is a major factor in the nutritional profile [32]. Proteins obtained from animal sources are considered more significant than plant protein due to their balanced combination of amino acids. Although all animal proteins are equally healthy and nutritional for humans, fish protein is easier to digest due to the unique amino acid composition of fish muscles [33, 34]. Habitat differences also play a great role in the structural composition of protein. Fish require less structural support to move compared to land animals, and therefore fish muscles contain less connective tissue, which makes fish more tender and delicious. Furthermore, cold- and warm-blooded variations also show differences in the protein lipids of terrestrial and aquatic animals [32].

Lipids. The meager presence of fats in seafood has increased its market demand. However, the presence of long-chain polyunsaturated fatty acid n-3 in fish muscles increases the nutritious index of fish. Therefore, fish should make up an essential part of the human diet [35]. Co-specific species may have contrasting lipid compositions due to variations in environmental conditions, maturity, and age [36]. Hui *et al.* observed that pelagic fish store lipids in the head and muscles, while demersal species keep them in their livers and below the skin [28].

Carbohydrates. The role of carbohydrates in fish is essential because their deficiency may cause growth retardation [47]. Moreover, carbohydrate deficiency can limit the function of macronutrients in the fish body. Nevertheless, cultured fish usually have a carbohydrate-rich diet, which is consequently consumed by humans. Carbohydrates have always been considered an excellent source of human nutrition with great biological importance. In fish, however, the importance of carbohydrates varies over time depending on their ecosystem [38]. Mayer *et al.* reported a versatile range of marine carbohydrate structures [39]. One of the primary functions of carbohydrates is to provide energy by cellular respiration, which is a fundamental constituent of protoplasm. Carbohydrates participate in energy release and storage [40]. A wide range of marine carbohydrates is used in applied sciences to produce nutrient supplements, cosmetics, and pharmaceuticals. Most importantly, carbohydrates play a biomedical role, providing benefits for human health against viral diseases and hematological effects that reduce the risk of hemorrhage [41].

Ash is inorganic matter that remains after the incineration of the organic content. It promotes the physiological and structural growth of the human body. Ash estimation is important for presenting the total amount of essential elements in fish meat [42, 43].

Moisture is one of the major bio-constituents of seafood. Moisture is around 80% in fresh fish muscles and slightly lower in fattier fishes. The protein structure in fish can hold moisture tightly even under high pressure. However, prolonged storage of frozen or chilled fish may affect the ability of protein to hold moisture in fish meat [44]. Many species, particularly those containing large quantities of lipid fat in the flesh and under the skin, are replaced by water as the lipid energy reserve is depleted [33]. Elemental composition shows inorganic contents in the fish muscles, while proximate composition determines organic contents [45]. All elements are divided into non-essential and essential elements based on their harmful or useful effects on the environment and human health. Essential elements such as Cu, Mn, Ni, Fe, and Zn are useful for aquatic organisms, as well as humans, within permissible limits [46]. A comprehensive study of metal concentrations in the entire ecosystem of Hawks Bay, Karachi defined how biotic and abiotic components are linked in terms of metal sharing [14]. Further, the authors elaborated the vulnerability of the important coastal ecosystem. According to Adewumi *et al*., increased concentrations of essential elements, which go beyond the permissible limits, cause them to accumulate in the human muscles, while their deficiency causes the failure of various body functions [45]. Such studies on metals in seafood are of great importance today and they are quite common throughout the world [21].

Fish is attaining great importance among healthy foods available on the global market since it contains a good combination of organic and inorganic essential elements and proximate nutrients [47]. Essential elements are responsible for numerous functions of the human body, including various enzymatic activities, as well as anabolic and catabolic functions of cells [48].

Health risk assessment. Metals pose a significant threat to people's health [49]. Metals are found in the edible tissues of fish species at the top of the aquatic food chain and are absorbed by humans through ingestion [50]. Therefore, one of our aims was to determine the health risks of Mn and Zn accumulations in the edible tissues of fishes from the Pakistan coasts.

STUDY OBJECTS AND METHODS

Fish sampling identification and laboratory handling. Two demersal and two pelagic fish species were seasonally (northeast monsoon, spring inter-monsoon, southwest monsoon, and autumn inter-monsoon) collected from the Karachi Fish Harbor. The samples were placed in an ice box and taken to the lab for analysis. Distilled water was poured over the samples to avoid any contamination. The species were identified by using the FAO's field guide and the Fishbase (Table 1) [51]. The scientific name, habitat, feeding habits, and gut contents were examined in the lab. The weight and length of the samples were measured. The stomach of each sample was removed, and the gut content was analyzed under a binocular microscope.

Essential elements analysis. To determine essential elements, 5 g of fish muscles (wet weight) was dried in an oven at a maintained temperature for 6 h and then homogenized to powder. Then, we shifted the homogenized sample to a beaker, added 5 mL of 65% HNO_3, heated the solution at 80–100°C until it became clear, and filtered it through Whatman filter paper. Distilled water was then added gradually to make up 100 mL of the solution. The sample was then transferred to a glass bottle and labeled for further analysis on an Analyst 400 flame atomic absorption spectrometer. The concentrations of elements were expressed as mg/L dry weight for comparison [52]. Na and K were determined by flame emission spectrometry, since their concentrations were beyond the highest standards of selected elements in atomic absorption spectrometry. However, Ca, Mg, Zn, and Mn were determined by flame atomic absorption in the presence of HCL lamps, as they provide precise values even at higher concentrations.

Biochemical composition analysis. Lowry's method was modified for protein analysis, as described by Esen [53].

The acid hydrolysis method was used for the AOAC lipid analysis [54]. The ash content was measured by incineration in a muffle furnace at 600-700EC for 5–8 h [55]. Moisture was determined as a difference between dry and wet weights [53]. Carbohydrates, %, were calculated by using the following Eq. (1) [52]:

$$\text{Total carbohydrates} = 100 - (\text{Protein} + \text{Fats} + \text{Moisture} + \text{Ash}) \quad (1)$$

Table 1 Fish identification, habitat, feeding habit, and gut content

Scientific name	Habitat	Feeding habit	Gut content
Nemipterus japonicus	Demersal	Carnivore	Fish, fish larvae, crabs, shrimps, mollusks
Epinephelus erythrurus	Demersal	Carnivore	Crabs, shrimps, squids, gastropods, bivalves, worms, sand and mud, small crustaceans, mollusks
Nematalosa nasus	Pelagic	Omnivore	Marine plankton, algae including weeds, nematodes, seaweeds, copepods, nauplius
Ilisha striatula	Pelagic	Carnivore	Fish eggs, copepods, copepod eggs, shrimp, crabs

Health risk estimation. The Pakistan Pure Food Laws cover 104 food items and regulate chemicals, heavy metals, as well as purity in raw food [56].

Fish muscle tissues were analyzed to evaluate the risk of Mn and Zn concentrations for human health. The daily intake of these metals from fish consumption was estimated for adults. The estimated daily intake (EDI) depends on metal levels and the amount of fish consumed. The EDI of Mn and Zn was determined using the equation below:

$$\mathrm{EDI} = \frac{C_{\mathrm{metal}} \times Wt_{\mathrm{fish}}}{\mathrm{BWt}} \quad (2)$$

where C_{metal} is the concentration of Mn and Zn in fish; Wt_{fish} represents the average daily consumption of fish according to the National Bureau of Statistics (Pakistan) and FAO's international consumption surveys (5.81 kg/capita/year), which is equal to 15.92 mg/kg/day; BWt is the adult body weight of 70 kg [57]. The estimated weekly intake (EWI) was obtained by multiplying the EDI values by 7.

To estimate the human health risk from consuming metal-contaminated fish, the target hazard quotient (THQ) was calculated as per Regional Screening Levels (RSLs) [58]. The THQ is an estimate of the risk level (non-carcinogenic) due to contaminant exposure. It was calculated as follows:

$$\mathrm{THQ} = \frac{C_{\mathrm{metal}} \times Wt_{\mathrm{fish}} \times 10^{-3} \times \mathrm{EF} \times \mathrm{ED}}{\mathrm{Rf.\,D.} \times \mathrm{BWt} \times \mathrm{ATn}} \quad (3)$$

where THQ is the target hazard quotient; C_{metal} is the concentration of Mn and Zn in fish, mg/kg; Wt_{fish} is the fish consumption rate, g/day; EF is the exposure frequency, day/year, or the number of exposure events per year of exposure; ED is the exposure duration, year; Rf .D. is the reference dose, mg/kg·day; BWt is the body weight, kg; and ATn is the averaging time, noncarcinogens, day/year. We used the reference doses established by the United States Environmental Agency and the Rik Assessment Information System [58, 59]. The values for Mn and Zn are 1.4×10^{-1} and 3.0×10^{-1}, respectively [58, 59]. The hazard index (HI) from THQs can be expressed as the sum of hazard quotients:

$$\mathrm{HI} = \mathrm{THQ}_{\mathrm{Mn}} + \mathrm{THQ}_{\mathrm{Zn}} \quad (4)$$

The health protection standard of lifetime risk for HI is 1 [58]. If HI = > 1.0, then the EDI of a particular metal exceeds the reference dose, indicating that there is a potential risk associated with that metal.

Statistical analysis. ANOVA was used to investigate the data throughout the season for both proximate composition and essential elements, except for an autumn inter-monsoon season ($p < 0.05$).

RESULTS AND DISCUSSION

We compared the essential elements and biochemical composition of two pelagic and two demersal fishes to understand their nutritional quality for human dietary demands. Fish habitat, feeding habits, and gut contents are given in Table 1. The average weight, length, and number of the sampled fishes are indicated in Table 2. The micronutrients, such as zinc (Zn) and manganese (Mn), and macronutrients sodium (Na), potassium (K), calcium (Ca), and magnesium (Mg) were extracted seasonally (Table 3). The concentrations of micro- and macroelements in our study show the same trends as in [60, 61], namely K < Na < Ca. The lowest value of K was observed during the autumn inter-monsoon season, while its highest value was found in the spring inter-monsoon season.

We found that food availability, season (winter-summer), pollution, and fishing pressures affect the levels of nutrients in the pelagic and demersal fishes. Further, the feeding habits and habitat of a fish can characterize

Table 2 Average weight, length, and number of fishes sampled in each season

Season	Species name	Length, cm	Weight, g	Pooled samples	Number of fishes in pooled samples
Northeast monsoon	*Nemipterus japonicus*	20.48	114.29	3	25
	Epinephelus erythrurus	28.18	308.29	3	25
	Nematalosa nasus	22.24	136.18	3	25
	Ilisha striatula	21.87	93.56	3	25
Spring inter-monsoon	*Nemipterus japonicus*	20.04	113.33	3	25
	Epinephelus erythrurus	28.10	304.38	3	25
	Nematalosa nasus	22.34	136.79	3	25
	Ilisha striatula	22.09	99.09	3	25
Southwest monsoon	*Nemipterus japonicus*	20.31	110.67	3	25
	Epinephelus erythrurus	28.18	304.38	3	25
	Nematalosa nasus	22.24	136.18	3	25
	Ilisha striatula	21.75	102.18	3	25
Autumn inter-monsoon	Nemipterus japonicus	20.36	110.22	3	25
	Epinephelus erythrurus	28.40	318.45	3	25
	Nematalosa nasus	22.40	140.39	3	25
	Ilisha striatula	22.15	102.00	3	25

Table 3 Macro- and micronutrients (mg/L) in dried fish samples expressed as mean and std

Seasons	Species name	Essential macronutrients				Essential micronutrients	
		Na	K	Ca	Mg	Zn	Mn
		Demersal fish					
Northeast monsoon	*Nemipterus japonicus*	287.54 ± 2.33	408.260 ± 1.762	80.48 ± 0.32	11.490 ± 0.436	1.82 ± 0.03	0.06 ± 0.03
	Epinephelus erythrurus	267.05 ± 28.83	456.62 ± 0.72	80.23 ± 0.15	11.870 ± 0.901	1.88 ± 0.23	0.120 ± 0.173
Spring inter-monsoon	*Nemipterus japonicus*	538.17 ± 1.17	584.56 ± 6.02	220.70 ± 5.80	11.62 ± 0.61	6.66 ± 0.55	0.16 ± 0.06
	Epinephelus erythrurus	425.06 ± 5.95	407.55 ± 57.50	177.86 ± 39.52	15.880 ± 0.508	2.95 ± 0.50	0.28 ± 0.05
Southwest monsoon	*Nemipterus japonicus*	338.03 ± 38.95	393.66 ± 85.65	130.46 ± 52.25	11.16 ± 0.68	3.15 ± 0.02	0.18 ± 0.08
	Epinephelus erythrurus	195.73 ± 2.80	284.02 ± 17.30	129.00 ± 23.32	11.83 ± 0.30	2.22 ± 0.01	0.28 ± 0.05
Autumn inter-monsoon	*Nemipterus japonicus*	220.33 ± 8.02	391.55 ± 84.55	61.86 ± 0.87	7.830 ± 0.005	1.53 ± 0.28	0.05 ± 0.02
	Epinephelus erythrurus	67.143 ± 0.88	105.13 ± 0.63	35.21 ± 0.10	7.05 ± 0.02	1.65 ± 0.56	0.03 ± 0.02
		Pelagic fish					
Northeast monsoon	*Nematalosa nasus*	241.13 ± 5.66	433.82 ± 1.07	81.48 ± 0.47	11.54 ± 0.45	2.04 ± 0.03	0.03 ± 0.02
	Ilisha striatula	178.130 ± 0.551	392.51 ± 0.42	80.65 ± 0.77	11.16 ± 0.15	1.47 ± 0.30	0.04 ± 0.01
Spring inter-monsoon	*Nematalosa nasus*	425.33 ± 25.44	490.29 ± 6.56	148.16 ± 16.91	11.53 ± 0.38	2.67 ± 0.24	0.41 ± 0.01
	Ilisha striatula	234.30 ± 46.07	436.53 ± 1.16	92.71 ± 0.46	11.69 ± 0.25	1.55 ± 0.20	0.12 ± 0.06
Southwest monsoon	*Nematalosa nasus*	225.10 ± 15.05	405.68 ± 1.51	119.50 ± 10.96	13.67 ± 0.29	1.26 ± 0.07	0.13 ± 0.01
	Ilisha striatula	440.70 ± 15.50	393.67 ± 5.32	127.23 ± 2.13	12.63 ± 0.35	17.66 ± 0.27	0.17 ± 0.01
Autumn inter-monsoon	*Nematalosa nasus*	138.00 ± 43.82	265.63 ± 2.04	46.72 ± 0.51	7.51 ± 0.13	2.40 ± 0.06	0.17 ± 0.01
	Ilisha striatula	175.16 ± 1.95	474.83 ± 4.93	56.29 ± 0.14	7.51 ± 0.06	2.94 ± 0.06	0.49 ± 0.33

the categorical composition of nutrients in its flesh. Total mineral contents in fish muscles range from 0.6–1.5% in wet tissues [62]. Our study provided extensive seasonal analytic data on the nutritional composition of two pelagic and two demersal fish species sampled from the Karachi fish harbor. The purpose of the seasonal analysis was to observe the trends and fluctuations in the concentrations of essential micro- and macroelements in the fish samples and their proximate composition.

Seasonal variation in essential elements among the four selected fish species was shown in the northeast monsoon, spring inter-monsoon, southwest monsoon, and autumn inter-monsoon seasons respectively. The concentrations of Na, K, and Ca were high in both the pelagic and the demersal fishes. Na had a higher concentration in the demersal fishes than in the pelagic species in our study. Its concentration was also higher than in the study conducted by Nordhagen *et al.*, who determined the same micro- and macroelements [63]. Higher concentrations of Na and K in the demersal fish are due to their diet and gender. Like crustaceans, they are a great source of K, Na, and Ca [62]. The minimum values of Na in the demersal species were lower than its minimum values in the pelagic species. Further, the concentrations of Na in both pelagic species were lower than those reported by Nordhagen *et al.* and higher than those determined by Lilly *et al.* [60, 63].

All the species were richer in K than Na, which may be because fish have a high capability of accumulating K. The concentration levels showed the same hierarchy as the one observed by Ersoy and Celik, namely K < Na < Ca < Mg [61]. Ca is found more in the demersal fishes than in the pelagic ones. However, its highest levels were observed in the spring inter-monsoon season, while its lowest values were found in the autumn inter-monsoon season. The Ca values in both the demersal and pelagic fishes were lower than the ones reported by Nordhagen *et al.* but higher than in the study conducted by Lilly *et al.* [60, 63]. In this study, Mg was found in low concentrations similarly to Ca [60]. The demersal species in our study showed the highest Mg levels during the spring inter-monsoon season. In the pelagic zone, Mg was only slightly higher in the southwest monsoon season compared to the spring inter-monsoon.

The high concentrations of Na, K, Ca, and Mg in all the four fishes in the spring inter-monsoon are due to the mixing of water and a high availability of food during this season, as well as heavy rainfall. Higher land runoff from different areas results in increasing these elements in a water body in particular seasons [61]. The same

levels of metals were found by Ersoy and Celik [61], namely K > Na > Ca > Mg. Stepanova and Lugovaya suggested that high concentrations of Na, K, and Ca in carnivore fish species were due to their diet and feeding on other small fishes and crustaceans, which are the greatest source of these elements [64]. Fish is usually richer in K than Na because seawater is responsible for the morphological alteration in fish.

Zn was greater in the pelagic fishes than in the demersal ones. Afandi *et al.* suggested that it is because pelagic fishes are more adapted to feeding at a higher level in the food chain and they can easily bio-accumulate Zn [65]. In our study, the concentrations of Zn were below the permissible limit of 30 mg/kg established by the Ministry of Agriculture, Fisheries and Food in all the four seasons [66].

Other essential elements such as Mg and Mn varied in the studied species. These micronutrients are necessary only in minute quantities since their high levels in the muscles or tissues can increase metabolic reactions [67]. Excessive concentrations of these essential elements can lead to health problems. For example, excessive consumption of Mn causes hemochromatosis and may cause thalassemia [68].

The spring inter-monsoon is the best season to utilize seafood with high bio-nutrients, while the autumn inter-monsoon season showed the lowest trends. Furthermore, various morphological and physiological factors of species, physicochemical factors of water, reproductive cycles, and anthropogenic activities can affect metal accumulation in fish muscles [69]. For example, anthropogenic activities are lower during the winter season, which can contribute to less metal accumulation in fish muscles.

The proximate composition of the fish samples was also investigated seasonally, including the contents of protein, lipids, carbohydrates, ash, and moisture (Table 4). Our results confirmed that fish is a source of both bio-nutrients and essential elements, but the bio-profile of species helps to understand which fish is good to consume in what season. The protein and lipid contents in the targeted demersal species in our study were higher than those in the study by Nordhagen *et al.* but similar to those indicated by Nurnadia *et al.* [63, 69]. The carbohydrates showed higher trends when compared to the study by Nurnadia *et al.* [69]. Both species showed higher bio-nutrients in the spring inter-monsoon season. *Nemipterus japonicus* is carnivorous and feeds mostly on crustaceans throughout the year, while *Epinephelus erythrurus* is a migratory species that consumes more food during the spring [8, 70]. Migration is due to an abundance of nutrients, which could explain the high protein-lipid and carbohydrate trends in the spring inter-monsoon season for both demersal species. Further, physiological, ecological, and physicochemical conditions could also be a reason for the fluctuation of bio-nutrients in the fish muscles [71]. The moisture content of the demersal species in our study was high, while the ash content was within the permissible limits, except during the northeast monsoon season [69].

The protein content in the pelagic species under study showed lower trends than in the study Nordhagen *et al.* and differed slightly from the study by Nurnadia *et al.* [63, 69]. The lipid content, however, showed similar trends to those in these two studies. The carbohydrate content in our study was higher than the one reported by Nurnadia *et al.* [69]. Both pelagic zone species, *Nematalosa nasus* and *Ilisha striatula*, showed higher bio-nutrient trends in the southwest monsoon season. This is due to a huge share of plankton consumption in their diet [71]. The pelagic species showed the highest levels of protein, lipids, and carbohydrates in the summer due to the abundance of macronutrients in the pelagic zone. Since summer is a euphotic period with more light penetration, it has ideal conditions for the growth of plankton [72].

Table 4 Proximate composition of wet fish muscles expressed in percentage and std

Seasons	Species name	Protein, %	Lipids, %	Carbohydrates, %	Moisture, %	Ash, %
			Demersal fish			
Northeast monsoon	*Nemipterus japonicus*	16.31 ± 1.13	21.39 ± 0.18	19.88 ± 1.19	90.22 ± 1.47	9.78 ± 1.01
	Epinephelus erythrurus	22.01 ± 0.69	20.48 ± 0.02	21.90 ± 1.55	96.68 ± 0.81	8.91 ± 0.60
Spring inter-monsoon	*Nemipterus japonicus*	21.52 ± 0.51	21.96 ± 0.34	24.57 ± 0.50	97.71 ± 4.57	2.29 ± 0.21
	Epinephelus erythrurus	22.87 ± 0.30	21.51 ± 0.08	24.49 ± 1.31	98.92 ± 0.90	0.28 ± 0.20
Southwest monsoon	*Nemipterus japonicus*	18.15 ± 1.00	20.74 ± 0.02	20.19 ± 0.55	97.08 ± 0.38	2.16 ± 0.32
	Epinephelus erythrurus	21.14 ± 0.52	20.43 ± 0.34	23.57 ± 1.39	97.12 ± 0.78	1.99 ± 0.44
Autumn inter-monsoon	*Nemipterus japonicus*	18.78 ± 0.67	20.61 ± 0.02	19.84 ± 0.41	90.21 ± 9.11	0.77 ± 0.11
	Epinephelus erythrurus	15.65 ± 0.31	20.29 ± 0.02	15.87 ± 0.52	90.93 ± 1.46	2.26 ± 0.53
			Pelagic fish			
Northeast monsoon	*Nematalosa nasus*	19.14 ± 0.79	21.65 ± 0.18	20.74 ± 0.71	89.40 ± 8.05	7.14 ± 0.90
	Ilisha striatula	18.99 ± 0.66	20.64 ± 0.17	20.22 ± 0.01	93.13 ± 4.01	2.59 ± 1.23
Spring inter-monsoon,	*Nematalosa nasus*	13.72 ± 0.25	20.68 ± 0.02	14.91 ± 0.17	93.40 ± 3.04	2.55 ± 1.16
	Ilisha striatula	20.56 ± 0.87	20.51 ± 0.02	20.97 ± 0.59	94.52 ± 1.93	3.88 ± 0.56
Southwest monsoon	*Nematalosa nasus*	19.32 ± 0.62	22.40 ± 0.01	22.96 ± 0.50	98.70 ± 1.12	1.30 ± 0.20
	Ilisha striatula	22.59 ± 1.20	22.47 ± 0.12	28.14 ± 0.91	98.93 ± 0.81	1.07 ± 0.53
Autumn inter-monsoon	*Nematalosa nasus*	15.02 ± 0.04	20.49 ± 0.13	16.39 ± 0.58	98.40 ± 0.52	1.41 ± 0.52
	Ilisha striatula	13.47 ± 2.49	20.48 ± 0.13	18.13 ± 0.92	98.92 ± 0.90	0.28 ± 0.20

This could be a reason for the high bio-nutrient content in the summer season. Monsoon causes advection (when warm air moves into a cool region) and upwelling, which generate ocean currents and cause the mixing of nutrients and photo-chemicals [73]. Also, there is a direct link between plankton abundance and high proximate composition values of pelagic species, as well as an indirect relationship between fats and moisture [44]. In our study, the moisture content was higher than in the study conducted by Nurnadia *et al.*, while the ash content was within the permissible limits with some variation [69]. Interestingly, we also found that the carnivorous species showed higher carbohydrate levels than to the omnivorous ones.

