Functional Value of Fermented Bovine Colostrum

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Abstract. Peptide composition, antioxidant activity, antimutagenic action and antimicrobial potential of defatted and fermented colostrum were compared. In colostrum samples fermented with *Lactobacillus acidophilus* the relative amount of fraction with a molecular mass (mr) under 10 kDa reached 7.5 %, which was directly correlated with the action of the bacterial proteolytic system. According to chromato-mass-spectrometry the fraction with mr 1200–1300 Da, or peptides consisting of 11–12 amino acid residues, predominated in fermented colostrum. The fluorimetric technique has revealed that antioxidant activity of fermented colostrum ultrafiltrate (fraction with mr≤10 kDa) is double that of defatted sample. The Ames test demonstrated 9.5–15.3 % reduction of mutation rate caused by fermented colostrum in strain *Salmonella typhimurium* TA 98 and 4.6–11.1 % decrease in strain TA 100, exceeding the corresponding effects induced by defatted colostrum. It was found by impedimetric method that colostrum peptides showed a greater antibacterial potential against Gram-negative test strain *Escherichia coli* ATCC 8739 versus Gram-positive strain *Staphylococcus aureus* ATCC 6538. Fermentation of first milk with *Lactobacillus acidophilus* resulted in enhanced antioxidant, antimutagenic activities and production of peptides with antibacterial capacity.

INTRODUCTION

Biologically active potential of fermented dairy products is determined by the presence of specific peptides responsible for hypotensive, antioxidant, antimicrobial, antimutagenic and other effects [1, 2]. The proteolytic activity of probiotic microorganisms catalyzes the production of fermented milk proteins with specific peptide composition and functional characteristics [3, 4]. Bovine colostrum, or first milk, is a promising source of bioactive peptides [5]. The development of new fermented food stuffs with confirmed functional advantages (antioxidant activity, antimutagenic effect and antimicrobial action) appears extremely relevant. The novelty of this research implies elucidation of new data on biological activities of bovine colostrum proteins fermented with probiotic bacteria. In the previous studies, certain aspects of splitting colostrum proteins with bacterial proteases (alcalase and neutrase) were studied, the molecular mass distribution of proteolysis products was clarified, the effect of physicochemical parameters of hydrolysates on their antioxidant, antimutagenic and antibacterial characteristics was evaluated [6, 7].

The aim of this research was investigation of biologically active properties of bovine colostrum fermented with *Lactobacillus acidophilus* bacteria.

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MATERIALS AND METHODS

Production of Fermented Milk Samples and Their Physical-Chemical Characterization

The samples of dry defatted colostrum and dry fermented colostrum were provided by All-Russian Research Institute of Dairy Industry (Russia); acidophilic bacilli (*Lactobacillus acidophilus* strain 630) were used for fermentation.

10 % solutions of defatted and fermented first milk were prepared in distilled water and centrifuged to precipitate insoluble particles at 6000 g and temperature 4 °C for 30 min. The obtained supernatants were fractionated using Spin-X UF Concentrator 20 filters (Corning, England) with a separation capacity 10 kDa. As a result, ultrafiltrates contained a fraction with a molecular mass (mr) inferior or equal to 10 kDa. The total protein content in the tested samples was determined according to ISO 8968–1:2014, the solids ratio was defined in compliance with ISO 6731:2010.

The proportion of low molecular mass protein fraction in colostrum samples was calculated by the formula:

$$DP = \frac{N_{UF} - N_C}{N - N_C} \times 100 \quad (1)$$

where DP is degree of proteolysis, %; N_{UF} is the amount of total protein in the sample of fermented colostrum after ultrafiltration, mg/ml; N is the amount of total protein in the sample of fermented colostrum, mg/ml; N_C is the amount of total protein in the sample of native colostrum ultrafiltrate (control), mg/ml. The research results were presented as the mean values for 3 independent experiments.

