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Physico-chemical properties and micronutrient profile of functional chocolate with mare's milk powder and resveratrol

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ABSTRACT

Cardiovascular diseases (CVDs) represent one of the most severe healthcare challenges for many countries, accounting for one-third of all deaths worldwide. One significant risk factor contributing to the increase in CVD prevalence is the disruption of dietary patterns, characterised by a deficiency of essential macro- and micronutrients in the population's diet. In this context, a particularly relevant direction in food biotechnology and preventive medicine is the nutritional prevention of CVD by developing new functional food products with pronounced health-promoting and cardioprotective properties. Mare's milk and resveratrol have a balanced chemical composition and can be utilised to prevent many chronic non-communicable diseases, including CVD. This study aims to develop functional milk chocolate enriched with dried mare's milk and resveratrol and determine its physicochemical properties and micronutrient profile. The replacement of cow's milk in chocolate with dried mare's milk and fortification using resveratrol powder resulted in significant changes in the product's properties, which varied depending on the quantity of added functional ingredients. These include the mass content of B vitamins, vitamins A and E, calcium, magnesium, essential amino acids, and polyunsaturated fatty acids. Sensory analysis revealed changes in the product's organoleptic properties following incorporating these functional components. Thus, adding 20% dried mare's milk and 0.10% resveratrol can enhance the biological value and improve the sensory qualities of the chocolate products.

Keywords: functional product, mare's milk powder, resveratrol, healthy diet, micronutrient profile, nutritional prevention.

INTRODUCTION

In recent decades, one of the acute problems in global public health has been the negative trend of increasing chronic non-communicable diseases, which account for about 70% of total mortality worldwide **[1]**. The widespread prevalence of non-communicable diseases is due to environmental and physiological factors and individual socio-behavioural correlates of population nutrition **[2]**. Cardiovascular diseases account for a significant proportion of these non-communicable diseases. According to statistics provided by the World Health Organization, 43% of mortality from non-communicable diseases is due to cardiovascular disease (CVD) (up to 17.9 million people annually) **[3]**. One of the primary factors in the development of CVDs is a nutritional status disorder characterised by a decrease in the levels of essential macro- and micronutrients, including enzymes, vitamins, macro- and microelements, crucial amino acids, polyunsaturated fatty acids, polyphenols, and other minor biologically active compounds.

Traditional drug prevention methods for these pathologies, characterised by the use of pharmacological agents to reduce the risk of CVDs, have several disadvantages due to undesirable side effects and high drug costs, which make it difficult for people in developing countries to access these treatments. A practical approach to addressing this issue is to optimise and correct the population's nutritional status by introducing functional food products into

preventive dietary regimes with specific component compositions and properties **[4]**. Developing new products with pronounced biomedical properties for preventing CVD is highly relevant in this context.

When developing functional food products, a primary condition for selecting a base product for fortification is its widespread consumption **[5]**. Confectionery products, particularly chocolate, certainly fall into this category. In recent years, there has been a steady increase in the popularity of confectionery products worldwide, not only among children but also among adults **[6]**. Milk chocolate holds a special place in the range of confectionery products. It is made from cocoa beans (*Theobroma cacao L.*) and cow's milk and is widely consumed globally due to its high sensory appeal. However, most chocolate products are characterised by unbalanced chemical composition and low biological value due to high levels of saturated fats, sugar, flavourings, and artificial additives, which can negatively impact various body systems **[7]**. Given these factors, a promising approach is to develop new types of chocolate products for healthy eating by incorporating non-traditional dairy raw materials and other biologically active substances into the chocolate matrix.

One promising raw material source for producing functional dietary preventive nutrition products is mare's milk **[8]**. The unique chemical composition of mare's milk includes an albumin component, a relatively low protein and fat content, and high levels of lysozyme and lactoferrin **[9]**. The fatty acid profile of mare's milk is distinguished by a high content of omega-3 and omega-6 polyunsaturated fatty acids, including linoleic, α-linolenic, docosahexaenoic, and eicosapentaenoic acids, which help reduce the risk of cardiovascular disease **[10]**. Mare's milk also has a balanced vitamin and mineral profile, containing high B vitamins, ascorbic acid, tocopherol, cholecalciferol, magnesium, potassium, calcium, phosphorus, and other elements **[11]**. Due to its unique properties, mare's milk is of great scientific interest in producing healthy food products.

Phenolic compounds, particularly resveratrol powders, have become particularly interesting in preventing cardiovascular diseases (CVD) **[12]**. Resveratrol is a natural bioactive compound within the plant polyphenol group, synthesised by some plants as a defence against parasites and microorganisms, and it possesses notable tonic, antioxidant **[13]**, anti-inflammatory **[14]**, and cardioprotective properties **[15]**. Japanese scientist Takaoka Michio first extracted resveratrol from the roots of a white hellebore (*Veratrum grandiflorum O. Loes*) **[16]**. The primary source of resveratrol is grapes (*Vitis vinifera*), with the highest concentrations found in the skin of dark-coloured grapes **[17]**. In nature, this bioactive compound exists in both free and glycosylated forms. Glycosylated resveratrol exhibits better solubility and stability, is well absorbed in the digestive tract, and is metabolised in the liver to form conjugates **[18]**. Scientific literature supports resveratrol's positive role in the treatment and prevention of obesity **[19]**, diabetes mellitus **[20]**, and several cardiovascular diseases **[21]**. Including resveratrol in the daily diet has been shown to reduce blood serum levels of glucose, cholesterol, free fatty acids, and triglycerides **[22]**.

Scientific Hypothesis

Given the high biological value, hypoallergenic nature, balanced content of essential micronutrients, and functional properties of mare's milk, we hypothesise that using non-traditional dairy raw material as a foundation for developing speciality food products may lead to the creation of new types of chocolate designed for a healthy diet, with targeted restorative and preventive properties.