Carnivorous species contain a high level of starch or carbohydrates, as observed by Moon [74]. Omnivorous species use carbohydrates as a source of energy, while carnivorous species show less or no use of carbohydrates as an energy source, so the selection of food may also be a cause of fluctuating carbohydrate levels between the species with different feeding habits [37].

Apart from the seasonal differences, we found some other interesting links, particularly a direct and indirect link between the feeding behavior and the proximate composition of the species.

Our study also showed an interesting link between the species with omnivorous and carnivorous feeding behavior. The omnivorous species are rich in lipids due to their feeding on zooplankton, including copepods. Zooplankton stores accumulate lipids in various body parts. This selection of food could be a reason for high lipid levels in *N. nasus* or omnivorous fish [73].

There is also an indirect link between protein and lipid levels, as well as a direct link between protein and carbohydrate values, which may be due to the changes in reproductive stages and the physiology of the fish body.

The sampled species from the demersal zone showed the highest levels of essential elements throughout the year, namely K > Na > Ca > Mg in the northeast monsoon, spring inter-monsoon, southwest monsoon, and autumn inter-monsoon, respectively (Fig. 1). Potassium and Sodium were the most abundant macronutrients found in both species throughout the study period. The demersal species *N. japonicus* and *E. erythrurus* showed the highest values of macro- and micronutrients during the spring inter-monsoon season and the lowest in the autumn inter-monsoon season. Although both *N. japonicus* and *E. erythrurus* are demersal species, the contents of Na, K, and Ca were high in *N. japonicus*. The concentration of K was significant in the spring inter-monsoon and lowest in the autumn inter-monsoon season. This may be due to the seasonal transition of feeding. The proximate composition of both demersal species showed the same trends throughout the year, with the highest values in the spring inter-monsoon and the lowest values in the autumn inter-monsoon (Table 4). Although *N. japonicus* and *E. erythrurus* are both demersal and therefore rich in protein, the levels of protein and carbohydrates were higher in *E. erythrurus*. The fat content was high in *N. japonicus*. Thus, our results showed a direct link between the protein and carbohydrate levels and an indirect link between the protein and lipid contents. The bio-nutrient profile of the selected species is shown seasonally in Fig. 2.

N. nasus, one of the most ecologically important species in Pakistan, showed the highest contents of macro- and micronutrients between the both pelagic zone species during the spring inter-monsoon season, with exception

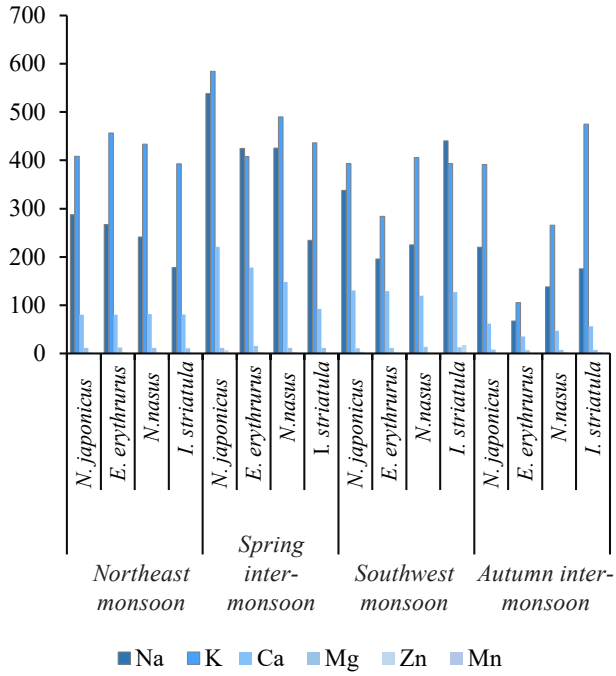

Figure 1 Essential elements in fish species by season, mg/L

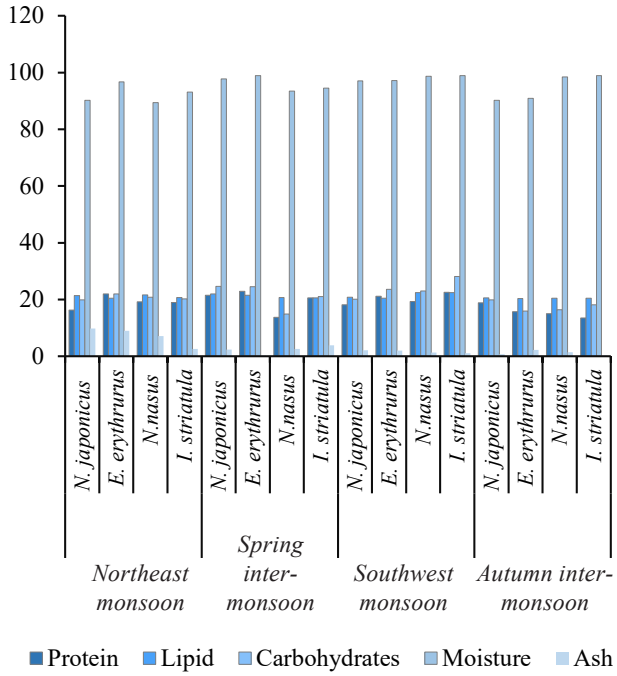

Figure 2 Proximate composition of fish species by season, %

Table 5 Estimated daily intake, estimated weekly intake, target hazard quotients, and hazard index for Zn and Mn (mg/kg wet wt.) for adults from the consumption of two demersal and two pelagic fishes

Seasons	Species name	Estimated daily intake		Estimated weekly intake		Target hazard quotients		Hazard index
		Zn	Mn	Zn	Mn	Zn	Mn	
				Demersal fish				
Northeast monsoon	*Nemipterus japonicus*	4.1×10^{-4}	1.4×10^{-5}	2.8×10^{-3}	9.5×10^{-5}	1.4×10^{-3}	9.7×10^{-5}	1.5×10^{-3}
	Epinephelus erythrurus	4.3×10^{-4}	2.7×10^{-5}	2.9×10^{-3}	1.9×10^{-4}	1.4×10^{-3}	1.9×10^{-4}	1.6×10^{-3}
Spring inter-monsoon	*Nemipterus japonicus*	15.0×10^{-4}	3.6×10^{-5}	1.1×10^{-2}	2.5×10^{-4}	5.0×10^{-3}	2.6×10^{-4}	5.3×10^{-3}
	Epinephelus erythrurus	6.7×10^{-4}	6.4×10^{-5}	4.7×10^{-3}	4.5×10^{-4}	2.2×10^{-3}	4.5×10^{-4}	2.7×10^{-3}
Southwest monsoon	Nemipterus japonicus	7.2×10^{-4}	4.1×10^{-5}	5.0×10^{-3}	2.9×10^{-4}	2.4×10^{-3}	2.9×10^{-4}	2.6×10^{-3}
	Epinephelus erythrurus	5.0×10^{-4}	6.4×10^{-5}	3.5×10^{-3}	4.5×10^{-4}	1.7×10^{-3}	4.5×10^{-4}	2.1×10^{-3}
Autumn inter-monsoon	*Nemipterus japonicus*	3.5×10^{-4}	1.1×10^{-5}	2.4×10^{-3}	7.9×10^{-5}	1.2×10^{-3}	8.1×10^{-5}	1.2×10^{-3}
	Epinephelus erythrurus	3.8×10^{-4}	6.8×10^{-5}	2.6×10^{-3}	4.8×10^{-5}	1.3×10^{-3}	4.8×10^{-5}	1.3×10^{-3}
				Pelagic fish				
Northeast monsoon	*Nematalosa nasus*	4.6×10^{-4}	6.8×10^{-5}	3.2×10^{-3}	4.8×10^{-5}	1.5×10^{-3}	4.9×10^{-5}	1.6×10^{-3}
	Ilisha striatula	3.3×10^{-4}	9.1×10^{-5}	2.3×10^{-3}	6.4×10^{-5}	1.1×10^{-3}	6.5×10^{-5}	1.2×10^{-3}
Spring inter-monsoon	*Nematalosa nasus*	6.1×10^{-4}	9.3×10^{-5}	4.2×10^{-3}	6.5×10^{-4}	2.0×10^{-3}	6.7×10^{-4}	2.7×10^{-3}
	Ilisha striatula	3.5×10^{-4}	2.7×10^{-5}	2.4×10^{-3}	1.9×10^{-4}	1.2×10^{-3}	1.9×10^{-4}	1.4×10^{-3}
Southwest monsoon	*Nematalosa nasus*	2.9×10^{-4}	2.9×10^{-5}	2.0×10^{-3}	2.1×10^{-4}	9.6×10^{-4}	2.1×10^{-4}	1.2×10^{-3}
	Ilisha striatula	4.0×10^{-3}	3.9×10^{-5}	2.8×10^{-2}	2.7×10^{-4}	1.3×10^{-2}	2.8×10^{-4}	1.4×10^{-2}
Autumn inter-monsoon	*Nematalosa nasus*	5.5×10^{-4}	3.8×10^{-5}	3.8×10^{-3}	2.7×10^{-4}	1.8×10^{-3}	2.7×10^{-4}	2.1×10^{-3}
	Ilisha striatula	6.7×10^{-4}	1.1×10^{-4}	4.6×10^{-3}	7.8×10^{-4}	2.2×10^{-3}	7.9×10^{-4}	3.0×10^{-3}
Total						4.1×10^{-2}	4.4×10^{-3}	

of Mg, which was highest in the southwest monsoon. *I. striatula* showed the highest values during the southwest monsoon season, except for K and Zn in comparison of both targeted pelagic zone species. The lowest levels of all essential elements between both pelagic species were observed during the autumn inter-monsoon season, except for K and Mn (Fig. 1). *N. nasus* showed the highest values of macro- and micronutrients during the spring inter-monsoon, except for Ca. *I. striatula* showed the highest macronutrient values (Na, Ca, and Mg) during the southwest monsoon, except for K (Fig. 1). *N. nasus* showed the highest proximate composition values during the southwest monsoon, except for the ash content. *I. striatula* showed high proximate composition values during the southwest monsoon, while ash was high in the northeast monsoon season. The protein and carbohydrate contents were found higher in *I. striatula*, while *N. nasus* showed a higher lipid content throughout the season (Fig. 2).

Risk assessment. Provisional tolerable weekly intake estimates the amount per unit body weight of a likely hazard contaminant in fish that can be consumed over a lifetime without risk of unfavorable health effects. Provisional tolerable weekly intake is meant to emphasize that long-term exposure is substantial for metals that accumulate in the body. Adverse effects for people are observed with many metals in the range of exposure. Provisional tolerable weekly intake should be compared to well-established and internationally accepted tolerance. Provisional tolerable weekly intake is established for metals that do not leave the body instantly and may remain there permanently. The provisional tolerable weekly intake safe level for Zn is 7, but the Joint Expert Committee on Food Additives has not established such a level for Mn [75, 76].

Assumptions are used in risk assessments. The US Environmental Protection Agency's Regional Screening Levels and the Risk Assessment Information System present methods for estimating the non-cancer risk [58, 59]. The theoretical and estimated lifetime target hazard quotients were calculated for adults exposed to Mn and Zn from the consumption of fish from Pakistan coasts of the Arabian Sea (Table 5).

The hazard index of less than 1 indicates that the estimated exposure is below the USEPA reference dose for the relevant metals for all seasons and both demersal and pelagic fish species. We found that the hazard index value for Mn and Zn was lower than standard 1 for all four fish species, demonstrating that the ingestion of these fishes from Pakistan coasts of the Arabian Sea will not result in overexposure to these contaminants. Thus, they have no adverse effects on the health of consumers.

The estimated daily intake was calculated by taking the weighted average of Zn and Mn in each fish species and multiplying it by the respective consumption rate. The daily intakes of Zn were estimated between 0.000286 and 0.004 mg for adults during all seasons, much lower than the Rf.D. value (0.3 mg/day) [58, 59]. The estimated weekly intakes for adults were between 0.002 and 0.028 mg/kg, respectively. The safe provisional tolerable weekly intake value for Zn is 7 mg per kg of body weight [75]. In our study, the Zn levels were quite below this safe value.

The daily intakes of Mn were calculated between 0.000011 and 0.00011 mg for an adult, which is well below the Rf.D. (0.14 mg/day) [58, 59]. The provisional

tolerable weekly intake value for Mn has not been estimated yet [76]. Although there is no specific assessment of Mn, it appears that Mn in fish contact materials does not cause any concern [76].

CONCLUSION

Our overall results validated that macronutrients such as K and Na were present in significant quantities in the fish inhabiting both pelagic and demersal zones. The spring inter-monsoon was found to be the season in which essential elements peaked. Therefore, this season can be suitable for fish consumption. Our study also found that the demersal zone showed a good bio-nutrient profile in the spring inter-monsoon season, while the pelagic species were high in bio-nutrients in the summer (southwest monsoon) season.

It cannot be ignored that feeding habits play a vital role in energy and nutrient flows in an ecosystem. Interestingly, the carnivorous fishes accumulated carbohydrates more sufficiently than the omnivorous fishes in our study. We also observed a direct link between protein and carbohydrates and an indirect link with lipids. We hope that our study will help to understand the nutritional dynamics and energy flow in different species and zones.

The risk assessment showed that the two demersal and two pelagic fish species in our study had Zn and Mn levels below the allowable values, and the estimation of non-carcinogenic risk revealed no possible adverse effects on human health.

CONTRIBUTION

All the authors were equally involved in the research analysis and manuscript writing.

CONFLICT OF INTEREST

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

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Properties of plant extracts and component composition: column chromatography and IR spectroscopy

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Abstract:
Medicinal plants contain various biologically active substances. This study aimed to investigate properties of plant extracts and component composition of plant raw materials from the continental part Kaliningrad region (Guards district). For this, we used column chromatography and IR spectroscopy.
The objects of the study were samples of plant extracts of *Eryngium maritimum*, *Hedysarum neglectum*, *Melilotus officinalis*, and *Aesculus hippocastanum*. To produce medicinal plant extracts, we prepared methanol extraction by the Soxhlet method for 8 h (15 cycles). The antioxidant activity of the studied samples was determined by their ability to reduce the radical 2,2-diphenyl-1-picrylhydrazyl. The disk-diffusion method was used to evaluate the antimicrobial activity of the plant extracts against such test strains as *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*.
In the extracts, 3,4-dihydroxybenzoic acid, astragalin, luteolin-7-glucoside, rosmarinic acid, and chlorogenic acid were identified. However, more research is needed to determine which of the individual phenolic compounds in *E. maritimum*, *H. neglectum*, *M. officinalis*, and *A. hippocastanum* are involved in exhibiting antioxidant activity. It was found that the plant extract of *H. neglectum* had activity against the bacterium *B. subtilis* and the mold fungus *C. albicans*, while the plant extract of *E. maritimum* was detrimental to the growth and development of both Gram-positive and Gram-negative bacteria.
Infrared spectroscopy can help in further studies to determine properties of medicinal plants to ensure the safety and efficacy of plant-based products.

Keywords: Medicinal plants, *Eryngium maritimum*, *Melilotus officinalis*, *Hedysarum neglectum*, *Aesculus hippocastanum*, extracts, infrared spectroscopy, antioxidant properties

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INTRODUCTION

Medicinal plants contain various phenolic compounds and their derivatives. These are important biologically active substances, which are powerful antioxidants used in the production of flavorings, synthetic dyes, protective agents against various oxidative stresses, and therapeutic agents for the human body [1]. Plants are sources of antioxidants which play an important role in preventing cell damage and controlling oxidative stress in the body [2]. Plant antioxidants, such as polyphenolic compounds, are considered important ingredients because of their beneficial properties [2, 3]. In Chinese phytotherapy, various parts of the plant (roots, bark, leaves, fruits, and seeds) are used to treat many human diseases [4, 5]. Benefits of antioxidants are stabilization or inactivation of free radicals, protection of cells during oxidation, and prevention of the destruction of cellular components [6].

Antimicrobial resistance poses a serious threat to human health worldwide [7]. The cost of developing a new generation of antibiotics from discovery to market is high, and the return on investment is low. For this reason, the development of new antibiotics has slowed dramatically. However, plants produce many bioactive secondary metabolites that exhibit antimicrobial activity in varying degrees and can help solve the problem [7].

Infrared (IR) spectroscopy is a powerful analytical technique for analyzing any medicinal plant sample under any conditions. IR spectra are used to measure the vibrations of molecules excited by infrared radiation and to identify organic, inorganic, and polymeric materials for scanning in infrared light [8].

We found no research on the use of high performance liquid chromatography to examine various biologically active substances from sea holly (*Eryngium maritimum* L.), sweet yellow clover (*Melilotus officinalis* Pall.), red root (*Hedysarum neglectum* Ledeb.), and horse chestnut (*Aesculus hippocastanum* L.). Additionally, important bioactive compounds were quantified using solvent extraction methods, and their antioxidant activity was evaluated using standard methods. Over the past decade, several analytical methods have been used to measure the freeradical activity of herbaceous plants [7]. Spin splitting methods for detecting free radical activity in tea trees and fruits have been demonstrated to be a more appropriate and advanced extraction process [9]. Biologically active compounds of these plants have a variety of high biological activity, such as antioxidant, free radical absorption activity, antimicrobial, anti-inflammatory, and anticancer activity. The Kaliningrad region is a unique region of Russia, primarily due to its geographical location and climatic conditions. Favorable conditions for vegetation growth are contributed by such factors as the natural characteristics of the region's mild maritime climate, the smooth course of temperatures throughout the annual cycle, the long frost-free period, rapid soil formation due to the constant ingress of organic matter and its rapid decomposition, as well as the abundance of precipitation [10–12].

Official medicine recognizes the general potential and gives priority to medicinal plants in the treatment of many diseases. In this regard, the study of the biological activity of *E. maritimum*, *M. officinalis*, *H. neglectum*, and *A. hippocastanum* as understudied plants is very relevant [12]. It is well known that these potentially beneficial plant species have a diverse composition of biologically active substances, pharmacological activity, but have received little attention [11].

E. maritimum contains essential and fatty oils, flavanoids, steroids, coumarins, terpenoids, polyacetylene compounds, and carbohydrates. *E. maritimum* is used as a diuretic, tonic, antibacterial, antispasmodic agent [13, 14]. The plant is used in the treatment of cancer due to the presence of biologically active substances which have antitumor activity, such as coumarins, xanthotoxins, and leucoanthocyanins [14]. *H. neglectum* is used in medicine for the treatment of cardiac, pulmonary, as well as cold and skin diseases of viral nature. It exhibits antituberculosis, antitumor, hepatoprotective, antibiotic, immunostimulating, choleretic, diuretic, hyperglycemic effects and stabilizes lysosomal membranes. *H. neglectum* has therapeutic properties due to a unique combination of biologically active substances, including xanthones, flavonoids, and polysaccharides [14]. *A. hippocastanum* is used to treat cardiovascular diseases, gastrointestinal disorders, a number of nervous disorders, skin, parasitic, and other diseases [15]. The key biologically active substances of *A. hippocastanum* are chicoric acid, ferulic acid, vitexin, epicatechin, dihydrocoumarin, water-soluble polysaccharide complexes, polyphenolic compounds of phenyl benzo-γ-pyrone, benzo-γ-pyrone, and phenolcarboxylic acid derivatives [15]. The plants mentioned above have unique properties due to the mild marine climate, slight temperature fluctuations, illumination, are a valuable source of new pharmaceuticals and the basis for the development of functional products. In addition, various parts of medicinal plants are increasingly used in the cosmetic industry [1]. There are modern ideas about oxidative stress and extreme radicals. Antioxidants help prevent these harmful effects. This article describes methods used in the quantitative description of antioxidants of medicinal plants of the Kaliningrad region.

The use of herbal medicines and supplements has increased over the past few decades. This is consistent with the growth of self-medication, so there is a trend toward returning to traditional and natural products. Consumers prefer herbal and natural products because they are safer and more likely to reduce the side effects of chemical drugs, improve health, and lower treatment costs [1]. This can have negative consequences for the consumer. Product quality assurance can be evaluated both qualitatively and quantitatively. Quantitative assessment of plant products focuses on phytochemical components found naturally in a sample as well as impurities that should not be present [2, 3].

Various analytical techniques, such as high-performance liquid chromatography (HPLC), ultra-high-performance liquid chromatography, liquid chromatography-mass spectrometry, gas chromatography-mass spectrometry, nuclear magnetic resonance (NMR), and thin layer chromatography, can be used to check the phytochemical content and impurities in medicinal herbs. However, the methods have a number of drawbacks. HPLC and NMR methods are commonly used methods to obtain data on the composition of plant components. However, the main disadvantage of these methods is the high cost and long time of research [16]. In addition, when analyzing plant-based medicines, one of the obstacles is the efficiency of analysis because the complex components of plant-based medicines can complicate the process. Furthermore, the analysis process can also damage the material [15].

In light of the foregoing, this study aimed to investigate properties of plant extracts and component composition by using column chromatography and IR spec-

troscopy plant raw materials from the continental part of the Kaliningrad region (Guards district). The results of an infrared spectroscopy study of biologically active compounds from *E. maritimum*, *M. officinalis*, *H. neglectum*, and *A. hippocastanum* are presented in this paper. This method is promising for the pharmaceutical industry as a future green line of highly effective plant antioxidants [17]. Extracts of *E. maritimum*, *M. officinalis*, *H. neglectum*, and *A. hippocastanum* exhibit significant antioxidant and antimicrobial activity.

STUDY OBJECTS AND METHODS

Objects of research. Medicinal plants of the Kaliningrad region, namely *Eryngium maritimum* L., *Melilotus officinalis* Pall., *Hedysarum neglectum* Ledeb., and *Aesculus hippocastanum* L. The biomaterial was confirmed by A.V. Pungin, the head of the herbarium of the Institute of Living Systems of Immanuel Kant Baltic Federal University (Protocol No. 6/2022). The above-ground parts of *E. maritimum*, *M. officinalis*, *H. neglectum*, and *A. hippocastanum* (stem, leaves, and flowers) were collected during the flowering period (July-August 2022). Herbs were dried in well-ventilated rooms in the shade under a roof.

Extract productions. To produce *E. maritimum*, *M. officinalis*, *H. neglectum* и *A. hippocastanum* methanolic extracts, we applied the Soxhlet method for 8 h (15 cycles). The extract was then vaporized under reduced pressure and then dissolved in a mixture of methylene chloride:methanol (1:1). Samples of *E. maritimum* for subsequent IR spectroscopy were obtained using preparative liquid chromatography. For this purpose, a silica gel suspension in methylene chloride was loaded into a glass chromatography column (Agilent Technologies Prep LC, Santa Clara, California, USA) in such an amount that the silica gel occupied no more than half of the column volume. The solvent composition for the elution was chosen by thin-layer chromatography on DC-Fertigfolien Alugram SIL G/UV_{254} plates; the sorbent was silica gel (Agilent Technologies Prep LC, Santa Clara, CA, USA). Elution was performed in the gradient mode in the methylene chloride:methanol system with a gradient of 0–100%, at the rate of 10% increase in methanol concentration every 10 fractions [18, 19]. The volume of the fractions was 4 mL. Some methylene chloride:methanol (8:2) fractions were selected.