Objectives

Primary objectives: This study aimed to formulate a functional chocolate product enriched with mare's milk powder and resveratrol and to evaluate its physicochemical properties and micronutrient composition comprehensively.

MATERIAL AND METHODS

Samples

Samples description: The experimental samples consisted of chocolate products made from mare's milk powder with varying concentrations of resveratrol, according to the recipe and production technology developed by the authors. A control sample of milk chocolate based on cow's milk, supplemented with chopped sultanas sourced from the retail market, was used for comparison. The formulations of both the control and experimental chocolate samples, which varied in their content of mare's milk powder (MMP) and resveratrol powder (RP), are shown in Table 1.

Table 1 Formulations of experimental samples of functional chocolate with varying contents of mare's milk powder (MMP) and resveratrol powder (RP) (%).

Ingredients	Control	Quantity, %		
		MMP 5/RP 0.05	MMP 10/RP 0.075	MMP 20/RP 0.10
Maltodextrin	–	49.675	44.650	34.625
Sugar	44.50	–	–	–
Cocoa butter	25.00	25.00	25.00	25.00
Cocoa mass	20.00	20.00	20.00	20.00
Cow's milk powder	10.00	–	–	–
Mare's milk powder	–	5.00	10.00	20.00
Soy lecithin	0.3	0.3	0.3	0.3
Crushed sultanas	0.2	–	–	–
Resveratrol powder	–	0.05	0.075	0.10

The pilot-scale batches of chocolate are shown in Figure 1.

Figure 1 Pilot-scale batches of chocolate.

Samples collection: Sample collection was conducted to ensure the representativeness and reliability of the data obtained. The experimental chocolate samples were prepared in strict accordance with the established recipe. The control sample was acquired from a retail store in Almaty, Kazakhstan.

Samples preparation: Sample preparation: The chocolate samples were produced according to the aforementioned methodology, cooled to 4 °C, and transported in a thermal container capable of maintaining the product temperature for 48 hours.

Number of samples analysed: 16

Chemicals

All reagents were of analytical grade and were purchased from Kazhimbaza (Kazakhstan). Standard vitamin solutions were prepared using Vitamin A, Vitamin E, ascorbic acid, and B vitamins (B1, B2, B3, B6, B9)

manufactured by Sigma-Aldrich (CAS, USA). To identify methyl esters of fatty acids, a standard solution from SUPELCO Corporation, 37 Component Fatty Acid Methyl Ester Mix (47885-U), was used, which includes 37 methyl esters of fatty acids.

Animals, Plants and Biological Materials

Resveratrol powder from black grapes (*Vitis vinifera*).

Mare's milk (SaumalBioTech LLP, Karaganda, Kazakhstan).

Instruments

Forming Machine DEDY (Germany), Cooling Tunnel DEDY (Germany), Tempering Kettles DEDY (Germany), Vibrating Tables DEDY (Germany), Packaging Machine KD-260 (China), Autonomous Thermo Tank TEM-100 (Russia), 20L Small chocolate conche refiner machine (China), Electronic Scales (210/0.001g) Sartorius (Germany), pH метр HI 2213 (Germany), High-performance liquid chromatograph: Perkin Elmer Series 200 – HPLC (USA), Atomic absorption spectrometer: Agilent 240 FS AA (USA), Gas chromatograph: Agilent 6890N (USA), Orbital shaker: ELMI S-3.02L A20 (Latvia).

Laboratory Methods

Preparation of Milk Chocolate Using Mare's Milk Powder and Resveratrol

The process of making chocolate with the addition of mare's milk powder and resveratrol includes the following technological stages: preparation of the raw materials, weighing of all ingredients according to the recipe (Table 1), preparation of the chocolate mass, moulding of chocolate products, wrapping, and packaging.

Preparation of the chocolate mass begins with loading grated cocoa, maltodextrin, cocoa butter, mare's milk powder, and resveratrol extract powder into the melanger. These components are ground for 20 minutes, after which the resulting mass is fed into a five-roll mill. The milled mass is then transferred to a conching machine. After conching, the chocolate mass enters an intermediate, intensively mixed collector. The chocolate mass then passes through a homogeniser and undergoes a second conching stage at a temperature of 65±5°C. After passing through a vibrating sieve, the finished chocolate mass, at a temperature of 65°C, is pumped to an intermediate collector, which is tempered intensively for 72 hours at a temperature of 45±5°C. At the end of the tempering process, the chocolate is cooled to 30±2°C.

Then, the chocolate mass is cast into moulds using an automatic moulding machine. After moulding, the chocolate products are cooled to 8±2°C, packed, and prepared for transportation and storage.

Determination of Physico-Chemical Characteristics of Chocolate

The physical and chemical characteristics of milk chocolate, in particular, the mass fraction of moisture, ash, protein, and lipid content, were analysed according to the methods of the Association of Official Analytical Chemists **[23]**.

The mass fraction of carbohydrates (%) was calculated according to the formula (1):

Mass carbohydrate fraction (%) = 100% - (moisture % + ash %, proteins % + lipids %) (1)

Caloric content was calculated using the formula (2):

Total caloric value, kcal/100 g = (Proteins x 4) + (Carbohydrates x 4) + (Lipids x 9) (2)

Sensory analysis

Sensory analysis of milk chocolate was conducted in the Laboratory of Food Biotechnology and Specialized Foods at the Kazakh Academy of Nutrition in Almaty, Republic of Kazakhstan. The analysis took place in a dedicated room free from extraneous odours. The chocolate parameters evaluated included appearance, taste and aroma, structure, shape, and texture. The analysis methods involved a visual assessment of the samples and tasting.

Determination of Vitamin Content

The vitamin content of chocolate was assessed using high-performance liquid chromatography (HPLC) on a Perkin Elmer Series 200 HPLC liquid chromatography. The HPLC system comprised a Flexar UHPLC pump connected to a photometric detector (wavelength range 190–700 nm), a column thermostat, and an autosampler for automated sample preparation and injection. HPLC separation was performed on a reversed-phase column, a Perkin Elmer Pecosphere Reduced Activity C8 LC Cartridge Column (PE-02580191). A mobile phase buffer solution was prepared using a 1:1 mixture of methanol and acetonitrile combined with KH_2PO_4 (25±3°C, pH 3.3±0.5). Retinol and tocopherol were extracted from the chocolate samples using supercritical fluid extraction. Vitamins B1, B2, B3, and B6 were extracted with 0.01 M HCl, while vitamin B9 was extracted with 0.1 M $NaHCO_3$ in water. Ascorbic acid was extracted using metaphosphoric acid (HPO_3) **[24]**.

Determination of Mineral Content

The analysis of mineral content in the studied chocolate samples was performed using an Agilent 240 FS AA atomic absorption spectrometer equipped with flame and electrometric atomisers. The process involved atomising the mineralised sample solution in an air-acetylene flame. The macro- and microelements in the mineralised solution are converted into an atomic state under the influence of the air-acetylene flame. The degree of light absorption at the wavelength corresponding to the resonance line is proportional to the metal concentration in the test sample **[25]**.

The results of mineral element concentrations in the solution (μg/cm³) and the content in the finished products (mg/kg) were processed according to the following formula (3):

$$X = \frac{C \times Y \times K - C_k \times Y_k}{m} \quad (3)$$

Where:

$C_к$ – contamination level in the control experiment, μg/cm^3;

K – dilution or concentration factor of the initial sample solution, equal to the ratio of the volume of the analysed solution to the volume of the aliquot taken for dilution or concentration;

Y – volume of the initial sample solution, cm^3;

Y_k – volume of solution in the control experiment, cm^3;

P – sample weight, g.

Determination of Amino Acid Content

A high-performance liquid chromatography method was used to determine the amino acid content of chocolate. Substituted and essential amino acids were analysed on a Perkin Elmer Series 200 HPLC liquid chromatograph. The analysis of amino acid content in the samples under study consisted of initial removal of lipids by extraction with a mixture of organic solvents, acid hydrolysis of proteins, preparation of DABS derivatives of amino acids and evaluation using high-performance liquid chromatography **[26]**. Amino acid analysis conditions: temperature 22±5°C, air humidity not more than 75%, mains voltage 220±20V, AC frequency 50±1Hz.

The mass concentration of each amino acid in the analysed chocolate sample was calculated according to the formula (4):

$$Ci = \frac{V1 \times V3 \times X}{Mprobe \times V2} \quad (4)$$

Where:

Ci – concentration of amino acid in the sample mg/100 g;

V1 – volume of solvent in which the sample was dissolved after hydrolysis, cm^3;

V2 – volume of solvent in which the sample was dissolved after hydrolysis, cm^3; V3 - volume of solvent in which the sample was dissolved after hydrolysis, cm^3;

V3 – volume of solvent in which DABS-derivatives are dissolved, cm^3;

Mprobe – sample weight g;

X – concentration of amino acid obtained from the calibration graph, 10-6 mg/cm^3;

100 – conversion coefficient of amino acid concentration per 100 g of product.

Determination of Fatty Acid Content

The content of fatty acids and fatty acid trans-isomers in the tested chocolate samples was determined using an Agilent 6890N chromatographic system equipped with a flame ionization detector, a helium carrier gas, a hydrogen generator, an air generator, and a compressor.

Sample preparation involved lipid extraction using solvents, solvent evaporation with a rotary evaporator, and the preparation of fatty acid methyl esters **[27]**. Lipid extraction was conducted by precipitating proteins and other impurities using a 2:1 chloroform-methanol solvent mixture. After mixing the sample with solvents, the aliquot was placed on an ELMI S-3.02L A20 orbital shaker at 200 rpm for 4 hours to ensure thorough mixing. After mixing, phase separation occurred, and the upper-phase supernatant was transferred to a 250 cm³ flask and evaporated in a rotary evaporator at 70±2°C to remove chloroform and methanol.

The obtained fat fraction was then used to prepare fatty acid methyl esters: 100 μl of the fat sample was placed into a 5 ml Eppendorf tube. For methyl ester formation, 100 μl of 2 N sodium methylate solution (2N $NaOCH_3$) was added. Next, 2 ml of hexane was added to the mixture and then centrifuged at 37±2°C and 10,000 rpm for 5 minutes. After centrifugation, the upper phase was transferred to 2 cm³ vials for fatty acid content analysis.

The analysis was performed on an Agilent 6890N gas chromatograph with a plasma ionisation detector and a 60 m DB-23 capillary column. A standard solution from SUPELCO Corporation (37 comp. FAME mix, 47885-U) containing 37 fatty acid methyl esters was used for qualitative identification of fatty acid methyl esters.

Description of the Experiment

Study flow: At the initial stage of the study, control and experimental samples of chocolate products were developed with varying ratios of dried mare's milk and resveratrol. In the next stage, changes in the physicochemical and sensory properties of the samples were assessed, and the contents of vitamins, minerals, amino acids, and fatty acids were determined. At the final stage, statistical analysis was conducted on the results obtained from the experiments.

Quality Assurance

Number of repeated analyses: 3

Number of experiment replication: 3

Reference materials: Vitamin A, Vitamin E, Ascorbic acid, and B group vitamins (B1, B2, B3, B6, B9) manufactured by Sigma-Aldrich (CAS, USA). 37 Component Fatty Acid Methyl Ester Mix (47885-U) from SUPELCO Corporation.

Calibration: To ensure the accuracy and reliability of measurements during the study, the calibration of instruments and methods was conducted. The calibration of laboratory equipment was performed in accordance with established standards and manufacturers' recommendations. To verify measurement accuracy, reference materials conforming to international standards, as well as secondary reference materials for additional data verification, were utilized.

Laboratory accreditation: The experiments were conducted in the testing laboratory of Nutritest LLP, accredited by the National Accreditation Center of the Republic of Kazakhstan (accreditation certificate No. KZ987702B056813929).