The eluted fractions were determined by the time of release of the maximum of its chromatography peak. HPLC parameters: reversed-phase, 2-channel (binary) pumps, flow rate 1.0 cm^3/min, injection volume 0.005 cm^3, temperature 40°C, UV spectrophotometric detection on a diode matrix at λ = 254 nm, approximate retention time 5 min. The wavelength was selected in accordance with State Standard R ISO 17735-2021 for chromatography with a UV detector. This is the optimal wavelength for the HPLC chromatograph used. Glass chromatography column operates in open access mode, the chromatograph is modular with possible switching of columns, using MassHunter Walkup software for identifying substances. Column parameters: analytical, 46 mm in diameter, densely packed, flow rate 1000 µL/min. The mobile phase is more polar than the graft phase and is supplied under pressure up to 200–500 atm.

All standards and reagents, graded chemically pure or higher, were purchased from AG Analitekspert, Moscow, Russia.

Preparation of *E. maritimum*, *M. officinalis*, *H. neglectum*, and *A. hippocastanum* extracts for IR spectrometry. KBr powder (m = 0.11 g) (Pike Technologies, Madison, Wisconsin, USA) was impregnated with the sample solution (V = 0.5 mL). Next, the powder with the sample was dried in a drying cabinet (Memmert GmbH, Memmert, Germany) at 50°C until the liquid evaporated completely for 40 min. The resulting dry powder was ground in an agate mortar to grind the fraction. The resulting mixture was further pressed into a transparent tablet. The IR spectrum of the prepared samples of plant extracts was measured on an IR spectrometer IRPrestige-21 (Shimadzu, Kyoto, Japan): the spectral range 500–4000 cm^{-1}, a spectral resolution of 2 cm^{-1}, frequency (wave number) 100 cm^{-1}, and the number of scans of 32. The spectra of the comparison samples were obtained under similar conditions. The relative standard deviation of the method was 1.97% and the signal-to-noise ratio was 60 times.

Determination of antioxidant activity of *E. maritimum*, *M. officinalis*, *H. neglectum*, and *A. hippocastanum* extracts. To study the antioxidant activity, the plant extracts were dissolved in 1 mL of dimethyl sulfoxide and sonicated (Sonorex Super RK 100 H, Bandelin, Germany) for 5–10 min until they dissolved completely. The samples were dissolved right on the day of analysis.

The antioxidant activity of the studied samples was determined by their ability to reduce the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH, $C_{18}H_{12}N_5O_6$, M = 394.33) [20]. The interaction reaction of antioxidants with DPPH-radical proceeds according to the scheme:

$$DPPH^* + AH \rightarrow DPPH–H + A^*$$

The reduction of the DPPH radical by an antioxidant results in a reduction of the purple-blue color of DPPH in ethanol, and the reaction was monitored by the change in optical density using the spectrophotometric method.

The samples of plant extract solutions were mixed with 2.85 mL of a freshly prepared 0.1 mM solution of 2,2-diphenyl-1-picrylhydrazyl for analysis. The mixture was incubated in dark at room temperature for 30 min. The decrease in optical density compared to the control (dimethyl sulfoxide solution) was recorded at 517 nm (UV-3600 spectrophotometer, Shimadzu, Kyoto, Japan). Ascorbic acid solutions of known concentration were used as standard solutions. The results were expressed in mg of ascorbic acid equivalent per gram of extract or individual compound (mg AA/g). In the absence of sample mass, the antioxidant activity was expressed by the EC_{50} value per µL of solution required to bind 50% of the DPPH radical [10].

The antioxidant activity of the samples was analyzed in three replicates.

Determination of antimicrobial activity of *E. maritimum*, *M. officinalis*, *H. neglectum*, and *A. hippocastanum* extracts. The antimicrobial activity of the plant extracts was studied by the disk-diffusion method, against such test strains as *Bacillus subtilis* (Gram-positive bacteria), *Escherichia coli*, *Pseudomonas aeruginosa* (Gram-negative bacteria), and *Candida albicans*. *E. coli*, *P. aeruginosa*, and *B. subtilis* strains were cultivated on solid LB nutrient media at 37°C [21, 22].

C. albicans microscopic fungi strains were cultivated on Ringer's medium (ammonium chloride, 0.4 g; sodium hydrogen carbonate, 0.005 g; calcium chloride dihydrate, 0.040 g; potassium chloride, 0.0525 g; sodium chloride, 1.125 g) at 25°C.

The concentration of the microbial suspension during the experiment was at least 1.5×10^8 CFU/mL. The disc diameter was 6 mm. Kanamycin (for bacteria) and fluconazole (for the yeast-like fungus, *C. albicans*) was used as a comparison in a concentration of 50 and 500 μg/disk, respectively. A mixture consisting of 1% trifluoroacetic acid (31%) and acetonitrile (69%) was used as control. For reliability of the results, the experiment was repeated three times. The average value was used as the result of the measurement [21].

All standards and reagents, graded chemically pure or higher, were purchased from AG Analitekspert, Moscow, Russia.

Statistical analysis. Each experiment was repeated three times, and the results were presented as mean ± standard deviation. Standard statistical methods were used to process the obtained data.

RESULTS AND DISCUSSION

Figures 1–4 show the results of preparative chromatography of *Eryngium maritimum* L., *Melilotus officinalis* Pall., *Hedysarum neglectum* Ledeb., and *Aesculus hippocastanum* L.

Peaks of the IR spectra of extracts of medicinal plants are presented in Table 1.

The qualitative composition of the extract fractions of *E. maritimum*, *M. officinalis*, *H. neglectum*, and *A. hippocastanum* are presented in Tables 2–5.

Figures 5–8 demonstrate IR absorption spectra of the *E. maritimum*, *M. officinalis*, *H. neglectum*, and *A. hippocastanum* extracts. They allow us to amplify data about the structure and properties of the extracts.

The values of antioxidant activity of the studied plants are presented in Table 6.

The antimicrobial activity of *E. maritimum*, *M. officinalis*, *H. neglectum*, and *A. hippocastanum* extracts is presented in Table 7.

The results of the studies (Table 7) show that the extracts had the greatest antibacterial activity against *Bacillus subtilis*. The experiment revealed that the diameter of the inhibition zone of *E. maritimum* was 14 mm. None of the extracts showed activity against *Escherichia coli*. The zone of inhibition of *H. neglectum* extracts was 7 mm. However, all these values did not exceed the inhibitory activity of the positive control, kanamycin, which had an inhibition zone diameter of 28 mm.

It was found that against the test strain of *P. aeruginosa*, the highest activity was observed in the plant extracts of *E. maritimum* and *A. hippocastanum*. The *H. neglectum* extracts showed little activity against *C. albicans*.

Next, in order to establish the minimum inhibitory concentration of the studied extracts, plant extracts that exhibited antimicrobial (antibacterial and fungicidal) activity against the test strains were selected. For such extracts, dilutions of 2, 4, and 6 times were prepared. However, none of the extracts of *A. hippocastanum*, *H. neglectum*, *E. maritimum*, and *M. officinalis* that were isolated showed activity against the test strains of *P. aeruginosa*, *C. albicans*, *E. coli*, and *B. subtilis*.

Analysis of Fig. 1 and the data obtained Table 2 shows that the main component of *E. maritimum* extract was

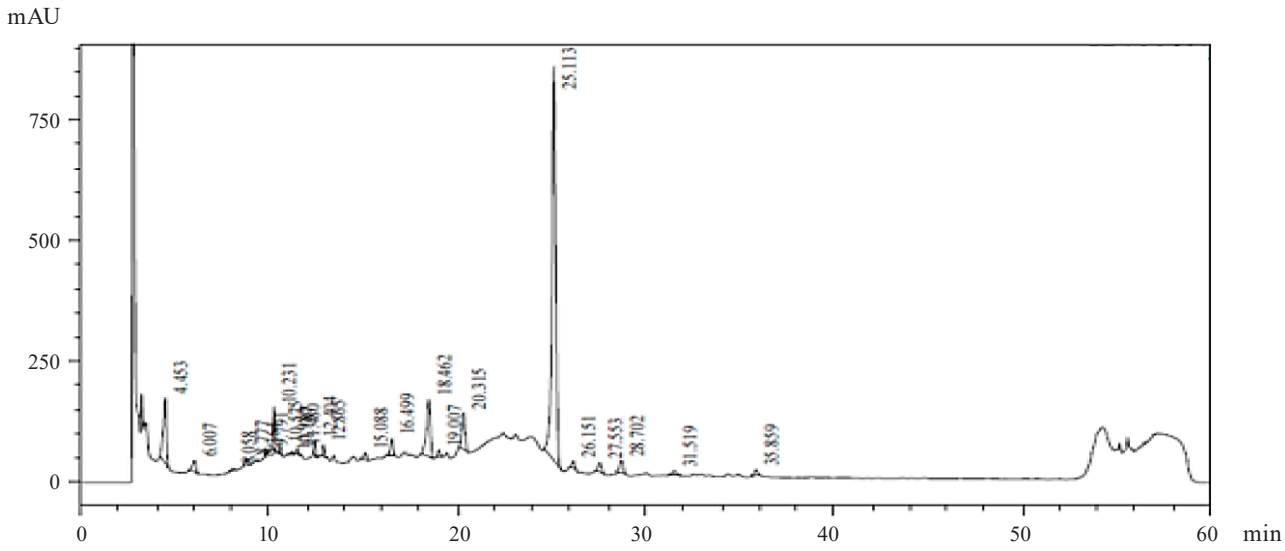

Figure 1 Chromatogram of the *Eryngium maritimum* L. extract

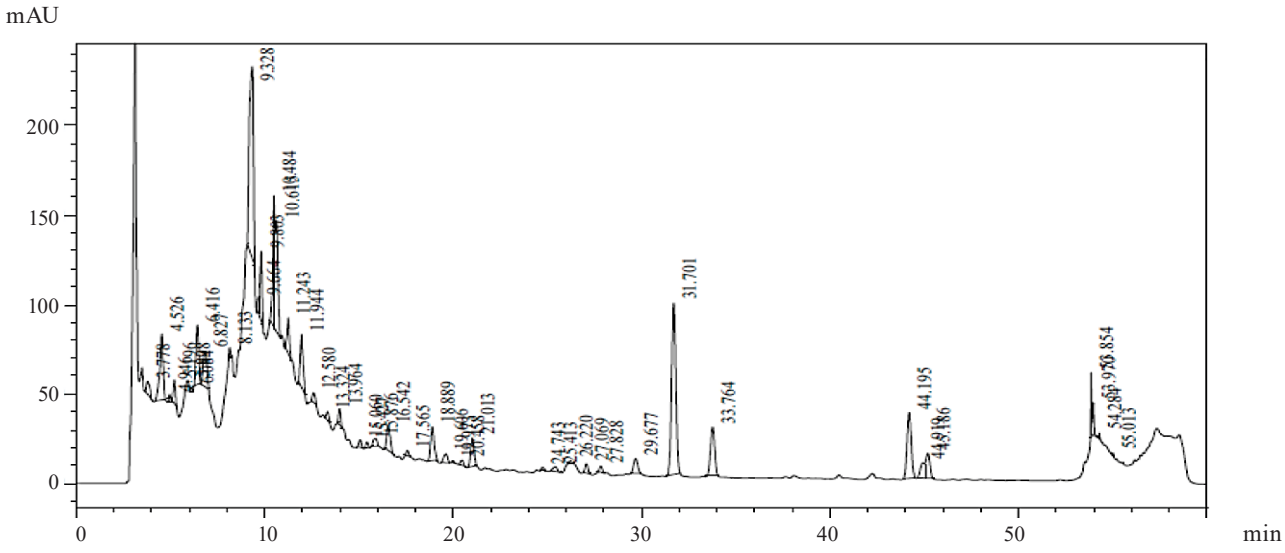

Figure 2 Chromatogram of the *Hedysarum neglectum* Ledeb. extract

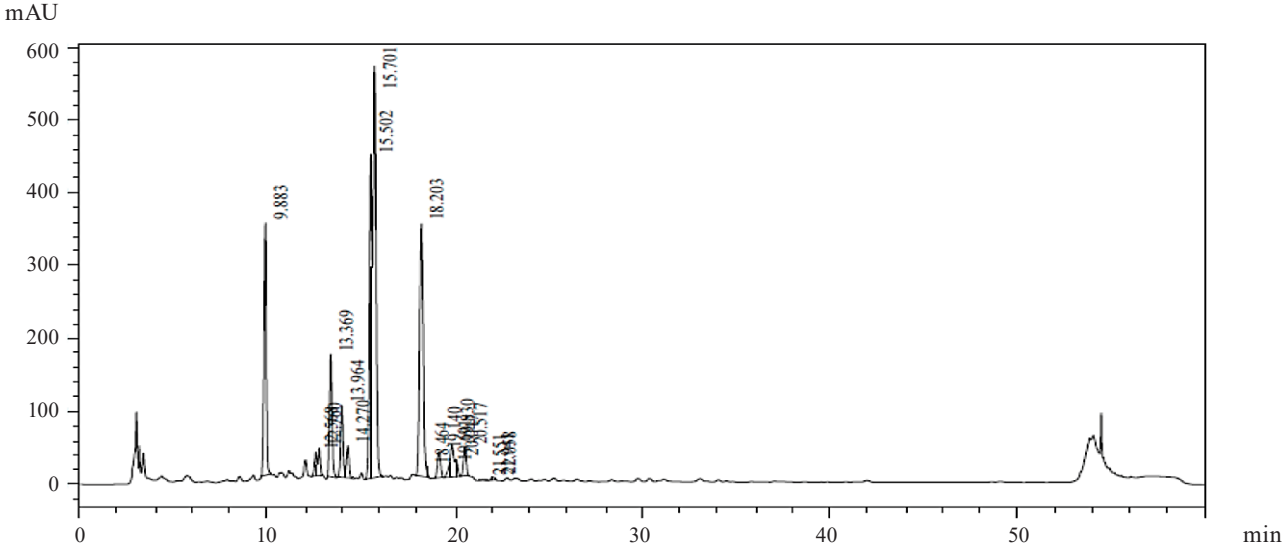

Figure 3 Chromatogram of the *Melilotus officinalis* Pall. extract

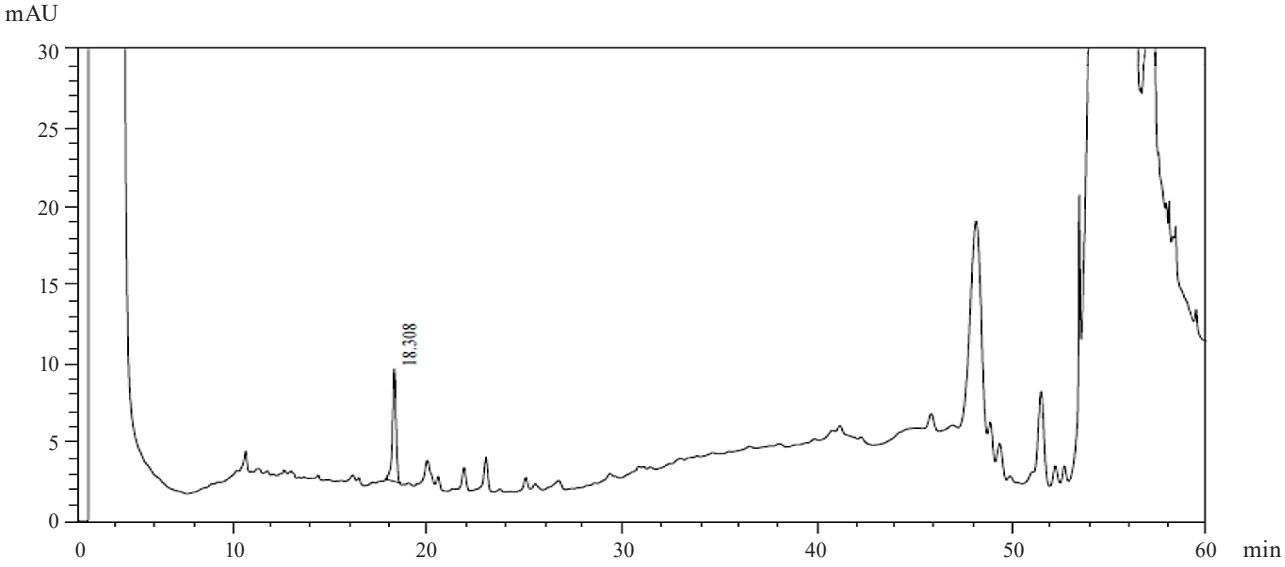

Figure 4 Chromatogram of the *Aesculus hippocastanum* L. extract

Table 1 Peaks of the IR spectra of plant extracts, cm^{-1}

Eryngium maritimum	*Hedysarum neglectum*	*Melilotus officinalis*	*Aesculus hippocastanum*
3400	3400	3400	3400
–	2950	2950	2956
2925	2925	2925	–
–	2850	2850	–
–	2370, 2353	2352	2352
2360	–	2348	2348
–	–	1725	1725
1640	1640, 1600	1650	1660, 1610
1525	–	1556	1520
1460	1475, 1430	1475	1450
1350	1360	1380	1390
1300	–	1325	–
1250	1275	1275	1275
1200	1200	–	–
1150	1160	–	–
–	1090	1090	1090
1060	1025	1050	1050
–	910, 900	900	–
820	840	–	825

Table 2 Content of phenolic components in *Eryngium maritimum* L. extracts

Biologically active substance	Retention time, min	Quantity, mg/kg
3,4-dihydroxybenzoic acid	6.007	1.133
Astragalin	25.113	80.317
Hyperoside or rutin	19.007	0.264*
Luteolin-7-glucoside	20.315	6.050
Chlorogenic acid	10.575	1.469
Rosmarinic acid	28.702	4.142

Table 3 Content of phenolic components in *Hedysarum neglectum* Ledeb. samples

Biologically active substance	Retention time, min	Quantity, mg/kg
Coumaric acid	14.270	0.460
Hyperoside or rutin	19.140	11.628*
Quercetin-3D-glucoside	20.517	10.410

* calculated for hyperoside

astragalin (peak 25.113 min, 80.31 mg per kg of plant material). In addition, 3,4-dihydroxybenzoic acid, astragalin, luteolin-7-glucoside, rosmarinic acid, and chlogenic acid were identified in the indicated fraction. In addition, peak 19.007 may correspond to hyperoside or rutin.

Coumaric acid and quercetin-3D-glucoside were identified in the obtained samples of *H. neglectum* extract (Table 3). The 19 140 peak may correspond to hyperoside or rutin. The quantitative composition of the fraction is shown in Table 1. Large peaks of 9.883, 13.369, 15.502, 15.701, and 18.203 min were unidentified (Fig. 2). Identification of trace organic compounds in multicomponent mixtures is one of the most difficult problems in

Table 4 Content of phenolic components in *Melilotus officinalis* Pall. extracts

Biologically active substance	Retention time, min	Quantity, mg/kg
Astragalin	26.220	0.506
Luteolin-7-glucoside	21.013	2.703
Apigenin-7-O-glucoside	25.413	0.626
Rosmarinic acid	27.828	0.420
Catechin	9.803	16.195

Table 5 Content of phenolic components in *Aesculus hippocastanum* L. extracts

Biologically active substance	Retention time, min	Quantity, mg/kg
3,4-dihydroxybenzoic acid	5.078	13.302
Astragalin	25.128	1.804
Apigenin-7-O-glucoside	26.675	1.496
Kaftaric acid	9.113	Traces
Chlorogenic acid	10.833	1.363
Catechin	9.709	Traces

ecoanalytical and biochemical studies, and control of impurities in process samples. In these cases, the nature of the objects excludes the possibility of preparative isolation of components and the study of individual substances. However, the spectra in many cases are insensitive to the isomerization of the carbon skeleton of molecules and conjugated systems, so some compounds remain unrecognized [23].

Astragalin, luteolin-7-glucoside, apigenin-7-O-glucoside, rosmarinic acid, and catechin were identified in the extract samples obtained from *M. officinalis* (Table 4). The extract samples of *A. hippocastanum* contained 3,4-dihydroxybenzoic acid, astragalin, apigenin-7-O-glucoside, chlorogenic acids, and kaftaric acid and catechin traces (Table 5). Major peaks (6.386, 13.451, and 28.388 min) were not identified (Fig. 4).

The peak recorded in the spectra of all the studied samples at 3400 cm^{-1} is associated with absorption due to strain vibrations of the -OH group [24]. The peaks located in the region of 2950 to 2850 cm^{-1} are attributed to the asymmetric stretch of the $-CH_2$ group [24].

The wavelength region from 4000 to 2500 cm^{-1} is not used for determining individual phenolic compounds because it contains primarily broad bands associated with stretching vibrations of hydroxyl groups, as well as bands associated with stretching vibrations of aromatic ring groups-C-H in all spectra.

The best region for identification of phenolic compounds by infrared spectroscopy is the so-called fingerprint region, which is located in the range from 1725 to 820 cm^{-1}. The absorption peak at 1725 cm^{-1} was due to the stretching vibration of the carbonyl group and was present only in the spectra of *M. officinalis* and *A. hippocastanum*. Furthermore, IR spectra of all the samples revealed peaks in the range of 1660–1600 cm^{-1}

(Figs. 5–8), which is typical of hydroxybenzoic acids and/or hydroxycinnamic acids [25].

Chlorogenic acid gives an absorption band around 1725–1720 cm^{-1}, which could be related to the, β-unsaturated aliphatic ester vibrations, which register between 1740 and 1705 cm^{-1}, or the carbonyl CO stretch of a protonated carboxylic acid [24]. Consequently, the band located at 1725 cm^{-1} may be related to the presence of this acid in the *M. officinalis*, and *A. hippocastanum* extracts. In the medicinal plant *M. officinalis*, the content

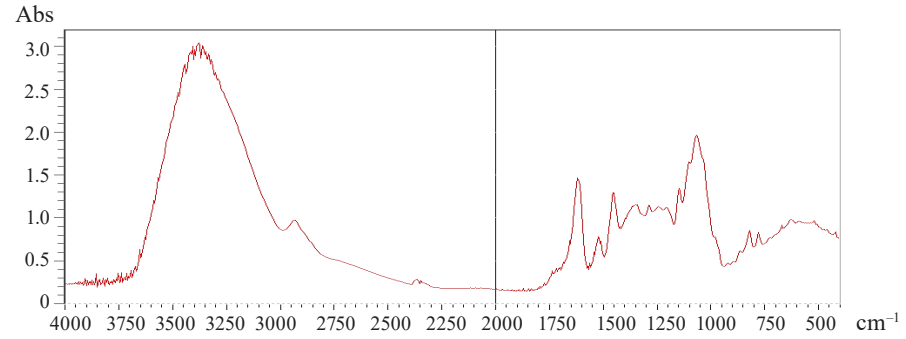

Figure 5 IR spectra of *Eryngium maritimum* L. extract

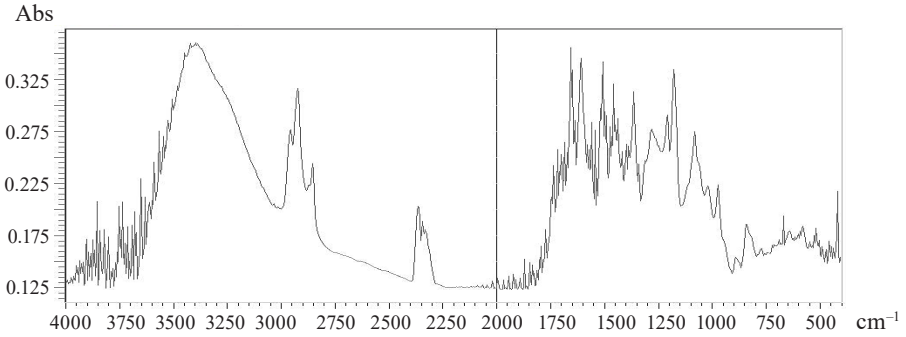

Figure 6 IR spectra of *Hedysarum neglectum* Ledeb. extract

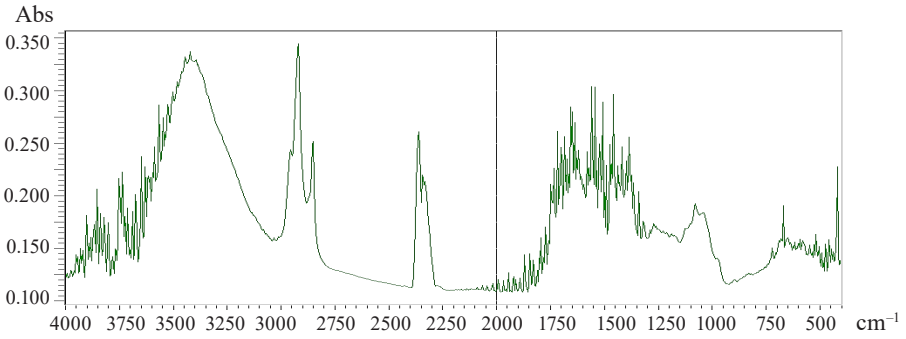

Figure 7 IR spectra of *Melilotus officinalis* Pall. extract

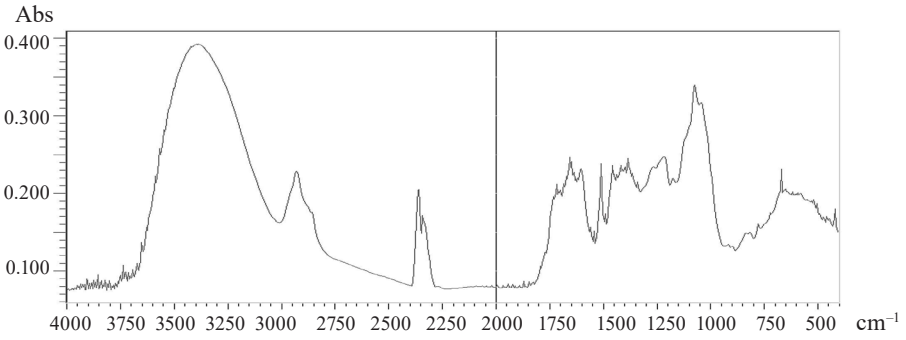

Figure 8 IR spectra of *Aesculus hippocastanum* L. extract

Table 6 Antioxidant activity of the studied medicinal extracts

Extract	Antioxidant activity, mg AA/g
Eryngium maritimum	59.53 ± 1.10
Hedysarum neglectum	55.85 ± 1.10
Melilotus officinalis	57.93 ± 1.10
Aesculus hippocastanum	53.86 ± 1.10

Table 7 Antimicrobial activity of plant extracts

Plant extract	Diameters of growth inhibition zones of test microorganisms, mm			
	Escherichia coli	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Candida albicans*
Aesculus hippocastanum	0	7.0 ± 0.2	0	0
Hedysarum neglectum	0	0	7.0 ± 0.2	9.0 ± 0.3
Eryngium maritimum	0	9.5 ± 0.3	14.3 ± 0.4	0
Melilotus officinalis	0	0	0	0
Kanamycin 50 μg	17.0 ± 0.5	12.0 ± 0.3	28.0 ± 0.8	–
Fluconazole 500 μg	–	–	–	20.0 ± 0.6

of coumarins reached 1.5–2%, among them coumarin, dihydrocoumarin, dicoumarol, etc. In the IR spectra of coumarins, as in the structure of α-pyrones, the bands of stretching vibrations of the carbonyl group lie in the region of 1750–1700 cm^{-1}. The variability depends on the structural features of coumarins.

The absorption peaks at 1200–1025 cm^{-1} were associated with C-OH, C-O-C, and C-C vibrational stretching, indicating the presence of a ring structure, a heterocyclic oxygen-containing (pyrane) ring [26].

The aromatic nature of the compounds was also confirmed by the intensive absorption of all the extracts at 900–820 cm^{-1}. The bands between 1200 and 1275 cm^{-1} represent the deformation vibrations of the C-O phenols and carboxyl group [27]. Thus, the IR spectra confirmed the presence of the biologically active substances in the extracts indicated in Tables 2–5.

Infrared spectroscopy can be used in qualitative and quantitative analysis. This method provides information on compound content in complex samples with small levels. The complexity of the information provided can be solved using chemometric methods. Chemometric methods can help in extracting information from spectra through multivariate analysis. Spectroscopy is considered a fast, time-saving, cost-effective, accurate, and non-destructive analytical tool [28]. Infrared spectroscopy has been successfully used for quantitative analysis in various fields, including pharmaceutical, food, agricultural, and biological evaluation. The root mean square error of calibration (RMSEC), root mean square error of prediction (RMSEP), root mean square error of cross-validation (RMSECV), and coefficient of determination (R^2) are used to assess the success of this method [29]. Several factors influence the good linearity and accepted standard errors of the prediction model. The choice of pretreatment spectra and variables can affect the accuracy and precision of infrared spectroscopy methods.

Analysis by infrared spectroscopy is ideal. If the evaluation results show R^2 values approaching the value of 1, low standard error (RMSEC, RMSECV, RMSEP) or the predicted square of the sum of residual errors, and relative percent difference. Values should be at least 2.4 or higher [29, 30]. Calibration R^2 values describe the linearity of the calibration curve generated from the infrared spectrum data (x-value) with these concentrations measured using the reference method (y-value). R^2 values close to 1 indicate that the infrared spectrum data are able to explain the concentration of compounds as the dependent variable. Most of the analysis of plant materials using infrared spectroscopy is performed on solid samples, which makes them prone to scattering [31–33]. Scattering causes irrelevant changes in the spectral data. If the pre-processing spectra are missing, a mixture of information and noise will occur, and this may lead to a decrease in the predictive ability of the model. Scattering typically reduces the number of mid-infrared reflectance measurements than near-infrared reflectance. This is due to the inhomogeneity of the particle size during measurement [31]. The particle size is inversely correlated with the scattering coefficient, according to studies by Otsuka [34].

The choice of spectra preprocessing methods is very important for successful calibration model generation because it can reveal or exclude important information related to the content measurement [35–37]. However, the success of the influence of spectra preprocessing on the formation of the calibration model can only be known after model validation [38–40]. It is important to compare several spectra preprocessing methods and their combinations to determine the more appropriate method for the data being analyzed. Preprocessing methods are appropriate if they reduce standard errors by reducing or minimally preserving model complexity [41–43].

The selection of CARS variables consists of three steps. The first step is to randomly sample the datasets and then the number of these variables will be reduced again using the exponential decreasing function method where the number of variables decreases rapidly. The remaining variables that lead to a low RMSECV value will be selected as informative variables included in the calibration model [44, 45]. This method can make the calibration model simpler and more efficient [35]. Unlike GA based on random selection of a subset of variables followed by calibration, the PLS model allows for the selection of the variable with the greatest influence. In addition, crosses and mutations are performed to form new variables, and recalibration is performed to decide which variable is the most appropriate [36, 37].

Our findings are consistent with those of Baltacıoğlu *et al.* [46]. When studying aqueous extracts of *E. maritimum*, it was found that such phenolic compounds and flavonoids as gallic acid, chlorogenic acid, caffeic acid, vanillic acid, catechin, rutin, quercetin and luteolin were isolated [47]. The extracts were examined by infrared spectroscopy. Phenolic compounds derived from *E. maritimum* play a very important role in the biological activities of medicinal plants, such as anti-inflammatory, antitumor, antioxidant, and antimicrobial effects [48].

We investigated the total content of phenols and flavonoids as well as the antioxidant properties of *E. maritimum*. IR analysis of *E. maritimum* extracts revealed a significant amount of phenolic compounds. The total content of phenols and flavonoids was higher in leaves than in roots and stems. This can be explained by the influence of the plant development stage on the distribution of phytocomponents. These observations are consistent with the findings of Markl *et al.* [49]. The antioxidant properties of *E. maritimum* evaluated using DPPH free radical scavenging activity showed that the *in vitro* activity is different for raw materials of different parts of the plant. The leaf extract showed the greatest antioxidant activity compared to other extracts. This agrees with the results of Nagy *et al.*, who revealed high antioxidant properties of *Erythronium caucasicum* leaf extract in comparison with the flower extract [50]. According to [46], the important antioxidant abilities of *E. maritimum* extracts were primarily due to the good correlation between phenolic and flavonoid content and antioxidant activity. This conclusion is consistent with the results of Sisay *et al.*, who have demonstrated such linear correlation [51]. In addition, the difference in the radical scavenging activity of *E. maritimum* may be related to the nature of their six phenolic components (gallic acid, catechin, chlorogenic acid, vanillic acid, caffeic acid, and cinnamic acid) and three flavonoids (rutin, quercetin, and luteolin). The therapeutic potential of these compounds has been reported in many papers [52]. For example, chlorogenic and caffeic acids are powerful antioxidants, both *in vivo* and *in vitro* [53]. Palmer *et al.* suggested that rutin, quercetin, luteolin, and caffeic acid derived from *E. maritimum* leaf extract may be responsible for their antioxidant properties [53].

A study made by Pauli *et al.* showed that gas chromatography-mass spectrometry analysis of *n*-hexane extract of *M. officinalis* revealed twelve compounds with a total peak area of 98.33%, mainly (9Z,12Z)-octadecadienoic acid (20.22%, 366 ppm), 14-methylpentadecanoic acid (19.52%, 353 ppm), and (9E)-octadecenoic acid (15.94%, 289 ppm) [54]. Two compounds, namely, *cis*-coumaric acid-2-O-*β*-D-glucopyranoside (*cis*-melilotoside, 1) and 1,2-benzopyron (coumarin, 2), were isolated from MeOH extract of *M. officinalis*. The structures of the isolated compounds were determined by spectroscopic methods such as NMR, UV-visible region, and Fourier-transform infrared spectroscopy (FTIR). The MeOH extract of *M. officinalis* was tested for its antioxidant activity using the DPPH assay. MeOH extraction of *M. officinalis* resulted in the isolation of *cis*-melilotoside and coumarin [54]. The MeOH-extract of *M. officinalis* showed an antioxidant activity of 54.06 ± 1.27 mg AA/g. In our case, extraction with methanol resulted in a value of 57.93 ± 0.70 mg AA/g of antioxidant activity of the extract. This discrepancy can be explained by the nature of the extractant. Methyl alcohol derivatives more efficiently extract BAS such as astragalin, luteolin-7-glucoside, apigenin-7-O-glucoside, rosmarinic acid, and catechin, which have increased antioxidant activity (Table 6).

According to Sacher *et al*., extraction of *M. officinalis* with chloroform resulted in the isolation of biologically active substances such as hexadecanoic acid, lupanone, lupeol, betulinic acid, oleanolic acid, and campferol-3-O-glucopyranoside, which were identified by infrared spectroscopy [55]. The antioxidant activity of these extracts was 52.38 1.2 mg AA/g, which is by 9.6% lower than the antioxidant activity of the *M. officinalis* methanol extract obtained in our work.

Zaborenko *et al*. proved the anticoagulant and antioxidant activity of ethanolic *M. officinalis* extracts [56]. It was noted that coumarin is a natural phenolic product obtained from sweet yellow clover (*M. officinalis*) by maceration in ethanol solvent. The crude extract was analyzed by high-performance liquid chromatography, then divided into two parts. The first part was characterized by FTIR and examined for anticoagulant and antioxidant activity, and the second part was fractionated by column chromatography. The results of the anticoagulant activity of the crude extract were revealed to be dose-dependent. The value of the antioxidant activity of *M. officinalis* was 53.61 ± 1.10 mg AA/g, which is by 7.5% lower than what our results. *M. officinalis* was found to be a good source of coumarin and coumarin derivatives. In addition, the advantage of FTIR analysis over column chromatography, which are considered valuable separation and characterization methods, was demonstrated [56].

Dmitrieva *et al*. described the antioxidant properties of *H. neglectum* (a forage plant) [57]. The xanthone glycoside mangiferin was extracted from this plant and

used for the drug Alpizarin. In addition to substances of xanthone nature (mangiferin and isomangiferin), *H. neglectum* contains sugars, vitamins and provitamins, tannins; the underground part contains oligomeric catechins, isoflavonoids, butylphenols, alkaloids, tannins, flavonoids, saponins, coumarins, carbohydrates, and vitamin C. The researchers have found that in order to select the optimal biologically active substances fractionation schemes, it is necessary to resort to multistage grouping schemes: broad (preliminary) isolation and preparative accumulation. In some cases, it is important to consider the presence of accompanying substances, as well as the efficiency and selectivity of the sorption and chromatographic technology used. According to the results of the authors, the use of silica gel and Sephadex LH-20 for the isolation of the flavonoid and gallic acid complex is the most effective method for selecting the optimal option for the preparative isolation of total biologically active substances in μg/mL. The results of the study enabled the isolation of the desired biologically active substances with a degree of extraction of at least 80%: xanthone, flavonoid, and gallic acid fractions. The value of the antioxidant activity of *H. neglectum* biologically active substances was 54.90 ± 1.10 mg AA/g. The data obtained in the study by Dmitrieva *et al.* are almost identical to ours (55.85 ± 1.10 mg AA/g) [57]. Slight differences are explained by differences in the approaches to determining the biologically active substances composition.

Infrared spectrometry revealed that *A. hippocastanum* aqueous extracts contain the coumarin glycosides scopolin, fraxin, and esculent [57]. A number of quercetin and camperfol flavonoid glycosides are also found in leaf tissue. Escin, as well as leucanthocyanins, *cis*-, *trans*-polyprenols, amino acids, fatty acids, and sterols were also found. According to the results of the FTIR analysis, the inhibitory effect on free radicals and oxidative processes in the human body is due to the phenolic components of *A. hippocastanum* polyols. Our results are confirmed by Küp *et al.* [58]. The value of the antioxidant activity of *A. hippocastanum* in a study by Kumar *et al.* was 53.86 ± 1.10 mg AA/g, which is by 4.0% less than in our study [59].

In a study by Konstantinovitch *et al.*, extracts of *A. hippocastanum* bark generally had weak antibacterial activity (MPC ≥ 0.625 mg/mL) and bacteriostatic potential (minimum bactericidal concentration/minimum inhibitory concentration ≥ 16) against both Gram-positive and Gram-negative bacteria, which confirms our studies [60]. Dahash *et al.* demonstrated that *A. hippocastanum* extract exhibited a significant antibacterial effect on tested Gram-negative and Gram-positive bacteria compared to negative controls: $p < 0.05$ for *Klebsiella pneumoniae* and *P. aeruginosa* and $p < 0.01$ for *E. coli*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* [61]. On the other hand, the extract had a relatively lower antibacterial effect on *K. pneumoniae*, *E. coli*, and *P. aeruginosa* compared to the positive control ($p < 0.01$), while the *A. hippocastanum* extract showed a comparable effect to the positive control for Gram-positive *S. epidermidis*, $p = 0.05$. In addition, the extract showed a concentration-dependent antibacterial effect against Gram-positive *S. epidermidis* and *S. aureus* compared to different concentrations ($p < 0.01$). The minimum inhibitory concentration and minimum bactericidal concentration of *A. hippocastanum* extract were high for Gram-negative bacteria and low for Gram-positive bacteria compared to the minimum inhibitory concentration of positive controls.

According to Ehsani *et al.*, aromatic *M. officinalis* is high in essential oils with antimicrobial properties [62]. The chemical components of essential oils were identified using chromatography-mass spectrometry analysis, and the antimicrobial activity of essential oils was evaluated using disk diffusion analysis, as well as determining the minimum inhibitory concentration and minimum bactericidal concentration against four important food bacteria: *Salmonella typhimorium*, *E. coli*, *Listeria monocytogenes*, and *S. aureus.* The key results revealed that *M. officinalis* has a strong antimicrobial effect on the bacteria tested. *S. aureus* with the lowest MPC value (0.12 mg/mL) was the most sensitive bacterium, although the antibacterial effect of *M. officinalis* was quite strong [63, 64].

Mosyagin *et al.* indicated that medicinal plants used in cosmetology are subject to mandatory quality control [65]. Physical and chemical methods of research are the most common. A comparative study of mint (*Mentha piperita* L.), thyme (*Thymus serpyllum* L.), and celandine (*Chelidonii herba* L.) infusions was performed using infrared spectroscopy for the first time. The results of mid-wave spectroscopy revealed infrared absorption peaks in different wavelength ranges for infusions of different medicinal plants. Thus, they amounted to 2900.41, 2830.12, 2123.51, and 1223.12 cm^{-1} for the gel based on thyme infusion; 3527.17, 2879.64, 2822.89, 1449.25, 1393.66, 1290.91, 1116.51, and 1083.03 cm^{-1} for celandine; and 3863.60, 3724.47, 2904.68, 2835.10, 1453.00, 1271.44, 1111.11, 1102.16, and 1034.30 cm^{-1} for mint.

The data obtained in the infrared spectrum of medicinal plant infusions allowed for the identification of stretching bands of carbonyl, hydroxyl, carboxyl, methyl, ether, acetal groups, compounds with aromatic ring, heterocyclic rings, in particular pyridine and quinoline rings, as well as mineral substances containing mainly phosphorus and sulfur compounds.

These groups and compounds are typical of some chemical compounds with biological activity (terpenoids, carotenoids, alkaloids, flavonoids, vitamins, acetylsalicylic, ursolic, oleanolic, and other organic acids and compounds). The results of the study can be used as reference identification IR spectra for rapid identification of medicinal plant infusions. The use of FTIR spectroscopy to study the chemical composition of medicinal plant infusions can have a wide practical value as a method of rapid analysis of medicinal plant infusions [65, 66].

CONCLUSION

This study examined *Eryngium maritimum* L., *Hedysarum neglectum* Pall., *Melilotus officinalis* Ledeb., and *Aesculus hippocastanum* L. extracts. IR spectroscopy determined that the main component of *E. maritimum* extract samples was astragalin. Also, 3,4-dihydroxybenzoic acid, astragalin, luteolin-7-glucoside, rosmarinic acid, and chlorogenic acid were identified in the extract. In addition, peak 19.007 may correspond to hyperoside or rutin. Coumaric acid and quercetin-3D-glucoside were identified in the *H. neglectum* extract. Peak 19.140 may correspond to hyperoside or rutin. Large peaks of 9.883, 13.369, 15.502, 15.701, and 18.203 min were unidentified.

Astragalin, luteolin-7-glucoside, apigenin-7-O-glucoside, rosmarinic acid, and catechin were identified in *M. officinalis* extract samples. The extract samples of *A. hippocastanum* contained 3,4-dihydroxybenzoic acid, astragalin, apigenin-7-O-glucoside, kaftaric and chlorogenic acids, and catechin. The peak recorded in the spectra of all the studied samples at 3400 cm^{-1} is associated with absorption due to deformation vibrations of the -OH group. The absorption peak at 1725 cm^{-1} was due to the tensile vibration of the carbonyl group and was present only in the spectra of *M. officinalis* and *A. hippocastanum*. Furthermore, the IR spectra of all samples recorded peaks in the range 1660–1600 cm^{-1}, which is typical of hydroxybenzoic acids and/or hydroxycinnamic acids.

We suggeste that phenolic compounds contained in *E. maritimum*, *H. neglectum*, *M. officinalis*, and *A. hippocastanum* extracts may be responsible for their antioxidant properties. Thus, the results indicated that these plant extracts might be recommended as natural antioxidants required to treat diseases caused by the presence of free radicals. However, further studies are required to determine which of the individual phenolic compounds in the *E. maritimum*, *H. neglectum*, *M. officinalis*, and *A. hippocastanum* leaves are involved in exhibiting antioxidant activity.

The plant extract of *H. neglectum* was found to be active against the bacteria *Bacillus subtilis* (diameter of the inhibition zone was 7.0 mm) and the mold fungi *Candida albicans* (diameter of the inhibition zone was 9.0 mm); the plant extract of *E. maritimum* was found to be detrimental to both Gram-positive and Gram-negative bacteria growth and development. It was found that *E. maritimum* extract had no fungicidal activity (test strain *C. albicans*).

Infrared spectroscopy is useful for the quantification of phytochemical components and impurities in plant-derived medicines and supplements. This analysis can help in further studies to determine the quality of nutritious plants to ensure the safety and efficacy of plant-based products. It has been found that chemometrics can overcome the complexity of the chemical composition of herbs. Optimization of preprocessing methods and variable selection and their combination are usually valuable in improving the predictive ability of models. This is indicated by an increase in the linearity value (in calibration and validation) and a decrease in the standard error in the model on spectra that undergo preprocessing, variable selection, and a combination of both compared to unprocessed spectra. A validation value (close to 1) indicates that the measurement of compound concentrations using the FTIR method gives an accuracy proportional to the measurement results of the reference method.

CONTRIBUTION

Conceptualization: O.O. Babich and S.A. Sukhikh; methodology and analyzed and interpreted the data: I.G. Samsuev, A.V. Tcibulnikova, E.S. Zemlyakova, V.A. Larina and A.D. Popov; formal analysis: O.O. Babich, S.A. Ivanova, S.Yu. Noskova and S.A. Sukhikh; writing, review and editing: O.O. Babich, S.A. Ivanova and S.A. Sukhikh; project administration: S.A. Sukhikh.

CONFLICT OF INTEREST

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

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Seasonal changes in the fatty acid profile of Kyrgyz khainak milk

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Abstract:
Khainak milk is a traditional source of nutrition for people living in the highlands of Kyrgyzstan. It is consumed both in its natural form and in the form of butter, cheese, and cottage cheese. We aimed to determine the composition of fatty acids in khainak milk, as well as its seasonal changes, since such data is lacking in literature.
Fatty acids were determined by gas chromatography qualitatively and quantitatively in the milk from five lactating khainaks farm-bred in the Issyk-Kul region. The milk samples were collected and analyzed in the spring, summer, autumn, and winter seasons over three years (2019, 2020, and 2021).
Kyrgyz khainak milk fat was mostly represented by saturated fatty acids, with a maximum content of 73.10 ± 2.19 g/100 g in winter. C14:0, C16:0, and C18:0 dominated in their composition, exceeding 5 g/100 g, with C16:0 (palmitic acid) reaching almost 35 g/100 g in winter. The flora of mountain pastures favorably contributed to monounsaturated fatty acids in khainak milk, especially oleic acid, whose content reached 26.85 ± 0.81 g/100 g in spring and then gradually declined to 18.90 ± 0.56 g/100 g, following changes in vegetation. Polyunsaturated fatty acids were found in small quantities varying from 3.25 ± 0.09 g/100 g in winter to 4.28 ± 0.12 g/100 g in summer.
The seasonal changes in the fatty acid profile of Kyrgyz khainak milk are most likely due to differences in the animals' diet. Our data can be used to optimize the process parameters for the production of full-fat products from khainak milk (cheese, butter, sour cream, etc.).