Data Access

The data are available upon request from the corresponding author due to confidentiality, as they contain personal information of the study participants, which is protected by patents.

Statistical Analysis

The obtained results were statistically analyzed using the software program “Statistica” v. 12.0 (StatSoft Inc., USA), calculating the arithmetic mean, standard deviation, and standard error of the mean for each parameter. Student's t-test was employed for comparisons, and differences were considered significant at $p \leq 0.05$. The presented results are based on repeated experiments conducted to evaluate the physicochemical parameters of the samples, as well as their vitamin, mineral, amino acid, and fatty acid compositions

RESULTS AND DISCUSSION

Effect of adding mare's milk powder and resveratrol on physicochemical and sensory parameters of chocolate

Experimental samples of functional chocolate with the addition of mare's milk powder at levels of 5%, 10%, and 20% (MMP 5, MMP 10, MMP 20) and resveratrol powder at concentrations of 0.050%, 0.075%, and 0.10% (RP 0.050, RP 0.075, and RP 0.10) were developed to determine the optimal levels of these components in the chocolate composition. The dependence of the physicochemical and sensory parameters of the chocolate on the dosage of the biologically active components was investigated in the analysed samples. Chocolate's physicochemical and sensory parameters were analysed with varying contents of mare's milk powder and resveratrol powder. The results of the study on the physicochemical parameters of the standard formulation (control) and the experimental samples with the addition of mare's milk powder and resveratrol in different ratios are presented in Table 2.

Table 2 Physico-chemical parameters of chocolate.

Parameters	Control	Quantity, %		
		MMP 5/RP 0.050	MMP 10/RP 0.075	MMP 20/RP 0.10
Protein, %	8.25±0.11	6.81±0.14	7.12±0.16	7.40±0.11
Fats, %	43.34±1.11	42.53±1.08	39.22±1.07	37.84±1.16
Carbohydrates, %	43.08±1.05	45.45±1.12	48.38±1.18	49.42±1.09
Moisture, %	4.12±0.08	4.10±0.09	4.04±0.10	3.96±0.08
Ash, %	1.21±0.03	1.11±0.03	1.24±0.06	1.38±0.03

Protein intake stimulates fundamental metabolic processes and is particularly important in a healthy diet **[28]**. In addition to providing the body with essential amino acids, proteins and peptides may also have pronounced anti-inflammatory **[29]**, antihypertensive **[30]**, antithrombotic **[31]**, and hypocholesterolemic effects **[32]**. Even though the total protein level in the experimental chocolate samples decreased slightly compared to the control (from 10.30% to 17.45%), replacing cow's milk with mare's milk powder in chocolate is of considerable interest. The protein component of mare's milk is represented by albumin and globulins **[33]**, as well as low molecular weight peptides **[34]** and free amino acids **[35]**, with low levels of high molecular weight proteins, which contribute to the immunobiological, detoxifying, and antioxidant properties of this product **[36]**. However, it should be noted that these are only preliminary data, and further, more detailed studies are required to investigate the protein composition of these milk types.

Lipid levels in food intake and daily diet, especially low-density lipoprotein (LDL), are significant determinants of cardiovascular and other diseases **[37]**. The replacement of cow's milk with dried mare's milk resulted in changes in lipid levels in the experimental samples. Thus, in the MMP 5/RP 0.050 sample, there was a slight decrease in total fat by 1.87% ($p<0.05$) compared with the control. The MMP 10/RP 0.075 sample showed a 9.51% decrease in lipid levels ($p<0.05$). The most significant reduction in total lipid content was observed in the sample containing 20% mare's milk powder and 0.10% resveratrol powder (MMP 20/RP 0.10), amounting to 12.69% ($p<0.05$) compared with the control sample.

Changes in the carbohydrate profile of the chocolate products were also observed in the experimental samples. Due to the high lactose content in mare's milk, the carbohydrate content in the experimental samples increased significantly by an average of 24.0% ($p<0.05$) compared to the controls, despite the high maltodextrin content.

One of the main criteria for assessing the consumer properties of a product is the analysis of its sensory indicators **[38]**. The sensory analysis included a comparative characterisation of the control and experimental chocolate samples. Important sensory indicators such as appearance, colour, shape, taste, aroma, structure, and texture were evaluated **[39]**. Sensory analysis of the indicators of the developed speciality chocolate products was conducted through visual and tasting assessments based on a ten-point scale for each qualitative characteristic. The results of the analyses are shown in Figure 2.