Keywords: Milk, khainak, season, fatty acids, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, gas chromatography

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INTRODUCTION

Kyrgyzstan has large highland pastures at an altitude of 2500 m or more above sea level which are only suitable for yaks (*Bos grunniens* L.) or their hybrids to graze. Other types of domestic animals are almost impossible to keep under these climatic and forage conditions [1, 2]. There are currently about 50 000 yaks in Kyrgyzstan and they are the main source of meat and milk for local farmers. While yak meat can be bought in the city markets, there are very few products made from yak milk. Yaks are kept in plain-like areas in winter and moved to alpine meadows (syrts) to graze in the summer months. Such transhumance is determined mainly by the climate rather than a range of grass species, since lowlands with hot temperatures are uncomfortable for yaks [3].

In addition to breeding yaks, farmers are increasingly crossing them with cattle (*B. grunniens* × *Bos taurus*), and the resulting hybrids show clear signs of heterosis, or hybrid vigor [4]. The hybrids in the first generation are referred to as "khainags" in Mongolia and Buryatia, "khainaks" in Tajikistan, "zo" in Pakistan, and "dzo" in Tibet [5]. In Kyrgyzstan, khainaks are called "argyns" or "artyns" (Fig. 1), which means a "hybrid". In our

Figure 1 Cattle-yak hybrids bred in the Issyk-Kul region of Kyrgyzstan

study, we used the term "khainak" as the most common term, as well as the term "Kyrgyz khainak" to show regional affiliation.

Similarly to yaks, khainaks graze in mountain pastures and do not require any special care. However, unlike yaks, they can withstand higher temperatures (30–32°C) and survive in a wider range of mountain zones (2000–5000 m above sea level), not only alpine ones. Khainaks are important livestock species providing highlanders with meat, fur, fuel (manure), and other resources. They are just as good as cows in terms of milk yield, or even better. Yaks and their crossbreeds produce 300 and 470 kg of milk, respectively [6]. Yak milk is twice as rich in fat as cow milk (7.22 and 3.55 g/100 cm^3, respectively) [7]. The quality of khainak milk is also better than that of cows, with a higher content of fat, protein, calcium, and phosphorus [8–10]. Recently, there has been growing interest in ethnic foods and alternative dairy products with functional properties, for example, yak and khainak milk products in Kyrgyzstan [11].

Yak milk is rich in a variety of bioactive substances [6]. Although Tibetans live on the highest plateau in the world and therefore should be prone to altitude diseases such as premature aging, edema, atherosclerosis, or carcinogenesis, there have been very few reports of their health problems [12]. This may be due to their diet consisting mostly of yak milk products: butter, yogurt, cheese, and kula (skimmed, fermented, and powdered milk) [13, 14].

Due to grazing conditions, yak milk fat may contain some unique fatty acids lacking in the milk of other mammals [15]. Therefore, there is an urgent need for studying the milk of animals found in mountain regions. A lot of research has been done by Chinese scientists, since more than 13 million domestic yaks live on the Qinghai-Tibetan Plateau in China [16].

The composition and content of fatty acids are two main factors that determine the quality of yak milk and its products, especially butter [15].

Since yaks and khainaks usually feed on natural pastures, the quantity and quality of their milk depend on the season and feeding conditions [17].

Landscape description. The Issyk-Kul region is located in the northeastern part of the Kyrgyz Republic and covers an area of 43 100 km^2. Its climate is affected by Lake Issyk-Kul, a large ice-free lake, and has some marine features such as mild winters, relatively warm summers, and smoothed annual temperatures. The mountain steppe accounts for 40.4% of the landscape, dominating in all of its subclasses (high-mountain, mid-mountain, low-mountain, inter-mountain basin, and mountain-valley). The landscape is formed by bunch-grass steppes: feather grass, ptilagrostis, white grass, and fescue. Intensive livestock grazing changes the vegetation of mountain steppes, reducing grass crops and forbs. The mountain steppe is dominated by turf grasses, including *Kobresia capilliformis*, *Ptilagrostis*, *Festúca valesiáca*, *Cárex*, and *Festuca tianschanica*. The upper parts of the slopes have higher moisture and therefore their meadow-steppes are rich in various forbs and tall-grass plants, particularly *Hordeum*, *Leucopoa albida*, and *Kobresia* [18].

The fat content in the milk of yaks found on the Qinghai-Tibetan Plateau is determined by the season, amounting to 6.2% during the vegetative stage of plants (May), 5.4% in the flowering stage (August), and 6.7% in the resting stage (December) [19]. Total fat in yak milk ranges from 5.6 to 8.6 g/100 g [20].

There are a number of studies on seasonal changes in fatty acids in the milk of different mammals, but little is known about the effect of the season on the fatty acid profile of khainak milk [21]. Liu *et al.* used gas chromatography to determine fatty acids in the milk of yaks found on the Qinghai-Tibet Plateau at different times of the year [15]. They found that the concentrations of *cis*-9 C18:1, *cis*-11 C18:1, *cis*-9, *trans*-11 C18:2, and C18:3n-3 were higher in summer (25.00, 26.00, 1.50, 1.46, and 0.33 g/100 g of total fatty acids, respectively) than in winter (22.00, 17.00, 0.77, 1.27, and 0.28 g/100 g of total fatty acids, respectively). Ding *et al.* confirmed that the summer season, when animals graze on green pastures, ensures the best fatty acid profile and highest milk yield. In another study, the contents of polyunsaturated fatty acids and conjugated linoleic acids in the

milk of different species in India increased during the winter, which may be due to the seasonal availability of green feed [22, 23]. However, saturated fatty acids were by 13–14% higher in the summer. On the whole, the contents of monounsaturated fatty acids, polyunsaturated fatty acids, and conjugated linoleic acids were significantly altered by the season and the milk type. According to Marquardt *et al.*, the location of pastures at a certain altitude above sea level influences the fatty acid composition of milk [24]. In particular, the contents of oleic, linoleic, and linolenic acids in ghee were the lowest in the highest pasture (4500 m), with the highest concentrations of condensed tannins in feed.

We aimed to study seasonal changes in the fatty acid composition of milk from khainaks living in the highlands of Kyrgyzstan in order to identify the best period for obtaining a biologically complete product. We know of no studies to date that provide such information.

STUDY OBJECTS AND METHODS

Chemicals and reagents. Methanol and sodium methoxide for lipid transmethylation and hexane for chromatography were obtained from VWR International (USA). A standard solution of fatty acid methyl esters was purchased from Sigma-Aldrich (USA).

Milk collection. For research the milk of khainaks (first-generation hybrids) bred by farms in the Issyk-Kul region of the Kyrgyz Republic (N420432, E771827) was used. Milk was sampled in the Spring (May), Summer (August), Autumn (October), and Winter (December) of 2019, 2020, and 2021. The lactating khainaks (n = 5) had the same calving periods and were kept under pasture conditions. Milk samples (n = 3) were collected into clean containers. The samples were filtered through a fabric filter and poured into special sterile bags. They were frozen immediately after sampling and stored at −18°C. The frozen milk samples were delivered in insulated containers to the laboratory for analysis.

Sample preparation for fatty acid analysis. Milk samples were thawed at 4–6°C for at least 12 h. To extract total lipids, 100 cm^3 samples were placed in 50-cm^3 tubes and centrifuged at 10 000 min^{-1} for 15 min. The separated fat fraction was transferred to a 250-cm^3 glass and blended with 150 cm^3 of hexane for 1 min. The separated hexane layer with dissolved fat was transferred to a 25-cm^3 round-bottomed flask to distill the solvent off at 70 ± 2°C. The resulting fat fraction was used to prepare fatty acid methyl esters.

Fatty acid methyl esters were prepared using a 2 M solution of sodium methoxide in methanol. For this, 0.10 ± 0.02 g of the fat fraction was sampled into a test tube and dissolved in 2 cm^3 of hexane. Then, 0.1 cm^3 of sodium methoxide in methanol was added to the resulting solution. After vigorous stirring for 2 min, the reaction mixture was left for 5 min and then centrifuged. The resulting solution was used for analysis immediately after preparation.

Determination of fatty acids. The composition of fatty acids was determined by using a Cristallux 4000M gas chromatographer equipped with a flame ionization detector (Meta-Chrome, Russia). Fatty acid methyl esters were separated on a Supelco SP2560 capillary column (100 m long, 0.25 mm internal diameter, 0.20 μm film thickness) (Sigma-Aldrich, USA), with an HP 6890 injector. The parameters of gas chromatography were as follows:

– injected sample: 1 mm^3;
– flow division: 1:10;
– initial column temperature: 140°C;
– isothermal part: 5 min;
– temperature increase at 4°C/min to 240°C (analysis for 50 min);
– detector temperature: 260°C;
– carrier gas: nitrogen (constant flow of 1 cm^3/min).

Fatty acid methyl esters were identified by comparing the retention times of peaks obtained with a standard Supelco 37 Component FAME mix (Sigma-Aldrich, USA).

Method validation. Linearity and operating ranges were assessed by dividing working fatty acid methyl ester standards from 1 to 200 μg/cm^3. Response factors for quantifying individual fatty acids were determined by comparing the standard and the test solutions. Individual fatty acid percentages were calculated using the chromatograph's software.

The method was validated according to the guidelines for validating chromatographic methods (ICH 2014). For this, we calculated the response linearity, range, detection and quantification limits, as well as precision. The accuracy of the quantitative method was assessed through the repeatability (n = 2) and reproducibility (n = 1) of the experiment.

Statistical analysis. Microsoft Office Excel 2010 was used for statistical processing. An arithmetic mean of two measurements was taken as a measurement result. Statistical significance of differences between data samples was determined by confidence intervals. Differences between means were compared at a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

Fatty acid methyl esters were identified by comparing the retention times of the peaks obtained by injecting a standard mix of methyl esters. Figure 2 presents a typical chromatogram for the separation of fatty acid methyl esters from khainak milk.

In particular, Fig. 2 shows the fatty acid methyl esters detected and identified with acceptable resolution. The mass fraction of fat in khainak milk was 4.50 ± 0.07, 5.00 ± 0.07, 5.20 ± 0.07, and 5.70 ± 0.14 g/100 g of milk in spring, summer, autumn, and winter, respectively.

The seasonal changes in the fatty acid composition of fat from khainak milk are presented in Table 1.

As can be seen from Table 1, khainak milk contained a significant amount (4.37%) of short-chain butyric acid ($p < 0.05$), which changed slightly in summer, autumn, and winter. According to [26], butyric acid (C4:0) is the most common short-chain fatty acid in yak, cow, sheep, and goat butter. One of its main functions is to

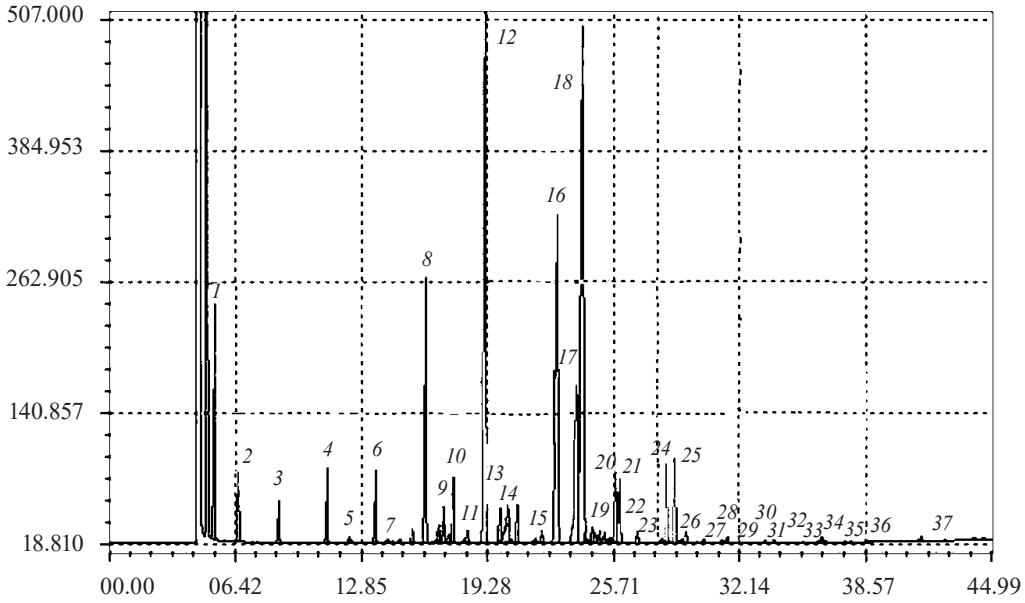
stimulate the production of epithelial mucin-2. Also, butyric acid reduces inflammation and lowers the pH of the colon, promoting beneficial bacteria and increasing mineral absorption [27, 28]. Short-chain fatty acids improve glucose, lipid, and energy metabolism. They also exhibit antimicrobial properties and reduce the risk of gastrointestinal disorders, certain cancers, and cardiovascular diseases [29, 30].

Caproic acid C6:0 dominated among the middle-chain fatty acids found in the milk of the Kyrgyz khainak. In the autumn, its content exceeded 3 g/100 g of total fatty acids. Like short-chain fatty acids, middle-chain fatty acids are involved in several regulatory and signaling functions of the human body.

According to Table 1, saturated fatty acids C14:0, C16:0, and C18:0 dominated in khainak milk, with a content of over 5 g/100 g. Palmitic acid (C16:0) reached almost 35 g/100 g of total fatty acids in the winter time.

The content of very-long-chain fatty acids was slightly higher than 1 g/100 g of total fatty acids. It hardly changed throughout the year.

Saturated fatty acids had a higher content in winter (73.10 ± 2.19 g/100 g of total fatty acids) compared to the other seasons. Their increase is mainly associated with changes in myristic (C14:0) and palmitic (C16:0) acids (8.50 ± 0.25 and 33.57 ± 0.99%, respectively). Khainak spring milk had a lower content of saturated fatty acids (59.32 ± 1.77%) than cow milk (62.60 ± 0.78%) [31]. The



1 Methyl butyrate (C4:0)
2 Methyl hexonoate (C6:0)
3 Methyl octonate (C8:0)
4 Methyl decanoate (C10:0)
5 Methyl undecanoate (C11:0)
6 Methyl dodecanoate (C12:0)
7 Methyl tridecanoate (C13:0)
8 Methyl myristate (C14:0)
9 Methyl myristoleate (C14:1)
10 Methyl pentadecanoate (C15:0)
11 Methyl *cis*-10 pentadecanoate (C15:1)
12 Methyl palmitate (C16:0)
13 Methyl palmitoleate (C16:1)
14 Methyl heptadecanoate (C17:0)
15 Methyl *cis*-10 heptadecanoate (C17:1)
16 Methyl stearate (C18:0)
17 Methyl *trans*-9 eladiate (C18:1n-9t)
18 Methyl *cis*-9 oleate (C18:1n-9c)
19 Methyl linolelaidate (18:2n-6t)
20 Methyl linoleate (18:2n-6c)
21 Methyl arachidate (C20:0)
22 Methyl-gamma-linoleate (18:3n-6)
23 Methyl *cis*-11-eicosenoate (C20:1)
24 Methyl linolenate (C18:3n-3)
25 Methyl heneicosanoate (C21:0)
26 Methyl *cis*-11,14-eicosadienoate (C20:2)
27 Methyl behenate (C22:0)
28 Methyl *cis*-8,11,14-eicosatrienoate (C20:3n-6)
29 Methyl erucate (C22:1n-9)
30 Methyl *cis*-11,14,17-eicosatrienoate (C20:3n-3)
31 Methyl *cis*-5,811,14-eicosatetraenoate (C20:4n-6)
32 Methyl tricosanoate (C23:0)
33 Methyl *cis*-13,16-docosadienoate (C22:2)
34 Methyl *cis*-5,8,11,14,17-eicosapentaenoate (C20:5n-3)
35 Methyl lignocerate (C24:0)
36 Methyl nervonate (C24:1)
37 Methyl *cis*-4,7,10,13,16,19-docosahexaenoate (C22:6n-3)

Figure 2 Chromatogram for separation of fatty acid methyl esters lipid fraction from Kyrgyz khainak milk (4.5% fat)

Table 1 Fatty acid composition (g/100 g of total fatty acids) of milk from khainaks, cows, and yaks by season

Fatty acids	Khainak raw milk (own data)					Cow milk [25]	Yak milk [25]
	Spring (May)	Summer (August)	Autumn (October)	Winter (December)	Mean		
C4:0	4.25 ± 0.12[a]	3.63 ± 0.11[a]	3.74 ± 0.11[a]	3.57 ± 0.11[b]	3.79 ± 0.11	1.23 ± 0.02	2.05 ± 0.03
C6:0	2.03 ± 0.06[b]	2.36 ± 0.07[b]	3.09 ± 0.09[b]	2.10 ± 0.06[b]	2.39 ± 0.07	1.75 ± 0.03	3.42 ± 0.05
C8:0	0.79 ± 0.02[a]	1.10 ± 0.03[b]	1.69 ± 0.05[c]	0.94 ± 0.03[a]	1.13 ± 0.03	1.20 ± 0.02	1.61 ± 0.01
C10:0	0.96 ± 0.03[a]	1.62 ± 0.05[a]	2.29 ± 0.07[a]	1.78 ± 0.05[a]	1.91 ± 0.05	2.15 ± 0.02	2.03 ± 0.04
C12:0	1.07 ± 0.03[a]	1.85 ± 0.05[b]	1.24 ± 0.04[a]	1.67 ± 0.05[a]	1.45 ± 0.04	2.42 ± 0.03	1.34 ± 0.01
C13:0	0.070 ± 0.002[a]	0.210 ± 0.006[a]	0.120 ± 0.003[a]	0.060 ± 0.002[a]	0.110 ± 0.003	0.14 ± 0.01	0.12 ± 0.01
C14:0	5.11 ± 0.15[b]	7.75 ± 0.23[b]	7.82 ± 0.23[c]	8.50 ± 0.25[a]	7.29 ± 0.21	10.00 ± 0.19	8.10 ± 0.13
C15:0	1.26 ± 0.03[a]	1.43 ± 0.04[a]	1.27 ± 0.03[a]	1.83 ± 0.05[a]	1.45 ± 0.04	1.19 ± 0.01	1.76 ± 0.02
C16:0	21.64 ± 0.64[b]	24.37 ± 0.73[c]	27.48 ± 0.82[a]	33.57 ± 0.99[a]	26.76 ± 0.80	27.80 ± 0.28	25.50 ± 0.17
C17:0	0.98 ± 0.02[b]	0.87 ± 0.02[b]	1.32 ± 0.04[b]	1.13 ± 0.03[b]	1.07 ± 0.03	0.65 ± 0.02	0.89 ± 0.02
C18:0	17.47 ± 0.52[a]	16.77 ± 0.50[a]	15.05 ± 0.45[a]	15.96 ± 0.47[a]	16.31 ± 0.48	13.50 ± 0.13	15.00 ± 0.26
C20:0	0.320 ± 0.009[a]	0.180 ± 0.005[b]	0.63 ± 0.02[a]	0.280 ± 0.008[a]	0.35 ± 0.01	0.17 ± 0.01	0.40 ± 0.00
C21:0	1.98 ± 0.06[c]	2.21 ± 0.06[c]	1.96 ± 0.06[c]	1.45 ± 0.04[c]	1.90 ± 0.06	0.03 ± 0.00	0.06 ± 0.00
C22:0	1.18 ± 0.03[b]	0.090 ± 0.003[b]	0.190 ± 0.006[a]	0.110 ± 0.003[a]	0.39 ± 0.01	0.08 ± 0.00	0.27 ± 0.01
C23:0	0.090 ± 0.002[a]	0.080 ± 0.002[a]	0.050 ± 0.001[a]	0.050 ± 0.001[a]	0.070 ± 0.002	0.02 ± 0.00	0.12 ± 0.00
C24:0	0.120 ± 0.003[a]	0.100 ± 0.003[a]	0.200 ± 0.006[a]	0.100 ± 0.003[a]	0.130 ± 0.004	0.04 ± 0.00	0.11 ± 0.00
Total SFAs*	59.32 ± 1.77	64.62 ± 1.93	68.14 ± 2.04	73.10 ± 2.19	66.28 ± 1.98	62.60 ± 0.78	65.90 ± 0.77
C14:1	0.180 ± 0.005[b]	0.41 ± 0.01[b]	0.59 ± 0.02[a]	0.77 ± 0.02[a]	0.48 ± 0.01	–	–
C16:1	1.37 ± 0.04[c]	1.26 ± 0.04[a]	2.27 ± 0.07[a]	0.070 ± 0.002[a]	1.24 ± 0.03	0.94 ± 0.03	1.07 ± 0.03
C18:1n-9, *trans*	7.89 ± 0.23[b]	6.86 ± 0.21[b]	4.52 ± 0.13[b]	3.74 ± 0.11[c]	5.75 ± 0.17	1.09 ± 0.01	3.06 ± 0.03
C18:1n-9, *cis*	26.85 ± 0.81[a]	23.32 ± 0.69[a]	20.82 ± 0.62[a]	18.90 ± 0.56[a]	24.47 ± 0.67	28.7 ± 0.18	20.81 ± 0.21
C20:1n-9, *cis*-11	0.180 ± 0.005[a]	0.200 ± 0.005[b]	0.370 ± 0.011[a]	0.080 ± 0.002[b]	0.210 ± 0.006	0.03 ± 0.00	0.03 ± 0.00
C22:1n-9	0.010 ± 0.001[c]	0.030 ± 0.001[c]	0.080 ± 0.002[c]	0.080 ± 0.002[c]	0.050 ± 0.001	0.01 ± 0.00	0.03 ± 0.00
C24:1n-9	< 0.001[a]	0.001 ± 0.000[a]	0.001 ± 0.000[b]	0.001 ± 0.000[c]	0.001 ± 0.000	0.01 ± 0.00	0.03 ± 0.00
Total MUFAs*	36.48 ± 1.09[a]	32.08 ± 0.96[a]	28.65 ± 0.85[a]	23.64 ± 0.71[a]	30.21 ± 0.90	33.00 ± 0.30	27.00 ± 0.32
C18:2n-6, *trans*	0.33 ± 0.01[b]	0.33 ± 0.01[b]	0.36 ± 0.01[b]	0.25 ± 0.01[c]	0.31 ± 0.01	0.13 ± 0.01	0.18 ± 0.01
C18:2n-6, *cis*	1.90 ± 0.05[b]	1.49 ± 0.04[b]	1.42 ± 0.04[b]	1.62 ± 0.05[b]	1.61 ± 0.05	1.73 ± 0.01	1.62 ± 0.03
C18:3n-3 (ALA)	1.78 ± 0.05[a]	1.36 ± 0.04[a]	1.32 ± 0.04[a]	1.25 ± 0.04[a]	1.42 ± 0.04	0.16 ± 0.01	1.37 ± 0.01
C20:3n-3, *cis*-11,14,17	0.020 ± 0.001[c]	0.010 ± 0.001[c]	0.010 ± 0.001[c]	0.020 ± 0.001[c]	0.015 ± 0.001	0.01 ± 0.00	0.02 ± 0.00
C20:3n-6, *cis*-8,11,14	0.020 ± 0.001[a]	0.010 ± 0.001[b]	0.020 ± 0.001[b]	0.020 ± 0.001[a]	0.017 ± 0.001	0.09 ± 0.00	0.02 ± 0.00
C20:4n-6 (ARA)	0.120 ± 0.003[a]	0.040 ± 0.001[a]	0.040 ± 0.001[b]	0.030 ± 0.001[b]	0.060 ± 0.001	0.19 ± 0.01	0.16 ± 0.01
C20:5n-3 (EPA)	0.070 ± 0.002[b]	0.040 ± 0.001[b]	0.030 ± 0.001[c]	0.050 ± 0.001[a]	0.050 ± 0.001	0.01 ± 0.00	0.08 ± 0.00
C22:6n-3 (DHA)	0.030 ± 0.001[a]	0.010 ± 0.001[a]	0.030 ± 0.001[c]	0.010 ± 0.001[a]	0.020 ± 0.001	0.01 ± 0.00	0.04 ± 0.00
Total PUFAs*	4.28 ± 0.12	3.29 ± 0.09	3.23 ± 0.09	3.25 ± 0.09	3.51 ± 0.10	3.05 ± 0.06	4.91 ± 0.09
SCFAs	8.03 ± 0.24	8.71 ± 0.26	10.81 ± 0.32	8.39 ± 0.25	8.98 ± 0.26	–	–
MCFAs*	30.62 ± 0.92	37.28 ± 1.11	40.76 ± 1.22	46.47 ± 1.39	38.81 ± 1.16	–	–
LCFAs*	59.92 ± 1.84	53.69 ± 1.62	47.87 ± 1.45	44.78 ± 1.35	52.22 ± 1.56	–	–
VLCFAs*	1.42 ± 0.04	0.31 ± 0.01	0.55 ± 0.02	0.35 ± 0.01	0.66 ± 0.02	–	–

* SFAs – saturated fatty acids; MUFAs – monounsaturated fatty acids; PUFAs – polyunsaturated fatty acids; SCFAs – short-chain fatty acids; MCFAs – middle-chain fatty acids; LCFAs – long-chain fatty acids; VLCFAs – very-long-chain fatty acids

Means ± SD with the same letter are not significantly different at $p = 0.05$

variations in the saturated fatty acid contents in Kyrgyz khainak milk (59.32–73.10%) were similar to those reported by Liu *et al.* for yak milk (67.78–70.67%) [15]. The total saturated fatty acids in khainak milk were higher than in cow and yak milk. Saturated fatty acids have long been associated with cardiovascular disease, leading to reduced consumption of saturated fatty acid-rich foods such as butter [17]. However, many authors have reported a lack of strong evidence to support the association of saturated fatty acids with cardiovascular disease and found their protective effects against stroke [19]. Therefore, moderate consumption of foods rich in saturated fatty acids is essential for human health. Milk fat is the main dietary source of saturated fatty acids for humans.

Oleic acid (C18:1n-9, *cis*) is the most abundant monounsaturated fatty acid in the milk of ruminants, including yak [25, 27]. In our study, the content of oleic acid reached 26.85 ± 0.81 g/100 g of total fatty acids in spring, gradually decreasing to 18.90 ± 0.56 g/100 g as the pas-

ture vegetation changed, which was consistent with the general pattern [32]. Oleic acid is one of the most essential fatty acids for human metabolism. It regulates blood pressure, prevents diabetes, and has antioxidant properties [33, 34].

Evidence suggests that the fatty acid composition of milk is independent of the stage of lactation, so its differences may depend on the geographic region and a variety of grasses growing there [35]. We found that in the spring and summer periods, fresh subalpine grasses contributed to high concentrations of oleic (C18:1 *cis* – 26.85 and 23.32%, respectively) and vaccenic (C18:1 *trans* – 7.89 and 6.86%, respectively) acids in the milk of the Kyrgyz khainak.

Polyunsaturated fatty acids are vital components for normal body growth, the development of the nervous system and immune functions, as well as for preventing cardiovascular diseases [36, 37]. Of particularly high biological value are n6 polyunsaturated fatty acids (linoleic – 18:2n-6, gamma-linolenic – 18:3n-6, and arachidonic – 20:4n-6) and essential n-3 polyunsaturated fatty acids (alpha-linolenic – C18:3n-3, eicosapentaenoic – C20:5n-3, and docosahexaenoic – C22:6n-3), which contain isolated double bonds and are less susceptible to oxidation. Many studies have shown the potential benefits of conjugated linoleic acid and its isomers, which exhibit antidiabetic and anticarcinogenic action and have a positive effect on the immune function [38–40]. Eicosapentaenoic and docosahexaenoic acids are especially important for the proper functioning of the brain, heart, and retina [41–43].

Yak milk fat contains about 3.2 times more polyunsaturated fatty acids than cow milk fat [6, 44]. According to [32], spring milk of a cattle-yak hybrid contains abundant polyunsaturated fatty acids, but their content decreases towards the end of lactation in line with changes in the phenological phase of pasture grass.

Of all polyunsaturated fatty acids, conjugated linoleic acid (C18:2n-6, *trans* and *cis*) has the strongest positive effect on human health, exhibiting anticarcinogenic (breast, stomach, skin cancer), antiatherogenic, antidiabetic, and immunomodulatory properties, as well as suppressing osteoporosis [25, 27, 45–48].

Conjugated linoleic acid is a powerful antioxidant that protects structural lipids from free radicals [49]. Its antioxidant activity is higher than that of other essential fatty acids and alpha-tocopherol. The content of this acid in khainak milk varied from 0.24 to 1.85 g/100 g of total fatty acids, reaching a maximum in spring.

Of significant interest are also some other polyunsaturated fatty acids with three or more double bonds in the molecule. Spring milk of the Kyrgyz khainak ($p < 0.05$) contained more α-linolenic (1.78 ± 0.05 g/100 g of total fatty acids), arachidonic (0.120 ± 0.003 g/100 g of total fatty acids), and eicosapentaenoic (0.070 ± 0.002 g/100 g of total fatty acids) acids than in the other seasons. According to [15], yak milk had a higher content of α-linolenic acid in August (0.34 g) than in the autumn months. This was probably due to abundant spring forbs on mountain pastures and a higher content of linoleic acid in fresh grass compared to fading grass. High levels of linoleic acid are associated with the synthesis of long-chain unsaturated fatty acids [49–51]. In another study, the milk from mountain and alpine pastures in the Aosta Valley (northwestern Italy) had a higher content of α-linolenic, vaccenic, and conjugated linoleic fatty acids than the milk from lowland pastures, which was associated with the presence of terpenoids in the grass and the grazing altitude [52].

Increasing the content of α-linolenic acid in the diet has been reported to reduce the risk of cardiovascular diseases [53]. However, reliable evidence is needed to confirm this conclusion and recommendations need to be revised before α-linolenic acid can be taken to prevent this pathology. In our study, the content of α-linolenic acid in khainak milk gradually decreased from a maximum in spring (1.78 ± 0.05 g/100 g of total fatty acids) to a minimum in winter (1.25 ± 0.04 g/100 g).

Arachidonic acid (C20:4n-6) is mainly obtained from food or synthesized from linoleic acid. It is involved in biological functions and significantly influences the electrical activity of ion channels in excitable tissues such as those of the brain, heart, and muscles. Also, arachidonic acid controls the central nervous system, as well as visual and hearing impairment in premature infants [54, 55]. In our study, khainak milk contained a small amount of this acid (less than 1 g/100 g of fatty acids).

Studies have generally confirmed the beneficial effects of n-3 polyunsaturated fatty acids on depression and cardiovascular health, as well as on the risk of preterm birth [56]. According to [57], the content of eicosapentaenoic acid in yak milk reached 4.91 g/100 g. However, the milk of the Kyrgyz khainak in our study contained negligible amounts of this fatty acid (0.07 g/100 g of fatty acids), which needs further research.

The qualitative and quantitative differences in the fatty-acid compositions of cow and yak milk may be associated with the animals' feed, breed, breeding methods, lactation stage, season, habitat, altitude, and other factors [15, 58]. Compared to the milk from penned cows, the milk from grazing cows contained more functional fatty acids and lipophilic antioxidants (tocopherols, retinol, and carotenoids), as well as smaller amounts of saturated fatty acids [31].

The ratio of unsaturated to saturated fatty acids is an important criterion for the quality of milk fat. This ratio for yak milk is 0.78 [57]. The higher the unsaturated to saturated fatty acids ratio, the more health benefits in fat. The oil of oceanic fish is commonly rich in health-benefitting fatty acids such as oleic (C18:1), linoleic (C18:2), conjugated linoleic (C18:2 *cis*-9, *trans*-11), arachidonic (C20:4), and eicosapentaenoic acids [59]. Khainak milk fat, like any fat of animal origin, has a far lower content of these fatty acids. However, the unsaturated to saturated fatty acids ratio in the spring khainak milk was about 0.69, which is comparable to yak milk. In cow milk, this ratio is only 0.57 [25].

The biological value of khainak milk fat can also be assessed by the efficiency of metabolism of essential fatty acids – the ratio of arachidonic acid (the main polyunsaturated fatty acid in membrane lipids) to all other polyunsaturated fatty acids with 20 and 22 carbon atoms. In khainak milk fat, this ratio reaches its maximum in spring (0.77), gradually decreasing to its minimum in winter (0.29), with 0.57 in summer and 0.42 in autumn.

The determination of fatty acids in khainak milk may stimulate the development of food additives, alternative dietary sources of fat, and balanced dairy products.

CONCLUSION

Our study was the first attempt to develop a scientific basis for processing the milk of khainaks, the first-generation cattle-yak hybrids living in the highlands of Kyrgyzstan. We described seasonal changes in the composition of fatty acids in hybrid milk and found that spring was the most favorable period for obtaining a biologically complete product with a high content of physiologically functional ingredients. In this period, khainak milk had the minimum content of saturated fatty acids (59.32 ± 1.77 g/100 g of total fatty acids) and the maximum content of monounsaturated (36.48 ± 1.09 g/100 g of total fatty acids) and polyunsaturated (4.28 ± 0.12 g/100 g of total fatty acids) fatty acids. Thus, khainak milk can become an alternative source of high-quality animal fat for a wide range of food products.

CONTRIBUTION

R.Sh. Elemanova prepared the original draft and edited the manuscript. M.M. Musulmanova reviewed and edited the original manuscript. T.Sh. Dzhunushalieva and E.A. Yurova reviewed the manuscript. All the authors made a substantial contribution to the concept and design of the manuscript, as well as approved its final version for publication.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Nutritional and therapeutic potential of functional components of brown seaweed: A review

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Abstract:
Seaweed has a unique chemical composition with an abundance of bioactive substances. In Russia, brown seaweed grows in the coastal areas of the Pacific Ocean (Far East) and the seas of the Arctic Ocean.
This review focuses on the therapeutic and nutritional potential of functional components of brown seaweed. It was based on a systematic analysis of research and review articles published from 2010 to 2023 and indexed in Scopus, Web of Science, and eLIBRARY.RU. Our particular interest was in seaweed's bioactive components such as polysaccharides, phenolic compounds, vitamins, lipids and fatty acids, proteins, peptides, and amino acids.
Compounds extracted from brown seaweed exhibit antioxidant, antiglycemic, antitumoral, neuroprotective, anti-inflammatory, anticoagulant, antibacterial, and immunostimulating properties. Brown seaweed and its derivatives are used as structural modifiers, antioxidants, preservatives, moisture-retaining agents, and sources of vitamins and minerals in the development of functional and preventive food products. They are also used as ingredients in meat, dairy, bakery and flour products, as well as in food additives and beverages, to provide potential health benefits and essential nutrients.
Studies have proven the functional effectiveness of food products containing brown seaweed and its derivatives. The incorporation of seaweed components into functional foods could contribute to global food security. More research is needed to develop new competitive products based on seaweed and to investigate them for the presence of substances hazardous to humans and the environment.

Keywords: Brown seaweed, bioactive substances, bioactivity, functional foods, functional ingredients, essential nutrients

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INTRODUCTION

Nowadays, there is an increasing interest in the production of healthy food products, with special attention paid to balanced therapeutic and functional products. This is associated with their positive impact on human health and preventative action against various diseases. Food formulators focus on the use of novel and renewable sources of commercial food raw materials that are rich in bioactive substances with therapeutic potential. Brown seaweed is a promising source of bioactive components since it has a high growth rate, a large increase in biomass, and an abundance of fermentable carbohydrates. In addition, seaweed does not need fresh water for cultivation.

Based on pigmentation, seaweeds belong to three different groups: *Phaeophyta* (brown algae), *Chlorophyta* (green algae), and *Rhodophyta* (red algae) [1–3]. Brown seaweeds, which number over 2000 species, have predominated in global seaweed production (some of the most common of them are shown in Fig. 1) [4]. From 1950 to 2019, seaweed production annually increased by 11% to reach about 35 million tons [5]. In 2019, two main brown seaweeds (*Laminaria saccharina* and *Undaria pinnatifida*) accounted for about 47% of global seaweed production [6].

In Russia, brown seaweeds grow in the coastal zone of the seas of the Arctic Ocean and in the coastal waters of the Pacific Ocean in the Far East [7, 8]. Commercial

stocks of brown seaweeds in the Arctic Ocean basin (the White and Barents Seas) are represented by the families of laminaria (*L. saccharina*, *Laminaria digitata*, *Laminaria hyperborea*, *Alaria esculenta*) and fucus (*Fucus vesiculosus*, *Fucus distichus*, *Fucus serratus*, *Ascophyllum nodosum*) [9]. Their total reserves exceed 900 000 tons. *L. saccharina* is commercially cultivated on the littoral of the White and Barents Seas. The Far-Eastern seas are home to over 160 species of brown seaweed, with their total reserves estimated at 25–28 million tons. In the coastal waters of the Peter the Great Gulf (the Sea of Japan), near the coast of Primorsky Krai, *Laminaria japonica* is cultivated in special engineering structures (seaweed plantations), with an annual yield of 600–800 tons.

Brown seaweeds are rich in carbohydrates, proteins, polyunsaturated fatty acids, and dietary fibers (Table 1), while being very low in lipids [10]. They are a plant source of vitamins, minerals (micro- and macronutrients), bioactive molecules and enzymes, as well as iodine [11]. These components may vary in content depending on the conditions of seaweed growth: temperature, water salinity, light exposure, degree of surfacing, depth of growth, type of substrate, and other factors [12, 13]. These changes are associated with the influence of external factors on the processes of photosynthesis, respiration, and permeability of seaweed shells [14–16].

Seaweeds are consumed in small quantities and therefore cannot be considered a major source of energy. However, brown seaweeds are a source of extracts rich in nutrients and bioactive substances. They have high potential for use as food additives and/or ingredients to enhance the nutritional and biological value of food products.

In this review, we analyzed and systematized data on the therapeutic and nutritional potential of the functional components of brown seaweeds. In the first part, we described their bioactive components, including polysaccharides, dietary fibers, phenolic compounds, pigments, lipids (fatty acids), proteins, peptides, amino acids, vitamins, and minerals. Based on current scientific publications, we evaluated possible uses of these compounds in the treatment and prevention of various diseases. The second part of the review is devoted to the use of bioactive substances extracted from brown seaweeds in food technologies for the production of foods with functional and preventative action.

STUDY OBJECTS AND METHODS

This study was carried out in the Chemistry and Technology of Marine Bioresources Laboratory at Murmansk State Technical University. We systematized data from original research articles and reviews on seaweeds, their bioactive components, as well as their nutritional and therapeutic potential and applications. For this, we employed a number of methods. First, bibliometric analysis was performed to determine the relevance of literature sources and clean the data of irrelevant or repeated sources. Then, the key scientific publications selected were exposed to in-depth analysis and systematization. A logical search strategy was employed for exploratory analysis to select publications covering

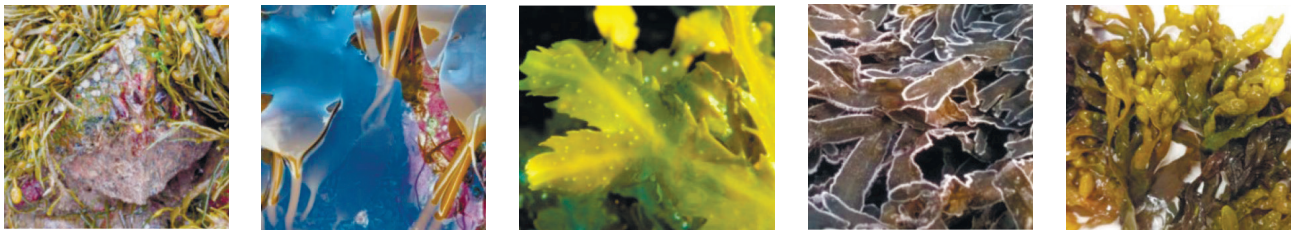

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Figure 1 *Phaeophyta* species (brown seaweed): a – *Ascophyllum nodosum*; b – *Saccharina latissimi*; c – *Fucus serratus*; d – *Fucus distichus*; e – *Fucus vesiculosus*

Table 1 Nutritional composition (g/100 g dry weight) of brown seaweeds

Brown seaweeds	Protein	Lipid	Carbohydrates	Dietary fiber	Ash	References
Himanthalia elongata	7.50 ± 1.43	1.00 ± 0.20	15.00 ± 2.56	36.00 ± 3.71	33.20 ± 3.22	[17]
Asperococcus ensiformis	2.90 ± 0.04	–	6.45 ± 0.50	58.81 ± 2.91	41.18 ± 2.91	[18]
Ascophyllum nodosum	11.40 ± 0.18	3.00	–	34.50 ± 2.37	29.50 ± 0.78	[19]
Saccharina latissima	15.20 ± 0.00	1.50	–	21.70 ± 3.04	39.90 ± 0.00	[19]
Lessonia nigrescens	10.42 ± 0.04	0.87 ± 0.02	–	10.54 ± 0.44	31.04 ± 0.05	[20]
Laminaria ochroleuca	6.26 ± 0.09	–	17.61 ± 0.96	–	35.48 ± 0.11	[21]
Carpophyllum flexuousm	5.90 ± 0.10	2.60 ± 0.10	–	–	8.70 ± 0.80	[22]
Carpophyllum plumosum	7.20 ± 0.20	1.70 ± 0.00	–	–	10.10 ± 0.80	[22]
Ecklonia radiata	7.60 ± 0.00	3.60 ± 0.20	–	–	9.20 ± 0.00	[22]
Undaria pinnatifida	12.50 ± 0.50	2.20 ± 0.00	–	–	11.40 ± 0.60	[22]

the entire range of research problems, from the description of properties of brown seaweeds to their consumption [23]. The search terms (keywords) were broad to provide maximum coverage and included various combinations with the term "brown seaweed". The search was limited to publications from 2010 to 2023 on three databases: Web of Science, Scopus, and eIBRARY.RU. The publication citation ranking was used to select publications from the generated list for in-depth analysis.

RESULTS AND DISCUSSION

Bioactive components of brown seaweeds and their therapeutic potential. Brown seaweeds have a unique chemical composition and are rich in various bioactive substances (Fig. 2) with physiological activity *in vitro* and *in vivo* [24, 25]. The main components of brown seaweeds are represented by minerals, including microelements, and organic substances, including proteins, free amino acids, lipids (fatty acids), pigments, polyphenolic substances, structural carbohydrates (alginic acids, fucoidan, cellulose), and spare carbohydrates (mannitol, laminarin). This composition results from complex metabolic processes occurring in seaweed.

Functional components extracted from brown seaweed have high potential in treating a number of chronic diseases due to their antioxidant, anticoagulant, antiglycemic, antitumorous, and neuroprotectant activities [26–32] (Fig. 3). These properties are key to potential nutraceutical and therapeutic applications of brown seaweeds [33, 34].

Figure 3 shows the main components of brown seaweeds that have biological activity and are widely used in various technologies.

Polysaccharides. Seaweed is an important source of polysaccharides, which are more diverse than those in land plants [35, 36]. Most carbohydrates in seaweed are sulfated and unsulfated polysaccharides. Brown seaweed is rich in polysaccharides such as laminarin, alginate, and fucoidan, which consist of monosaccharides such as glucose, rhamnose, galactose, fucose, xylose, mannose, as well as glucuronic and mannuronic acids [24, 37].

According to clinical studies, seaweed-derived bioactive components are effective in the prevention and treatment of COVID-19. Sulfated polysaccharides and polyunsaturated fatty acids obtained from seaweed also exhibit immunostimulating and antitumorous effects [33, 37, 38].

Alginates (or alginic acid) (Figs. 4a and b), which are part of cell membranes, are the most common

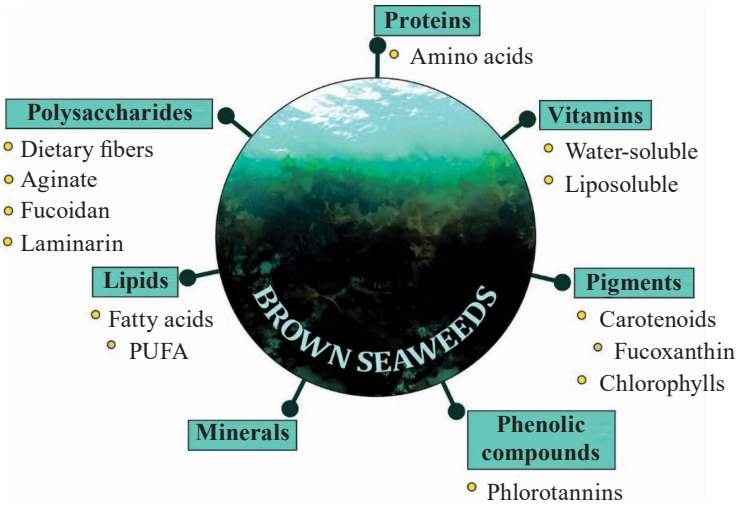

Figure 2 Bioactive components of brown seaweed

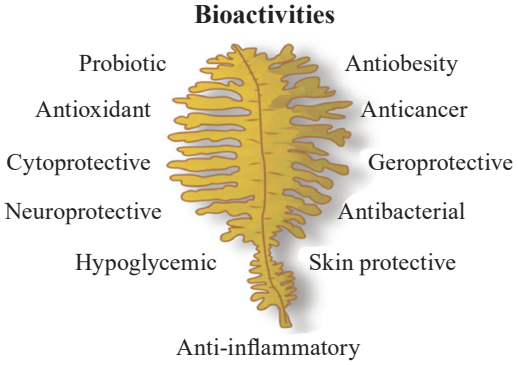

Figure 3 Components of brown seaweeds with bioactive properties

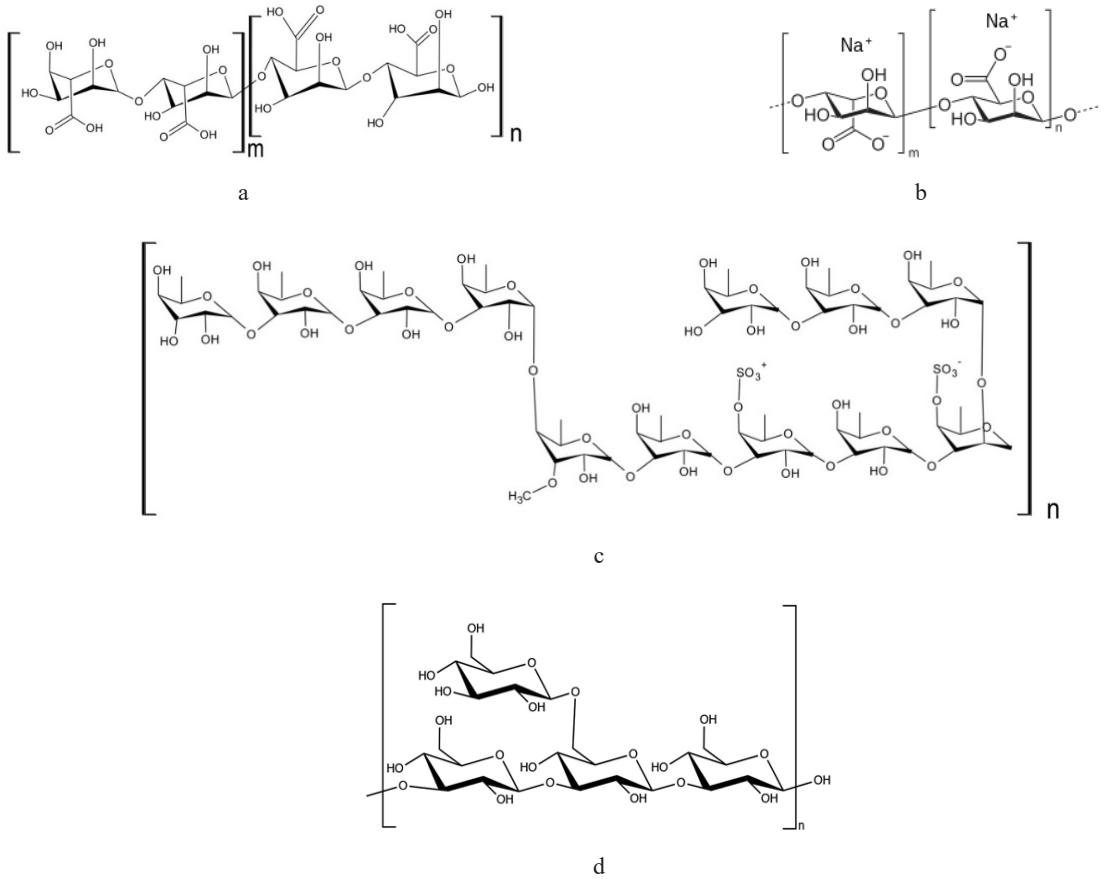

Figure 4 Chemical structure of brown seaweed polysaccharides: a – alginic acid; b – sodium alginate; c – fucoidan; d – laminarin

polysaccharide in brown seaweeds. Some of them contain up to 70% of alginic acid on dry basis [39]. Alginates can be obtained by chemical extraction, by microwave radiation, ultrasonic extraction, or a combination of enzymatic and traditional chemical extraction [40]. Alginates are extracted from such genera as *Laminaria*, *Ecklonia*, *Ascophyllum*, *Durvillaea*, *Lessonia*, *Macrocystis*, *Sargassum*, and *Turbinaria* [41]. *Laminaria* and *Macrocystis* are currently the main sources of alginates [42]. Alginates are widely used in the food and pharmaceutical industries to prevent bowel diseases and regulate blood sugar levels. Placebo-controlled studies in humans have shown that alginates have a positive effect on the appetite by increasing a feeling of fullness and reducing energy consumption by the body while maintaining its functionality [43–46].