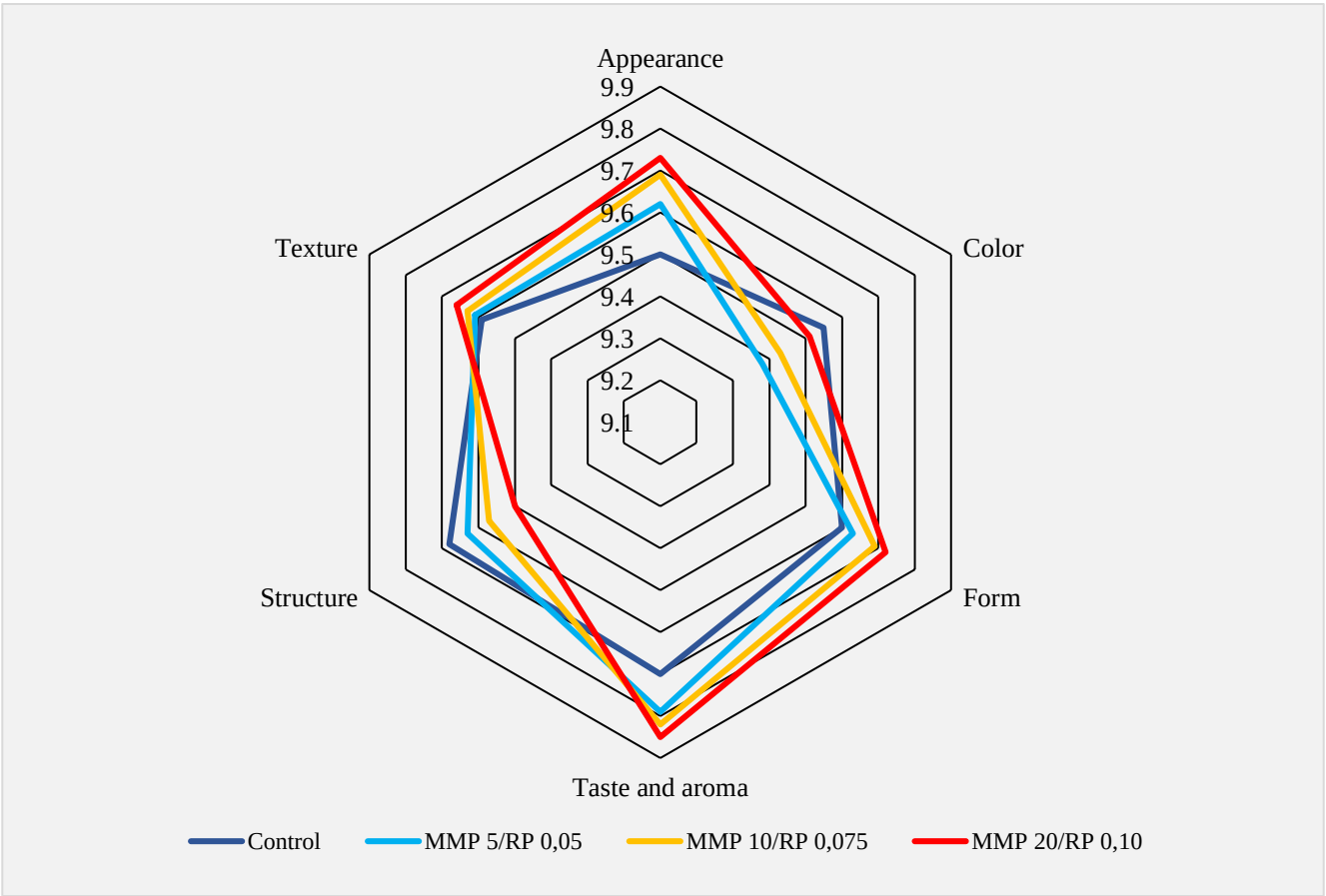

Figure 2 Profilogram of the effect of different concentrations of mare's milk powder and resveratrol on sensory parameters of chocolate.

The study's results on the physicochemical and sensory parameters of experimental chocolate samples with different contents of mare's milk powder and resveratrol, compared with the control, indicate changes in product properties after introducing these functional components. According to physicochemical parameters, the experimental chocolate samples were characterised by a balanced protein, lipids, and carbohydrate content. When mare's milk powder and resveratrol were added, protein content was decreased. The carbohydrate content in the experimental samples increased significantly with the addition of these ingredients. The mass fraction of carbohydrates in the MMP 5/RP 0.050 sample increased by 22.88% compared to the control. In the MMP 10/RP 0.075 and MMP 20/RP 0.10 samples, decreases in carbohydrate content of 23.30% and 24.02% were observed, respectively.

The sensory analysis results showed that the addition of resveratrol powder and mare's milk powder at concentrations of 0.050%, 5%, 0.075% and 10%, respectively, did not significantly affect the product's sensory characteristics. Incorporating this combination of functional ingredients at a concentration of 0.10% and 20% positively affected the chocolate's appearance, texture, and shape, providing the product with a pleasant aroma and flavour from the ingredients used. However, when the dosage of resveratrol and mare's milk powder was further increased, significant changes in the sensory properties of the samples were observed, particularly a disruption in the structure of the chocolate and the appearance of residual bitterness. Based on the analysis of physicochemical and sensory parameters, the optimal doses were 20% for the mare's milk powder and 0.10% for resveratrol.

Vitamin and Mineral Content

A food product's balanced vitamin and macro- and microelement content determines its biological value and preventative properties **[40]**. Vitamins can improve the function of the heart muscle and blood vessels, as evidenced by the results of numerous studies **[41]**, **[42]**. Table 3 shows the results of the vitamin and mineral composition analysis of chocolate products enriched with resveratrol powder and mare's milk powder in different ratios compared to the control sample.

The analysis of vitamin and mineral content showed that the samples under study had low levels of vitamins and macro- and microelements, which is typical for chocolate products in general **[43]**. Nevertheless, the results of the conducted research indicate a tendency to increase the content of both fat-soluble and water-soluble vitamins and macro- and microelements in the experimental samples. The analysis of the mineral composition of chocolate indicates that the increase in ash content in the experimental samples corresponds to an increase in the content of the studied mineral elements. A deficiency in multivitamins, as well as minerals, reduces the overall resistance of the organism to the effects of foreign compounds and increases the risk of developing various metabolic disorders **[44]**, cancer **[45]**, and cardiovascular diseases **[46]**. Relatively high concentrations of vital nutrients, such as B vitamins, vitamins A and E, calcium, magnesium, iron, and zinc, were observed in the MMP 20/RP 0.075 chocolate sample due to adding mare's milk powder to the chocolate composition.

Table 3 Vitamin mineral composition of chocolate per 100 g of product.