Alginates are also able to form hydrogels in the presence of metal cations. They are widely used in the food and pharmaceutical industries due to their high water-holding capacity and good adsorption properties [32].

Fucoidan (Fig. 4c) is a fucose-containing sulfated cell wall polysaccharide that protects brown seaweed against environmental impacts. Its content and composition vary depending on the species, as well as the season and stage of seaweed growth [47, 48]. Fucoidan can be extracted by conventional chemical, microwave, ultrasonic, or enzyme extraction [49]. *Saccharina latissima* and *Fucus evanescen*s are the most suitable sources of fucoidans [50].

Brown seaweed-derived fucoidan is the most promising anticancer agent due to its powerful antitumor activity against various types of cancer [51]. Also, fucoidan has a hypoglycemic effect and is therefore used to treat diabetes mellitus and prevent its complications [46, 52].

Fucoidan extracted from brown seaweed can also be used in aquaculture as a functional bioactive component in the diets of both fish and shellfish [53].

Laminarin. Beta-glucan (*β*-glucan), which is a polymer of glucose, is contained in the cell walls of plants, cereals, fungi, seaweed, and some species of bacteria [54]. Brown seaweed is the main source of laminarin (or laminaran), a type of *β*-(1→3)-glucan containing *β*-(1→6)-related branches (Fig. 4d) [55]. Laminarin was first discovered in kelp species [56]. It belongs to dietary fibers and is not digested in the upper gastrointestinal tract. Laminarin helps reduce the risk of colon cancer, obesity, and diabetes [47]. Oxidation and reduction processes enhance the antitumorous, antioxidant, and anti-inflammatory properties of laminarin [32]. In 2020, the beta-glucan market amounted to $403.8 million, and it is estimated to grow annually by 7.6% to reach $628.3 million by 2026 [57].

Dietary fibers. The human gut microbiota plays an enormous role in general health and disease prevention. Numerous current studies are aiming to strengthen the

immune system with the help of the gut microbiota and to treat a number of diseases, such as diabetes, cancer, and obesity [58]. Polysaccharides obtained from brown seaweed have been found effective in stimulating the gut microorganisms. Seaweeds contain 25–70% of total dietary fibers, of which 50–80% are soluble fibers [59]. Laminarin and fucoidan are typical soluble dietary fibers in brown seaweeds, while cellulose belongs to insoluble dietary fibers [60]. Sulfated polysaccharides, which are dietary fibers in seaweeds, are rarely found in terrestrial plants [61].

Dietary fibers have a prebiotic effect on human health. Brown seaweeds of the genera *Ecklonia*, *Sargassum*, *Laminaria*, *Ascophyllum*, *Fucus*, *Undaria*, *Saccorhiza*, and *Porphyra* have this effect due to the presence of polysaccharides, including dietary fibers (carbohydrates) which are not digested by human digestive enzymes. On the other hand, they are a nutrient substrate that stimulates the growth of beneficial microbiota (e.g., *Lactobacillus*, *Bifidobacterium*, and *Faecalibacterium*) [62, 63]. The prebiotic potential of seaweed has been confirmed by studies using a human colon model *in vitro* [17, 64]. As prebiotic compounds, dietary fibers induce an immune response by increasing the microbial activity of the gastrointestinal tract. This leads to fermentation and production of short-chain fatty acids, which, in turn, has several positive physiological effects. Seaweed's dietary fibers have antioxidant, anti-inflammatory, anticoagulant, and antiviral activities [65–68]. Ajanth Praveen *et al.* reviewed the structure of various seaweed polysaccharides, new methods their extraction and purification, as well as their immunomodulatory effects on the gut microbiota [69].

Dietary fibers of brown seaweed are widely used in food technology, mainly as thickeners, emulsifiers, gelling agents, and prebiotics [70]. Sulfated polysaccharides (dietary fiber) have been shown to play an important role in enhancing seaweed's antioxidant, immunomodulatory, anticarcinogenic, antiviral, and antimicrobial activities [41]. Dietary fibers extracted from brown seaweed show an excellent ability to swell and retain water due to the hydrophilic characteristics of sulfated polysaccharides [71]. This property is used in the production of meat products.

Phenolic compounds are among the most important bioactive components of seaweed. They include phenolic acids, tannins, flavonoids, catechins, and phlorotannins. Their composition varies depending on the type of seaweed. Brown seaweed contains mainly phlorotannins, which are complex polymers made up of phloroglucin links (1,3,5-trihydroxybenzene). Polyphenols account for 2 to 30% of seaweed's dry weight. Phenolic compounds in brown seaweed have been shown to have antihyperlipidemic and antihyperglycemic effects [72, 73]. Phlorotannins exhibit antioxidant, anti-inflammatory, antimicrobial, cytotoxic, and antitumorous activities [74, 75]. They can also be used as anti-aging agents [76]. Phlorotannins play a major role in cell wall creation and perform protective functions [56].

People with prediabetes, overweight, and obesity are recommended to consume extracts of brown seaweeds (*Ascophyllum nodosum* and *Fucus vesiculosus*), which contribute to a significant reduction in body weight, waist circumference, and overall fat mass, as well as have beneficial effects on insulin secretion [77]. Numerous studies have shown a positive effect of phlorotannins and polysaccharides from *Silvetia compressa* on the growth of probiotic bacteria and therefore the human microbiota [78]. The therapeutic effects and possible applications of polysaccharides and phenolic compounds from brown seaweeds are shown in Table 2.

Pigments. There are three groups of pigments in seaweed: chlorophylls, carotenoids, and phycobiliproteins [94, 95]. Brown seaweed contains chlorophylls "a", "c1 and c2", and fucoxanthin (Fig. 5), which give the cells the brown color, as well as *β*-carotene, neofucoxanthin, and other carotenoids [96]. Chlorophyll pigments have positive effects on human health. In particular, they contribute to chelation with some chemical carcinogens and mutagens, lower the risk of cancer, and exhibit high antioxidant activity [95]. Pigments can be extracted from seaweed by solvent extraction, liquid extraction under pressure, or microwave extraction [97].

Fucoxanthin is among the most common carotenoids in brown seaweed. It is absent in terrestrial plants [26]. Fucoxanthin exhibits strong antioxidant action against oxidative stress [45]. It is safe to use and has no side effects. Fucoxanthin protects the cardiovascular system and has anti-inflammatory, anti-cancerous, and neuroprotective effects. Furthermore, it is an effective chelator of toxic and heavy metals [98]. Fucoxanthin's antioxidant activity is associated with its neuroprotective, photoprotective, and hepatoprotective effects [26].

Lipids, fatty acids. Fatty acids with two or more double bonds are necessary for normal cell function, and they play a key role in cellular and tissue metabolism, regulating membrane fluidity, electron and oxygen transport, as well as temperature adaptation [99].

Lipids of seaweed mainly contain fatty acids with a long carbohydrate chain. Some brown seaweeds have a high content of total lipids in the range of 10–20 wt%. In particular, lipids account for 11.91 ± 2.00 wt% in *Dictyota bartayresii*, 10.80 ± 0.99 wt% in *Dictyota dichotoma*, 11.73 ± 0.49 wt% in *Spatoglossum macrodontum*, 12.8, 13.4, and 10.9 wt% (in April, May, and July, respectively) in *Costaria costata*, and 15.59 wt% (January) in *Cystoseira hakodatensis* [100]. Essential fatty acids and polyunsaturated fatty acids are found in large quantities in brown seaweed [101]. The main omega-3 polyunsaturated fatty acids are eicosapentaenoic acid (20:5 n-3), stearidonic acid (18:4 n-3), and *α*-linolenic acid (18:3 n-3), while the main omega-6 polyunsaturated fatty acid is arachidonic acid (20:4 n-6). Polyunsaturated fatty acids account for 51.28% of all fatty acids in *Saccharina japonica*. Arachidonic acid (C20:4 n-6) varies from 10.55% (*Undaria pinnatifida*) to 14.87% (*Sargassum horneri*), and eicosapentaenoic acid ranges from 8.36% (*C. costata*) to 13.04% (*Saccharina japonica*) [102].

Table 2 Therapeutic effects and applications of bioactive components of brown seaweeds

Seaweed genus	Bioactive component	Therapeutic effect	Application	References
Sargassum vachellianum, *Sargassum horneri*, *Sargassum hemiphyllum*	Polysaccharides	Antioxidant activity, inhibition of tyrosinase and elastase	Active components for skin protection	[79]
Eisenia bicyclis	*β*-glucan (laminaran)	Protection of the stomach (in case of gastric dysplasia)	–	[54]
Ascophyllum nodosum, Fucus vesiculos	Sodium alginate	Improvement of the human gut microbiota	Prebiotics	[80]
Laminaria japonica	Sodium alginate	Immunomodulatory and enterosorbent activities	Biogel in probiotics	[81]
Himanthalia elongata	–	Improvement of the human gut microbiota	Prebiotics	[17]
Sargassum polycystum, Turbinaria ornate, Padina boryana	Fucoidan, laminaran, and alginate	Antioxidant activity	Functional ingredients	[82]
Laminaria japonica	Fucoidan	Immunostimulating properties, antitumorous and anti-inflammatory effects	Medicines, adjuvants	[83, 84]
Fucus evanescens	Fucoidan	Immunoadjuvant activity	Adjuvants	[85]
Fucus evanescens	–	Hepatoprotective and antioxidant effects	Functional foods (bread)	[86]
Costaria costata, Undaria pinnatifida	–	Antiradical activity	Functional beverages	[87]
Sargassum glaucescens	Fucoxanthin	Men infertility treatment	–	[88]
Padina tetrastromatica	Fucoxanthin, lipids	Anti-inflammatory ability	Functional ingredients	[89]
Silvetia compressa	Phlorotannins, polysaccharides	Improvement of the human gut microbiota	Prebiotics	[78]
Ascophyllum nodosum	Phlorotannins	Reduction in DNA damage in obese individuals	Bioactive additives	[90]
Ascophyllum nodosum, Fucus vesiculosus	Polyphenols	Prevention of type 2 diabetes	Bioactive additives	[77]
Fucus vesiculosus	Polyphenols	Antihyperlipidemic, antihyperglycemic, anti-inflammatory effects	Bioactive additives	[72]
Sargassum pallidum	Polyphenols	Antioxidant and antiradical activities	Stress-protective substances and nutritional supplements	[91]
Sargassum pallidum, Ecklonia kurrome, Hizikia fusiforme and Undaria pinnatifida Suringar	Phenolic acids, flavonoids	Diabetes treatment, *α*-glucosidase inhibitors	Medicines	[92]
Ishige Okamura	Ishigoside (glyceroglycolipid)	Antioxidant and anti-inflammatory abilities	Functional foods to prevent the photoaging of skin	[93]

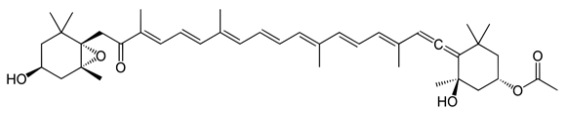

Figure 5 Chemical structure of fucoxanthin

The content of eicosapentaenoic acid is also high in *A. nodosum* (6.85%) and *S. latissima* (4.67%) [19]. *Sargassum pallidum* extract contains n-6 polyunsaturated fatty acids (41.3%) with C18 and C20 carbon atoms and such n-3 polyunsaturated fatty acids as *α*-linolenic (18:2; 7.8%), stearidonic (18:4; 7.3%), and eicosapentaenoic (20:5; 3.5%) acids.

Since the human body has a low ability to synthesize docosahexaenoic acid from linoleic acid, high intake of long-chain fatty acids and more unsaturated forms of linoleic acid (eicosapentaenoic and docosahexaenoic acids) is recommended to prevent cardiovascular disease [100].

Lipids containing n-6 and n-3 long-chain fatty acids are known to prevent atherosclerosis [103]. The lipid complex of *S. pallidum* introduced into the diet may have a hypolipidemic effect, restoring the liver's lipid metabolism and esterifying function, as well as regulating the lipoprotein content in plasma. The combined action of n-3 and n-6 polyunsaturated fatty acids in *S. pallidum*'s lipid complex has an antioxidant effect on

the organism [104]. *S. japonica*'s lipid complex reduces dyslipidemia and hypercholesterolemia, as well as normalizes the ratio of fatty acids in total lipids of blood plasma and erythrocyte membranes due to the presence of various polyunsaturated fatty acids [105].

Proteins, peptides, and amino acids. The protein content in brown seaweed ranges from 5.00 to 19.66%. For example, *Chnoospora minima*, *Dictyota menstrualis*, *Padina gymnospora*, and *Sargassum vulgare* contain from 10 to 15% of protein dry weight. Brown seaweed contains all essential amino acids in the quantities recommended by the Food and Agriculture Organization of the United Nations (FAO). Its levels of aspartic and glutamic amino acids, which give seaweed a unique taste and aroma, are higher than those in red or green seaweed [4, 47, 101, 106]. Peptides derived from seaweed are known to exhibit antioxidant properties. They are also effective in treating cardiovascular diseases and diseases associated with metabolic syndrome [47].

Glycoproteins obtained from brown seaweed have unique biological properties. For example, glycoprotein isolated from *U. pinnatifida* has an antioxidant effect and is active against inflammatory diseases and Alzheimer's disease due to cholinesterase inhibition [106, 107]. This glycoprotein inhibits the formation of toxic β-amyloid peptides by inhibiting β-secretase. In addition, it does not exhibit cytotoxicity in primary hippocampal cells and protects the cells from natural death. This glycoprotein was also shown to inhibit inflammatory mediators and nitric oxide, so it can be used as a dietary supplement to prevent inflammatory pathologies. Glycoprotein isolated from the brown seaweed *Laminaria japonica* has an antiproliferative effect on HT-29 colon cancer cells [108]. Glycoprotein was also shown to stimulate gastrointestinal cell growth in mice [109].

Brown seaweeds contain lectins, glycoproteins capable of reversibly and specifically binding to sugar residues [110]. Lectins are capable of specific recognition and can bind sugars (lactose, mannose, galactose, N-acetylgalactosamine, and N-acetylglucosamine) by noncovalent interactions. Lectin-carbohydrate interactions play an important role in such biological processes as cell adhesion, agglutination, opsonization, complement activation, and phagocytosis. Due to the specificity of mannose binding, lectins from brown seaweed are used to decipher and characterize complex mannose-containing glycans from the glycocalyx covering both normal and transformed cells. Lectins are also widely used as effective agents against the human immunodeficiency virus [111].

Seaweed lectins, often called phycolectins, are similar to plant lectins, but they also differ in some physical and chemical properties and have a unique carbohydrate specificity [112]. Phycolectins are monomeric proteins with a low molecular weight and an isoelectric point (pI) in the range of 4 to 6 [113]. Over 800 lectins from seaweeds have now been identified, of which 61% are from red seaweed, 22% from green seaweed, and 17% from brown seaweed. However, only about 40 lectins have been identified, purified, and sequenced [114].

Seaweed lectins are attracting attention because of their antiviral activity. Lectins can prevent the virus's invasion into host cells and spreading there. This is where they differ from most traditional antiviral agents which block the life cycle of a virus once it has entered the cell. In addition, lectins act as surface markers for tumor cell recognition, transmembrane signal transduction, cell adhesion, mitotic apoptosis, and cytotoxicity. Therefore, lectins can be used in cancer diagnosis and therapy [110].

Noteworthily, the consumption of brown seaweed that has not undergone deep processing can reduce the availability and digestibility of protein due to high contents of soluble fibers and polyphenols. For use in food, the protein of brown seaweed should be separated from non-protein components [115, 116]. Proteins can be extracted from seaweed by using enzymes, microwaves, ultrasound, pulsed electric fields, or supercritical fluids [4]. Enzymes that decompose polysaccharides are used to release flavor components that impart umami flavor, such as peptides and amino acids [117].

Vitamins. Seaweed contains both water- and fat-soluble vitamins. Brown seaweed is an excellent source of vitamins A, B_1, B_2, B_3, B_{12}, C, D, E, as well as pantothenic and folic acids.

Brown seaweed is rich in vitamins B_1 and B_2. In particular, *U. pinnatifida* and *S. japonica* contain 0.3 and 0.24 mg B_1/100 g dry weight, respectively, as well as 1.35 and 0.85 mg of B_2/100 g dry weight, respectively [118, 119]. According to [120], *U. pinnatifida* has even higher contents of vitamins B_1 and B_2, namely 5 mg of B_1/100 g dry weight and 11.7 mg of B_2/100 g dry weight. Vitamins B_1 and B_2 are contained in smaller amounts in *Eisenia arborea*, namely 0.06–0.12 and 0.65–0.92 mg/100 g dry weight, respectively [121]. Brown seaweed is also rich in vitamin C. Its contents in *U. pinnatifida*, *S. latissima*, and *F. vesiculosus* are 14.58, 61, and 40.9–51.7 mg/100 g dry weight, respectively [122–124].

Brown seaweed has a higher content of α-tocopherol, as well as β- and γ-tocopherols, compared to red and green seaweeds containing only α-tocopherol. The largest amount of vitamin E was found in *Macrocystis pyrifera* (132.77 mg/100 g) [119]. In *Durvillaea antarctica* and *U. pinnatifida*, the content of vitamin E amounted to 84.0 ± 0.5 mg/kg dry weight and 0.63 mg/100 g dry weight, respectively [125, 126].

Seaweed contains only provitamins of vitamin A. The brown seaweed *S. japonica* has a high content of β-carotene with vitamin A activity (2.99 mg/100 g dry weight calculated as 481 IU/100 g dry weight) [119]. According to [126], the content of vitamin A in *U. pinnatifida* is 4.73 IU/kg dry weight.

The bioavailability of vitamins is primarily related to their solubility, which ensures their absorption in the intestine. The bioavailability and absorption of some seaweed fat-soluble vitamins depends on whether they are taken with fat-containing foods or not. Fat-soluble vita-

mins are absorbed in the same way as dietary lipids [119]. Also, vitamins that are bound to fiber or some other carbohydrates in foods are less available than those taken in pure form.

Seaweeds are an important source of antioxidants since they can generate necessary compounds for protection against oxidation [127, 128]. Antioxidant activity is determined by several factors, such as the antioxidant's internal chemical activity against radicals, the location and reactivity of radicals, the antioxidant's concentration and interaction with other antioxidants, etc. Antioxidant compounds in seaweed include vitamin E (*α*-tocopherol), vitamin C (ascorbic acid), vitamin B_1, and nicotinic acid [119, 129]. Vitamins with strong antioxidant capacity can act as therapeutic agents to protect against cancer [51, 130].

Seaweed is the only non-animal source of vitamin B_{12}, which is important for vegetarians. Cobalamin is not synthesized in higher plants and is not required for their metabolism, so vegetables and fruits are low in this vitamin. Vitamin B_{12} deficiency is a common consequence of vegetarian and vegan diets that leads to pernicious anemia, a disease characterized by impaired hematopoiesis. Vitamin B_{12} is present in brown seaweeds of the genera *Ascophyllum* and *Laminaria*. Vitamin B_{12} also slows down the aging process [131, 132].

Vitamin E found in seaweed is a strong antioxidant that prevents the formation of free radicals. As reported in [133], vitamin E improves the condition of blood vessels and reduces their damage. Vitamin E has also been shown to lower the risk of lung and cervix uteri cancer by interacting with genotoxic radicals, reducing mutagenic activity, inhibiting the formation of carcinogenic nitrosamines, and protecting cell membranes from peroxidation [134, 135]. *α*-tocopherol is able to bind free radicals through the phenol group and plays an important role in the oxidation of biological membranes, lipoproteins, and fatty deposits, controlling or reducing lipid levels [136].

Ascorbic acid contained in seaweed is another effective antioxidant [119]. Due to its ability to neutralize free radicals, it is believed to play an important role in preventing cancer. In addition, ascorbic acid has prooxidant properties [137]. Several studies have established a correlation between vitamin C intake and lower incidence of stomach cancer [134, 138, 139]. They have also found a possible association with a decreased risk of developing cancer of the oral cavity, pharynx, lungs, and gallbladder in men. Vitamin C intake helps lower blood pressure in patients with hypertension, hyperlipidemia, and diabetes. A combination of vitamin C with other antioxidants (vitamin E, *β*-carotene) can provide a synergistic antihypertensive effect [140].

Minerals. Seaweed can accumulate micro- and macronutrients contained in seawater, which gave rise to the term "marine organic drugs". Brown seaweed absorbs minerals better than green or red seaweed due to its high content of alginic acid and its salts [141]. In brown seaweed, minerals and micronutrients account for 14–45% dry weight, depending on seasonal and climatic variations [142]. The mineral content in seaweed is up to 36% dry weight, which is 10–100 times higher than that of fruits and vegetables. Thus, seaweed can make an important contribution to the daily mineral intake [143].