Vitamins and minerals	Control	Quantity, %		
		MMP 5/RP 0.050	MMP 10/RP 0.075	MMP 20/RP 0.10
Vitamins				
Vitamin A, mcg	5.94±0.18	4.97±0.22	6.04±0.21	7.78±0.21
Vitamin E, mg	1.14±0.12	0.98±0.09	1.11±0.09	1.49±0.11
Vitamin B1, mg	0.28±0.01	0.19±0.01	0.27±0.01	0.38±0.02
Vitamin B2, mg	0.42±0.02	0.33±0.01	0.44±0.02	0.52±0.02
Vitamin PP, mg	0.51±0.01	0.41±0.01	0.53±0.02	0.67±0.01
Vitamin B6, mg	0.19±0.01	0.12±0.01	0.17±0.01	0.21±0.02
Vitamin C, mg	1.18±0.09	0.95±0.06	1.21±0.08	1.88±0.06
Minerals				
Ca, mg	223.46±1.45	189.53±1.39	226.80±1.48	272.00±1.51
Mg, mg	49.28±0.98	46.25±0.94	51.42±1.05	68.40±1.14
Fe, mg	1.18±0.15	1.01±0.12	1.20±0.16	1.44±0.17
Zn, mg	1.50±0.19	1.18±0.15	1.27±0.16	1.54±0.18

Amino Acid Content

The amino acid compositions of the studied chocolate samples were analysed. The content of amino acids in the experimental and control chocolate samples is presented in Table 4.

Table 4 Amino acid composition of chocolate in mg per 100 g of product.

Amino acids	Control	Quantity, %		
		MMP 5/RP 0.050	MMP 10/RP 0.075	MMP 20/RP 0.10
Essential amino acids				
Leucine	594.5±5.15	405.5±4.97	574.4±5.22	667.2±5.34
Isoleucine	191.2±2.11	168.5±2.23	185.4±2.09	204.3±2.15
Valine	238.4±2.34	197.5±2.27	224.6±2.31	299.1±2.45
Lysine	334.7±3.11	295.8±3.05	321.2±3.09	399.2±3.16
Methionine	83.7±0.86	61.8±0.71	72.2±0.75	95.6±0.93
Tryptophan	3.0±0.22	1.9±0.16	2.5±0.19	3.1±0.24
Threonine	234.9±2.21	189.2±2.05	222.4±2.09	279.9±2.34
Phenylalanine	202.7±2.35	194.6±2.24	231.2±2.29	279.9±2.39
Substituted amino acids				
Serine	192.5±2.04	168.8±1.89	189.5±1.94	224.9±2.21
Histidine	185.8±1.91	151.2±1.79	169.4±1.84	197.3±1.97
Arginine	346.6±3.24	302.2±3.19	343.5±3.21	405.9±3.53
Alanine	241.3±2.39	198.9±2.22	235.1±2.31	282.2±2.48
Cysteine	67.9±0.81	49.2±0.70	58.3±0.74	74.5±0.87
Aspartic acid	895.2±7.86	678.3±6.69	887.5±7.79	1026.1±8.05
Glutamic acid	984.5±9.09	786.2±8.01	905.8±8.99	1181.8±9.94
Proline	121.7±1.33	83.2±1.01	91.5±1.05	125.2±1.38
Glycine	133.7±1.28	98.2±1.05	138.4±1.34	189.1±1.68
Tyrosine	135.4±1.39	93.1±0.99	129.7±1.20	178.8±1.62

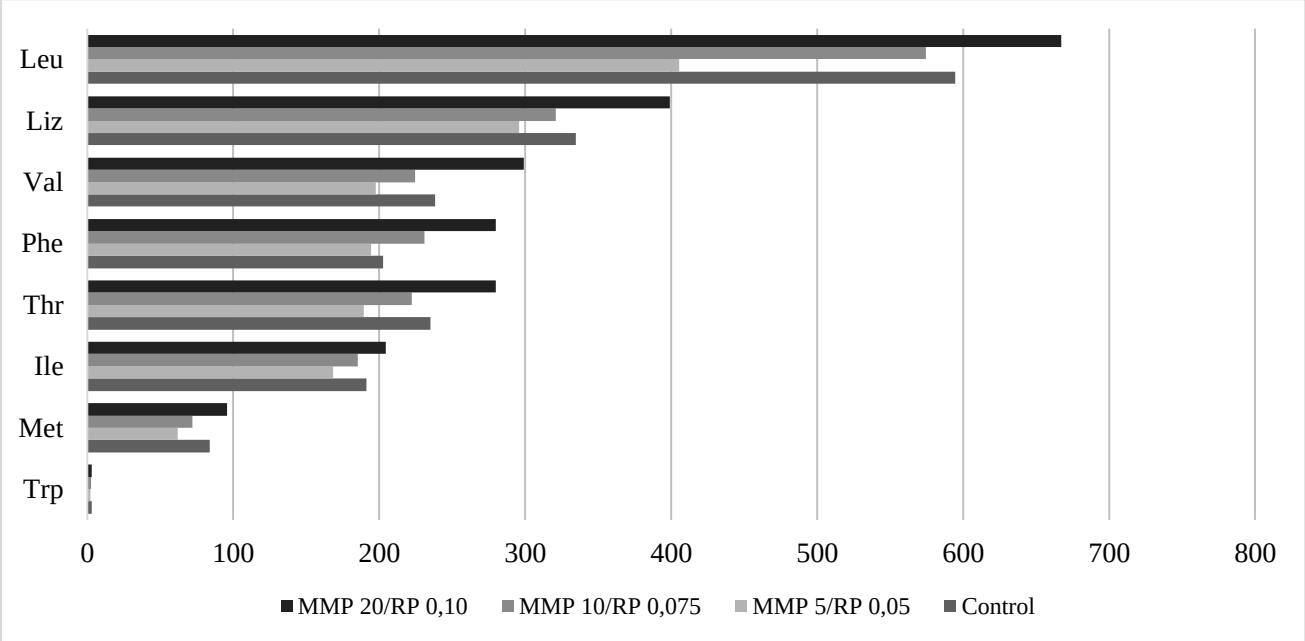

Figure 3 Essential amino acid content in experimental samples of specialised chocolate with the addition of dried mare's milk powder and resveratrol in different concentrations compared to the control.

The analysis of amino acid content in control and experimental samples of specialised chocolate showed that replacing cow's milk with mare's milk powder significantly increased the concentration of amino acids per 100 g of the product. Among the experimental chocolate samples, the highest total amino acid content (6114.1 mg/100 g of chocolate) was found in the experimental sample MMP 20/RP 0.10. The total content of essential amino acids amounted to 2228.3 mg/100 g of chocolate, which gives the products pronounced preventive properties.

The comparative analysis of essential amino acid content in experimental chocolate samples with the addition of mare's milk powder and resveratrol, compared to the control, is shown in Figure 3.

The results of the amino acid composition analysis showed that replacing cow's milk in chocolate with 20.0% dried mare's milk and 0.10% resveratrol increased the content of essential amino acids. In particular, there was a statistically significant increase in leucine by 12.23% ($p<0.05$), isoleucine by 6.85% ($p<0.05$), valine by 25.46% ($p<0.05$), lysine by 19.27% ($p<0.05$), methionine by 14.22% ($p<0.05$), tryptophan by 3.33% ($p<0.05$), threonine by 19.16% ($p<0.05$), and phenylalanine by 38.09% ($p<0.05$). The limiting amino acid is tryptophan at 3.1 g/100 g. The essential branched-chain amino acids (BCAAs) are particularly important—valine, isoleucine, and leucine **[47]**. A distinctive feature of BCAAs is that they are catabolized in extrahepatic tissues, rather than in the liver like other proteinogenic amino acids **[48]**. The balanced composition of these amino acids stimulates energy processes and ensures muscle contractions, which is an essential factor in preventive nutrition **[49]**.

Fatty Acid Content

The biological activity of fatty acids (FAs) is an important aspect that contributes to the maintenance of health and the prevention of various diseases **[50]**. Fatty acids can be classified into three main categories: saturated, unsaturated, and trans isomers, each of which has its own physiological effects on the body **[51]**.

The fatty acid profile of control and experimental samples of functional chocolate, with different contents of mare's milk powder (5%, 10%, and 20%) and resveratrol powder (0.05%, 0.075%, and 0.10%), was determined. All fat fractions of the tested samples were analyzed in three replicates and methylated on the same day. The DB-23 column (60 m) for chromatographic analysis was not changed throughout the experiment. The fatty acid composition of the control and experimental samples of functional chocolate is shown in Table 5.

Table 5 Amino acid composition of chocolate in mg per 100 g of product.

Fatty acids	Control	Quantity, %		
		MMP 5/RP 0.050	MMP 10/RP 0.075	MMP 20/RP 0.10
Saturated fatty acids (SFA)				
C4:0	ND	ND	ND	ND
C6:0	3.58±0.24	ND	ND	ND
C8:0	7.24±0.76	6.97±0.63	6.35±0.66	5.70±0.63
C10:0	2.89±0.18	2.62±0.19	2.35±0.13	1.77±0.11
C12:0	1.78±0.11	1.83±0.10	1.86±0.16	1.92±0.13
C14:0	3.86±0.21	2.91±0.19	2.12±0.22	1.51±0.17
C16:0	21.65±1.89	21.33±1.84	21.09±1.77	20.96±1.80
C17:0	1.64±0.09	1.70±0.08	1.75±0.11	1.80±0.09
C18:0	20.32±1.85	19.84±1.76	18.86±1.80	18.56±1.79
C20:0	14.74±1.26	14.86±1.22	14.92±1.19	15.08±1.27
C22:0	5.36±0.49	4.98±0.43	4.52±0.39	3.23±0.33
C24:0	2.45±0.16	2.25±0.13	1.61±0.09	0.94±0.07
∑ SFA	**85.51±7.24**	**79.29±6.57**	**75.43±6.52**	**71.47±6.39**
Monounsaturated fatty acids (MUFA)				
C16:1	2.23±0.15	2.94±0.16	3.43±0.19	4.47±0.19
C18:1	6.57±0.51	10.76±0.86	12.44±0.91	14.18±0.94
∑ MUFA	**8.8±0.66**	**13.7±1.02**	**15.87±1.10**	**18.65±1.13**
Polyunsaturated fatty acids (PUFA)				
C18:2 (ω-6)	3.04±0.26	4.18±0.32	5.85±0.34	7.16±0.48
C18:3 (ω-3)	1.43±0.08	1.74±0.12	1.91±0.18	2.08±0.19
∑ PUFA	**4.47±0.34**	**5.92±0.44**	**7.76±0.52**	**9.24±0.67**
Trans-isomers of fatty acids (TFA)				
C18:1 (trans-9)	1.22±0.07	1.09±0.06	0.94±0.05	0.64±0.03
C18:2 (trans-9,12)	ND	ND	ND	ND
∑ TFA	**1.22±0.07**	**1.09±0.06**	**0.94±0.05**	**0.64±0.03**