Brown seaweeds *Laminaria digitata*, *U. pinnatifida*, and *F. vesiculosus* are rich in minerals that are thought to improve glycemic control. These minerals include potassium (K; 2–15% dry weight), calcium (Ca; 0.1–3.0% dry weight), and magnesium (Mg; 0.1–1.5% dry weight) [31, 122, 144, 145]. These seaweeds also contain zinc (Zn) and chromium (Cr) in the amounts of 0.004–0.020 and 0.02–0.05%, respectively, due to improved circulating glucose levels [31, 122, 145–147].

According to scientific estimates, about one-third of the world's population is at risk of zinc deficiency, especially children under the age of five who need zinc to support their growth [148]. Zinc exhibits therapeutic effects in several chronic diseases, such as atherosclerosis, some cancers, autoimmune diseases, Alzheimer's and other neurodegenerative disorders, diabetes, depression, Wilson's disease, as well as aging [149]. Chromium is required for energy production from blood sugar, as well as for insulin function and lipid metabolism [150]. A daily intake of one gram of *U. pinnatifida* can meet the recommended physiological requirement for chromium [143].

Seaweed is one of the most important sources of calcium and phosphorus. It contains large amounts of Ca, Mg, Na, P, Zn, and I. Compared to other mineral-rich foods, seaweed contains more Ca, Cr, I, Fe, Mg, P, Se, Zn, K, and Na. However, it has a lower copper content compared to other foods such as raw meat or mushrooms [151]. The content of calcium in the brown seaweeds *A. nodosum* (575.0 mg/100 g raw weight) and *L. digitata* (364.7 mg/100 g raw weight) exceeds that of whole milk (115.0 mg/100 g raw weight). Thus, these seaweeds can be used as a source of calcium to prevent or treat osteoporosis in growing children, as well as in pre- and postmenopausal women. In addition, the non-digestible prebiotic carbohydrates in seaweed can increase calcium absorption and bioavailability [152–154]. *F. vesiculosus* also contains Ca and Mg in much higher concentrations than many other products. Particularly, its Ca values (2.175 mg per 100 g dry weight) are almost 20 times as high as in whole milk, while its Mg concentration (994 mg per 100 g) is about 5 times as high as in peanuts. The iron (Fe) content in *F. vesiculosus* can reach 49–52 mg per 100 g dry weight, so this seaweed may be useful in providing daily iron intake and preventing iron deficiency anemia [141, 154].

It has also been shown that one gram of seaweed can cover up to 57.6 % of the recommended daily intake of selenium, with the brown seaweeds *M. pyrifera* and *S. japonica* having the highest content [143].

Brown seaweeds are able to accumulate higher concentrations of sodium (Na) ranging from 0.4 to 9% dry weight and potassium (K) ranging from 2 to 15% dry weight than green seaweeds. The Na/K ratio in brown sea-

weeds is quite low (0.3–1.5), so they can support human cardiovascular health by reducing blood pressure [155].

Mg and Ca play a key role in the health of bones and teeth. In addition, Mg is also involved in cellular metabolism and enzyme systems, while Ca is involved in the regulation of heartbeat, nerve impulse transmission, muscle contraction, blood clotting, and activation of insulin and thyroid hormone calcitonin [156]. Brown seaweeds have been shown to contain higher amounts of calcium than tofu or cabbage [143].

Brown seaweeds are a source of iodine, especially in the genus *Laminaria*, which can accumulate iodine in amounts exceeding its content in seawater more than 30 000 times [157]. Iodine content in different seaweeds consumed as food can vary significantly (from 0.1 to 30 mg/g dry weight). Iodine is an important trace element necessary for the production of thyroid hormones thyroxine and triiodothyronine, which stimulate both metabolic regulation and normal development of the body. The bioavailability of iodine from brown seaweed is quite high, ranging from 31 to 90%, as shown by *in vivo* studies. However, excessive iodine intake can cause thyroid disorders and lead to both hyperthyroidism and hypothyroidism [158].

Uses of brown seaweeds in food technology. Among brown seaweeds, *F. evanescens*, *S. japonica*, *S. latissima*, *L. digitata*, and *U. pinnatifida* have the largest application in the food industry [159].

Seaweed is obtained in their natural habitat or grown on special farms. The world production of brown seaweed increased from 13 000 tons in 1950 to 16.4 million tons in 2019. Its average annual growth in 1950–2019 was higher than the growth of global aquaculture of all species. In 2019, the brown seaweeds *Laminaria saccharina* and *U. pinnatifida* accounted for 47.3% of the world's seaweed production [5]. The world market for seaweed products amounted to $4.7 billion in 2021 and is expected to reach $6.4 billion by 2026, with an average annual growth of 6.3% [160].

Seaweed is mainly cultivated and processed in East and Southeast Asia, where it is commonly used as a food product. Although it is a traditional product in the coastal communities, many countries consider seaweed a niche or a novel product and therefore consume it in small quantities. Functionally, seaweed is consumed:

– as part of vegetarian diet, as well as in therapeutic or preventative nutrition;
– as a seaweed-based food additive;
– for gastronomic purposes in exotic dishes of oriental cuisine;
– as organic, ecological bio-products, whose production is environmentally-friendly and reduces emissions of greenhouse gases; and
– in social nutrition as an affordable balanced product for the growing population, etc.

In addition to nutritional purposes, seaweed is also used to produce feed for farm animals (including aquaculture), pharmaceuticals and nutraceuticals, cosmetics, textiles, biofertilizers/biostimulants, bio-packing, biofuels, etc. [159, 161–163].

Brown seaweeds are widely used as a functional ingredient in food production to improve health and reduce the risk of developing diet-related diseases [24]. Due to their physical and chemical properties, as well as biological activity, brown seaweeds can be used for nutraceutical purposes [152]. Brown seaweeds and their components (polysaccharides, protein extracts, etc.) are increasingly used in food technologies [164–167]. Many regions where the use of seaweed is limited for various reasons are showing interest in functional foods, including seaweed products [168, 169]. The functional roles and uses of bioactive components of brown seaweeds in food products are shown in Table 3.

In 2021, the global market of functional foods reached more than $180 billion, and it is expected to grow by almost 3% per year in 2022–2027 [179].

Noteworthily, consumers are conditioned to use traditional, familiar products. Therefore, people in those countries where seaweed is not commonly used as a food and is not part of traditional cuisine are neophobic towards this product [176, 184]. However, seaweed is often used in sophisticated, trendy cuisine due to its unique sensory properties. Its chemical composition and functional effects also contribute to its growing popularity [191].

The main types of products where seaweed or seaweed-derived bioactive substances are used as functional ingredients are reviewed below.

Meat products. Modern meat products containing bioactive ingredients have a balance of nutritional and functional properties [35, 192].

F. vesiculosus is rich in phlorotannins, polyphenolic compounds with high antioxidant activity. *F. vesiculosus* extracts are used as natural preservatives in pork chops to protect their lipids and proteins from oxidation during storage [193].

Extracts of the brown seaweeds *A. nodosum*, *F. vesiculosus*, and *Bifurcaria bifurcata*, which are high in natural antioxidants, provide chilled pâté of lean pork liver with oxidative stability similarly to the synthetic antioxidant tert-butyl-4-hydroxytoluene [194].

Replacing sodium chloride with edible seaweed (*Himanthalia elongata* and *U. pinnatifida*) in meat sausages can lower the risk of chronic diseases by reducing the salt content [195].

Alginates are widely used as thickening and stabilizing agents to reduce the fat content in various restructured meat products. Their stabilizing properties are due to their ability to form complexes with proteins [42]. Replacing pork speck with sodium alginate in meat sausages decreases the fat content and the energy value of the final product [182].

The addition of dietary fiber derived from seaweed can clearly improve the quality, nutritional properties, and taste characteristics of processed meat products [4]. Seaweeds can also enhance the ability of sausages to bind water/lipids and contribute to a thinner

Table 3 Functional roles and applications of bioactive components of brown seaweeds in food products

Component	Functional role	Food product	References
Sodium alginate	Structural modifier for 3D printing	Rice paste (3D printing)	[170]
Sodium alginate	Structural modifier for 3D printing	Food ink (3D printing)	[171]
Sodium alginate	Structural modifier for 3D printing	Artificial steak (3D printing)	[172]
Sodium alginate	Fat-reducing agent	Meat sausages	[173]
Fucus vesiculosus extract (phlorotannins)	Food antioxidant	Pork chops	[174]
Undaria pinnatifida powder	Structure modifier, food antioxidant	Pork chops	[175]
Aqueous extracts *of Ascophyllum nodosum*, *Fucus vesiculosus*, *Bifurcaria bifurcata*	Food antioxidant	Pork liver pâté	[176]
Himanthalia elongata and *Undaria pinnatifida*	Sodium chloride reducing agent	Pork sausages	[177]
Dehydrated shredded *Himanthalia elongata* (phenolic compounds)	Food antioxidant	Cheese	[177]
Fucoidan (*Undaria pinnatifida*)	Food antioxidant, glycemic index reducing agent	Bread	[178]
Polyphenols *Ascophyllum nodosum* and *Fucus vesiculosus*	Food antioxidant	Bread	[179]
Alginate oligosaccharides (*Laminaria hyperborea*)	Food preservative	Yogurt	[180]
Fucoidan	Inhibitor of oral cancer cells	Shake	[187]
Fucoidan (*Fucus vesiculosus*)	Antimicrobial agent	Functional pasteurized apple drink	[182]
Sodium alginate	Fat and sugar substitute	Ice cream	[164]
Polyelectrolyte complex of sodium alginate and fish gelatin	Structure-forming agent	–	[183]
Complex of sodium alginate (*Fucus vesiculosus*) and gelatin	Gelling agent	Culinary, canned foods	[102]
Protein extract (*Himanthalia elongata*)	Foam former, emulsifier	Sausages, bread, cakes, soups, salad dressings	[184]
Components *of Saccharina latissima* and *Ascophyllum nodosum*	Flavor enhancer, sodium chloride reducing agent	Fish cakes	[95]
Extracts of *Ascophyllum nodosum* and *Saccharina latissima* with rich umami flavor and salty taste	Flavor ingredient, sodium chloride reducing agent	–	[95]
Laminaria ochroleuca	Anti-gluten agent	Gluten-free pasta	[185]
Ascophyllum nodosum and *Fucus vesiculosus* extracts	Antioxidant	Yogurt	[174]
Fucus vesiculosus powder	Functional food ingredient, antioxidant	Bread	[186]
Biomass of *Durvillaea antarctica* and *Laminaria digitata*	Functional food ingredient, a source of essential amino acids	Dietary supplement, essential amino acid *L-lysine*	[176]
Sargassum wightii powder	Food antioxidant	Coffee beverage	[177]
Powder of *Lessonia berteroana*, *Lessonia trabeculata*, *Macrocystis pyrifera*	Sodium chloride reducing agent	Dietary supplement	[173]
Fucoxanthin (*Dictyopteris polypodiodes*)	Food preservative	Marinated fish (sardine *Sardina pilchardus*)	[187]
Powder of *Ascophyllum nodosum*	Enhancer of functional properties, a source of dietary fiber	Whole-wheat bread	[180]
Aqueous ethanol extracts of *Sargassym pallidum* and *Saccarina japonica*	Cholesterol and cholesterol ester reducing agent, a source of neutral lipids and glycolipids	Bakery products	[188, 189]
Costaria costata, *Undaria pinnatifida*	Food antioxidant, a source of bioactive substances	Dry beverages	[87]
Alaria esculenta	Gelling agent, enhancer of nutritional value	Pastry fillings	[190]

and denser gel-like matrix of meat protein [196]. The addition of laminarin and fucoidan significantly delays lipid oxidation in pork chops during storage [197]. Further, dietary fiber derived from brown seaweed can be a real alternative to phosphates in the production of meat sausages [198]. Phosphate-free sausages treated with dietary fiber have an improved quality profile, which meets the demand for healthier meat products. Dietary fiber improves the texture of phosphate-free sausages and effectively slows down lipid oxidation during storage.

Dairy products. Bioactive components of brown seaweeds (*H. elongata*, *Laminaria ochroleuca*, *U. pinnatifida*) are used in the production of various dairy products to improve their quality indicators. In particular, phenolic compounds exhibit antioxidant properties and can therefore increase the antioxidant activity of cheese when added to the milk clot. Introducing dehydrated seaweed can also enhance the retention of whey in the milk clot and has a positive effect on the color and texture of cheese [199].

Alginate oligosaccharides have a potential antifungal effect against certain yeasts that cause milk spoilage. In yogurt starter culture, alginate oligosaccharides extracted from *Laminaria hyperborea* decreased the growth of microorganisms *Candida parapsilosis*, *Debaryomyces hansenii*, and *Meyerozyma guilliermodii*. Thus, these oligomers can ensure safe storage of dairy and milk-containing products whose shelf life is reduced by yeast [200].

The use of bioactive food foam containing sodium alginate can replace fat and sugar in ice cream. Such functional products can benefit consumers who are overweight, obese, or have other weight-related complications [201].

Bakery and flour products. In bakery and flour products (bread, noodles, cakes, cookies, etc.), seaweed is usually used in the form of fine powder [202–204]. Seaweed forms stable mixtures and emulsions with dough, improving the functional properties of the end products. Added to bakery and flour products, seaweed decreased the color values of lightness, redness, and yellowness [205].

When added to bread, polyphenol-rich brown seaweeds (*A. nodosum* and *F. vesiculosus*) significantly reduce carbohydrate digestion compared with the control bread. However, the heat treatment of seaweed in bread during baking lowers its polyphenol content, which may reduce the seaweed's ability to inhibit carbohydrates digestion *in vitro* [206].

Bread made from flour enriched with fucoidan extracted from the brown seaweed *U. pinnatifida* has a significantly high specific volume and softer crumb. Its improved quality is associated with a high production of CO_2 during proofing. Fucoidan's antitumorous activity *in vitro* is preserved even after baking [207]. The brown seaweed *F. vesiculosus* can be added to bread as a natural antioxidant, as well as to increase its nutritional value. The addition of *F. vesiculosus* powder increased the longitudinal viscosity of the dough, which decreased its sponginess at the end of proofing, compared to a typical wheat bread formulation [208].

Food additives are used to achieve a certain technological or sensory effect [209]. The growing consumer interest in natural products has led to an increased demand for natural food additives among food and beverage manufacturers. These additives are believed to have health benefits and are used as functional ingredients and natural sources of soluble dietary fiber.

Seaweed has a naturally salty taste due to its high content of minerals such as potassium, which can be used as a healthy substitute for sodium to reduce the risk of cardiovascular disease [95].

Food hydrocolloids produced from brown seaweed are biopolymers that are widely used as thickeners (in soups, gravies, salad dressings, sauces, and fillings), moisture-holding agents, stabilizers, emulsifiers, and gelling agents (in jam, jelly, marmalade, restructured foods, and low-fat products) [42, 166]. The global hydrocolloid market amounted to $9.7 billion in 2020 and is estimated to reach $13.36 billion by 2026 [210].

3D food printing is a new technology that can produce any food product a consumer might desire. For example, it can be used to develop a product with the exact nutritional value, the most beneficial nutrients, and without the ingredients a consumer is allergic to. It can even predict or personalize the taste, color, shape, and size of the food product [211]. Hydrocolloids are added to facilitate extrusion during 3D printing. Sodium alginate determines rheological properties and therefore is widely used in 3D food printing [212].

Drinks. Brown seaweed components are added to provide drinks with functional properties. For example, fucoidan, a water-soluble polysaccharide, is added to beverages such as tea, coffee, fruit drinks, etc. Fucoidan-rich seaweed is non-toxic and has antioxidant activity. Functional tea was developed from the brown seaweed *Sargassum binderi*. It was supplemented with lemon essence to mask the seaweed's unpleasant taste and smell and thus improve its consumer acceptance [213]. Another example is a functional pasteurized apple drink, in which fucoidan obtained from the brown seaweed *F. vesiculosus* exhibited biological activity as an antimicrobial agent against *Listeria monocytogenes* and *Salmonella enterica serovar Typhimurium* [214].

CONCLUSION

The production of functional food products based on brown seaweeds has enormous potential due to their unique biochemical composition. Brown seaweeds are rich in polysaccharides, dietary fiber, proteins, vitamins, minerals, and other nutrients, which contributes to high consumer interest. A number of authoritative *in vitro* studies have proven the effectiveness of food products based on brown seaweeds. Developing new competitive products is an important step in promoting seaweeds further and making them commercially viable. Also, more studies are needed to determine the safety of brown seaweeds during their harvesting, cultivation, and processing, including the environmental impact. In particu-

lar, new functional foods based on brown seaweeds should be thoroughly examined for the presence of pollutants, allergens, heavy metals or other substances that may pose a risk to both humans and the environment.

The incorporation of seaweeds into functional foods and a daily diet could potentially contribute to global food security in the future.

CONTRIBUTION

All the authors contributed equally to the study and bear equal responsibility for the information published in this article.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Quality management in animal farming

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Abstract:
The article introduces a long-term analysis of statistics and experimental research in domestic animal farming. Dairy cattle breeding is the dominant industry in Russian livestock farming. Its annual productivity has reached 6000 kg of milk per cow, while some farms and regions have brought this indicator up to 8000–10 000 kg. Unfortunately, the quality of milk, meat, and other livestock products is often neglected in favor of productivity.
Human health is believed to depend on nutrition. However, this dependence requires more specific evidence. For instance, the exact effect of livestock product quality management on the life and longevity of the population still remains unknown. Some studies revealed that pig breeds differ in the cholesterol status of fat; other research teams invented a high-quality green alfalfa dry feed, which provides animals with biologically active substances and normalizes metabolic processes.
This review highlights some promising scientific directions in fundamental and applied knowledge that can be beneficial to human health.

Keywords: Productivity, product quality, biologically active substances, technology, Holstein cows

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A state economy is effective when the life expectancy of the local population keeps increasing. Human longevity is known to depend on such factors as healthcare, lifestyle, climate, living conditions, genetic profile, etc., and other factors might be revealed in the future. In this respect, the effect of human diet on life expectancy is a very promising research direction. Most diets include livestock products. Livestock farming, which is almost as old as humanity itself, is one of the most significant factors that affect nutrition quality and, consequently, life span. For example, blood and colostrum are universal products vital for neonatal and postnatal fetal development. Chirvinsky and Maligonov formulated this correlation as a law, according to which blood and colostrum deficiency hinders fetal development [1]. Therefore, nutrition is a top priority factor that determines life expectancy, both in animals and humans.

In Russia, increasing milk and meat productivity has long been the most popular scientific direction, probably, as a result of the long-term shortage of animal food products. For many years, agricultural science gave priority to selection. However, it takes as long as 25–30 years to create a new highly productive breed of dairy or meat cattle. All efforts being concentrated on cross-breeding, while food quality management remained beyond the scope of scientific and industrial attention. As a result, cattle farming lacked funds to raise the quality of personnel and equipment in the sphere of milk and meat quality studies. Feeding technology, feed mixing, animal husbandry, and livestock raw material processing are important scientific areas related to product quality. Unfortunately, domestic agricultural science still fails to see them as priority areas. Nowadays, high milk yields are usually achieved by cross-breeding domestic cow breeds with Holsteins, and economic entities and government bodies tend to treat other economically important traits as insignificant. Naturally, contemporary Russian dairy and meat farming finds itself in a difficult situation: the high annual milk productivity, which exceeds 6000 kg per cow, is accompanied by the low quality of Holstein herds, where the reproduction has dropped to 76.4% calves per 100 cows and the productive longevity is as poor as 2.67 calving cycles per cow. Under such conditions, milk quality requires a comprehensive analysis, since cows' early retirement is most often associated with metabolic disorders, which inevitably affects the milk quality profile [2].

Colostrum is one of the most wholesome livestock products: it contains biologically active substances that provide newborns with vital nutrients and protect them from pathogenic microorganisms. Due to its immunomodulatory properties, expanded and long-term use of natural and frozen colostrum can improve the health of children and calves.

Colostrum composition in freshly-calved cows is known to depend on their diet. A balanced premix can double the amount of all amino acids in colostrum, compared to randomly purchased premixes. So far, no studies have featured further effects of colostrum on calves or children. Commercial livestock farming also knows selection methods that increase the productive longevity of dairy cattle. For instance, the Canadian company Immunity + has been selecting bull sires with increased immunity for 20 years and managed to increase the productive longevity in descendants.

Milk processing technology is another priority research topic. Thermal processing can be combined with other disinfecting methods or even excluded to preserve vitamins, enzymes, hormones, and other biologically active substances. Such studies open up new prospects for a more effective use of milk in children's or dietary foods.

Feed is the most effective way to change the composition and quality of milk, meat, and eggs. Unfortunately, this prospective area remains largely understudied. However, many years of production experience prove the supreme quality of Siberian milk and butter. In Siberia, cows feed on natural saline soil pastures and consume herb mix hay, which gives the resulting dairy products attractive sensory properties. Cattle that feed on monocultures grown on arable land produce livestock products of much inferior quality. Contemporary science cannot predict the exact effect of the abovementioned products on human health: this issue is a matter of some future comprehensive research.

The sensory profile of livestock products also depends on the microbial composition and count in the gastrointestinal tract of cows. The microbiota, in its turn, depends on the diet, namely, on vitamins, minerals, and easily digested carbohydrates [3].

The future of livestock farming lies with the combination of complex metabolism-regulating feed additives and breakthrough production technologies of high-quality roughage and succulent feed. In this respect, solar insolation and physical activity are the least-studied factors that affect livestock products.

Meat of young cattle, e.g., calves or foals, raised in line with a special technology, can improve children's metabolism. According to such technologies, milk-fed calves and foals graze on natural or artificial pastures from spring to late autumn, unlimited in movement and solar exposure. Such conditions provide especially valuable meat products. Free-range farming with temporary cattle barns is applied in many Russian regions, including Yakutia, Buryatia, Khakassia, Transbaikalia, Gorny Altai, etc. Veal and young horse meat are in high demand. This method is opposed to the traditional Italian technology that presupposes zero-grazing rearing and fattening of young cattle in industrial complexes. However, the resulting beef is of poor quality.

As the production of poultry and pork keeps increasing, the quality standards are becoming stricter. The current task of modern science is to determine the nutrients that define the quality of livestock products in order to use them in feeding farm animals.

The threshold level of productivity depends on the content of metabolic energy, proteins, easily digested carbohydrates, and some biologically active substances in the diet. All these nutritional elements correlate with feed quality. However, most agricultural producers in Russia have feed composition below average, i.e., of quality class II or non-class, while others do not bother do assess the quality of their feeds at all.

The livestock industry and the food science are currently facing with the fundamental task of establishing the relationship between the feed quality, the microbial development, and the quality of milk, meat, and eggs.

Of course, it is the task of economics to determine the priorities in assessing the elements of technology for livestock products. For example, modern top quality class feeds possess excellent digestibility and are extremely effective. They involve such breakthrough technologies as artificial drying of green alfalfa in a combined aerodynamic dryer, which combines six drying methods and provides high feed quality. Although the method could revolutionize the entire domestic cattle breeding, the Voronezh Region remains the only territory in Russia to use this technology so far [4]. As an alternative, farmers can provide cattle with natural or artificial microbial or enzyme additives that facilitate feed breakdown and digestion. Unfortunately, the current lack of experimental farms, physiologic al yards, and funds makes it next to impossible to perform reliable scientific experiments in animal husbandry.

The recent geopolitical events have proved that food independence is the most urgent task Russia has to solve, and this task requires combined efforts of fundamental science, applied studies, and production.

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