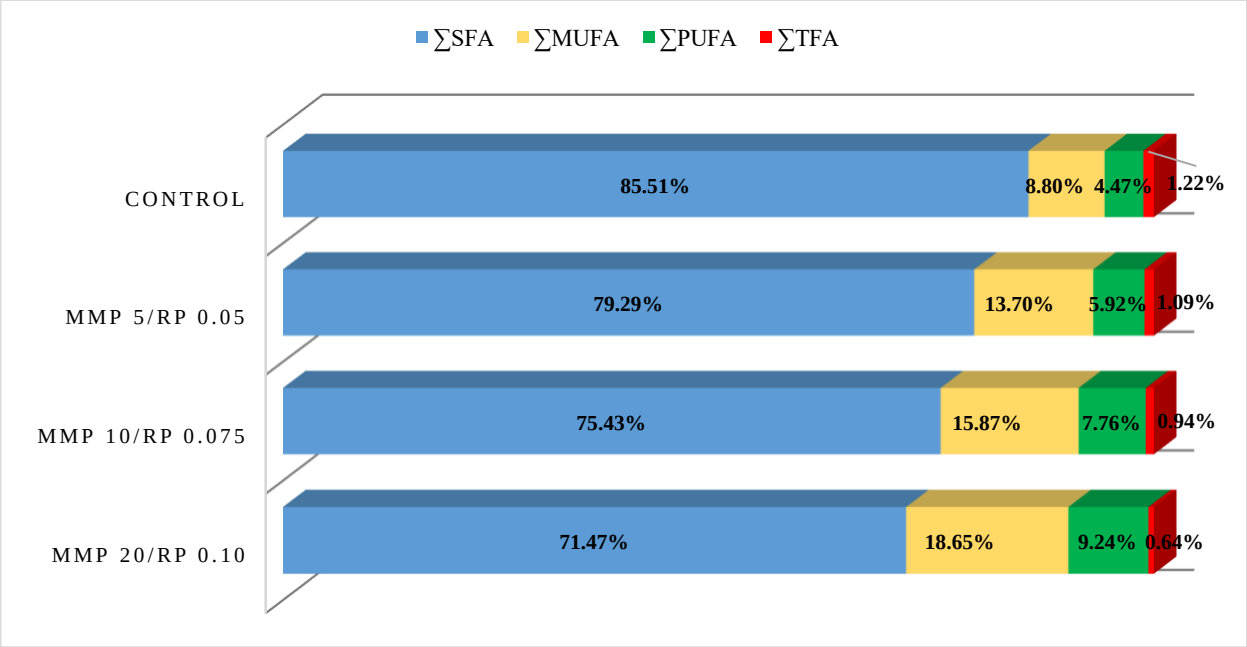

Figure 4 Comparative analysis of the fatty acid composition of control and experimental chocolate samples.

Figure 4 shows the saturated, mono-, polyunsaturated, and trans-isomer fatty acids ratios in the studied chocolate samples. In the control sample of chocolate based on dried cow's milk, the highest concentrations of fatty acids were represented by palmitic acid (C16:0) at 21.65%, stearic acid (C18:0) at 20.32%, and arachidic acid (C20:0) at 14.74%. The total saturated fatty acid content was 85.51%. The monounsaturated fatty acids in the control sample included palmitoleic acid (C16:1) at 2.23% and oleic acid (C18:1 cis-9) at 6.57%. Among the polyunsaturated fatty acids, linoleic acid (C18:2 all-cis-9,12) at 3.04% belonging to the ω-6 class and linolenic acid (C18:3 all-cis-9,12,15) at 1.43% belonging to the ω-3 class were detected in the control chocolate sample.

Replacing dried cow's milk entirely with mare's milk and adding resveratrol powder led to notable changes in the fatty acid profile of the chocolate products. As the dosages of mare's milk and resveratrol increased, individual saturated fatty acids and total fatty acid content decreased. In particular, the experimental sample "MMP 20/RP 0.10" showed a significant decrease in the concentrations of caprylic acid (C8:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), behenic acid (C22:0), and lignoceric acid (C24:0). The total fatty acid content of the "MMP 20/RP 0.10" chocolate sample was 71.47%. Thus, introducing mare's milk powder and resveratrol powder into the formulation of chocolate products revealed a statistically significant decrease in ∑SFA by 14.04% ($p<0.05$) compared to the control chocolate sample. Moreover, changes in the content of mono- and polyunsaturated fatty acids were observed in the experimental samples. Due to the high level of oleic acid (C18:1 cis-9) in mare's milk, the content of this fatty acid in the experimental sample "MMP 20/RP 0.10" increased by 7.61% ($p<0.05$), while the total MUFA content increased by 9.85% ($p<0.05$) compared with the control. Additionally, a significant increase in the total PUFA content in the experimental sample "MMP 20/RP 0.10" of 4.77% ($p<0.05$) was found. At the same time, a significant decrease in the level of trans-isomer fatty acids was observed in all experimental samples. These changes in the fatty acid profile indicate a high biological value for functional chocolate enriched with dried mare's milk and resveratrol powder, broadening its potential for inclusion in a healthy diet **[52]**.

The results of the polyunsaturated fatty acid content analysis in control and experimental chocolate samples demonstrated a significant increase. This effect is likely attributed to the addition of mare's milk, which contains a high level of polyunsaturated fatty acids, particularly linoleic (ω-6) and alpha-linolenic (ω-3) acids **[53]**. According to existing data, PUFA content in chocolate can vary significantly, ranging from 1% to 8%, depending on the sources of fats and other ingredients used in production **[54]**. The increase in PUFA content is essential due to its potential health benefits, including improved cardiovascular function and anti-inflammatory effects **[55]**. Furthermore, using natural fats, such as those found in mare's milk, is associated with a reduction in trans fats, enhancing the overall nutritional profile of chocolate.

Furthermore, the study observed a decrease in the level of trans-isomer fatty acids across all experimental samples. These findings align with data from other studies showing that natural fats, such as those in mare's milk, significantly reduce trans-isomer content **[56]**. In contrast, chocolate made with hydrogenated oils tends to have much higher trans-isomers.

CONCLUSION

To summarise the results of the study, we can conclude that replacing part of the cow's milk in chocolate with dried mare's milk and fortifying it with resveratrol powder had a significant effect on the physicochemical parameters, as well as the amino acid, vitamin, mineral, and fatty acid compositions of the chocolate. Sensory analysis indicated some changes in the product's properties following the introduction of these functional components. Thus, adding 20% mare milk powder and 0.10% resveratrol can enhance the biological value and improve the palatability of chocolate products.

Substituting cow's milk with mare's milk powder and incorporating resveratrol into the chocolate resulted in an enhanced fatty acid profile. Specifically, the concentration of saturated fatty acids decreased by 14.04%, while monounsaturated and polyunsaturated fatty acids increased by 9.85% and 4.77%, respectively. Furthermore, a significant increase in amino acid content was noted, including essential amino acids (2228.3 mg/100 g). The lipid content in the experimental samples decreased by 12.69%, and the concentration of trans isomers of fatty acids was also reduced. These findings demonstrate the improved nutritional quality of the chocolate, suggesting its potential as a beneficial product for healthy nutrition.

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