Peptide profile of colostrum samples was analyzed by chromato-mass-spectrometry. Agilent 1290 chromato-mass-spectrometric system with Q–TOF 6550 high-resolution mass spectrometric detector operating in positive electrospray ionization (ESI^+) mode was used to record high-resolution mass spectra. The spectrum detection range was 100–3200 m/z (mass to charge ratio). HPLC-analysis was performed with liquid chromatograph Agilent 1290 (Agilent, USA) using Hypersil Gold column (100×2.1 mm, 1.9 μm, Agilent, USA).

Assessment of Antioxidant Potential

Antioxidant activity (AOA) of test samples was evaluated by fluorimetric method, or ORAC (Oxygen Radical Absorbance Capacity)-assay. It is based on AOA determination of the samples via their ability to bind free radicals formed in Fenton system. The technique described in the article by E.I. Tarun [8] was applied. The results were presented as the mean values ± confidence interval.

Determination of Antimutagenic Activity

Antimutagenic activity of colostrum ultrafiltrates was assessed by modified Ames test according to [9]. *Salmonella typhimurium* TA 98 and TA 100 (*S. typhimurium* TA 98 and TA 100) indicator strains were used for short-term estimates. Ethidium bromide and sodium azide (10 μg per plate) were applied as direct mutagens for strains TA 98 and TA 100, respectively. The antimutagenic effect was checked in concentration range 0.11–1.70 and 0.04–10.88 mg of solids per plate for samples of defatted and fermented colostrum, respectively (in 3 replicates). Reduction of the mutation rate index (I_m, %) was calculated by the formula:

$$I_m = 100 - \frac{N_1}{N_2} \times 100 \quad (2)$$

where N_1 is the number of revertants in the experiment, N_2 is the number of revertants in the positive control. The statistical significance of the results was verified by the Dunnett's multiple comparison test.

Investigation of Antibacterial Action

Antibacterial activity of colostrum peptide fractions was determined by impedimetric method according to [10]. The final concentration of the solids in the tested samples was 1.0 mg/ml. Bacterial strains *Escherichia coli*

ATCC 8739 (*E. coli* ATCC 8739) and *Staphylococcus aureus* ATCC 6538 (*S. aureus* ATCC 6538) kindly provided by All-Russian collection of industrial microorganisms served as the test cultures. The studies were performed using BacTrac 4300 microbiological impedance analyser manufactured by (SY–LAB, Austria).

Percentage of inhibition index I (%) calculated by the formula (3) was chosen as the quantitative criterion for evaluation of antimicrobial activity:

$$I = \frac{IDT_2 - IDT_1}{IDT_2} \times 100 \quad (3)$$

where IDT_1 is the time of growth detection of the test culture in the control, h; IDT_2 is the time of growth detection of the test culture in the experiment, h. Any I value above zero signifies that the tested compound possesses antibacterial activity. I index under 15 % mark denotes a weak bactericidal impact on the test culture. I varying from 15 to 50 % stands for a moderate effect, while I exceeding 50 % means a strong antibacterial action. The results were expressed as the arithmetic means (n=3).

RESULTS AND DISCUSSION

Peptide and Protein Profile

Peptide composition of defatted and fermented defatted colostrum was examined based on the total protein content in the respective ultrafiltrates and chromato-mass-spectrometry data. Degree of proteolysis in the sample of fermented colostrum reached 7.5 %, the ratio of peptide fraction in defatted/fermented colostrum constituted 12.9/20.4 %. These data indicated 2.3 times increase of peptide proportion in the fermented variant.

According to chromato-mass-spectrometry proteolysis products with mr up to 1900 Da were detected in the fermented sample. The peak signal was detected at m/z values 1200–1300, which is commensurate with peptides consisting of 11–12 amino acid residues. The resulting peptide profile was determined by substrate and site specificity of enzymes from proteolytic system of *Lb. acidophilus*.

Antioxidant Properties

At the following stage, antioxidant activity of colostrum ultrafiltrate samples was described according to ORAC-method. Antiradical properties of milk are mainly due to the presence of casein and whey proteins [11].AOA of native proteins and peptides is associated with the reducing properties of amino acid radicals [12].

Antioxidant properties of test samples were evaluated by their ability to bind free radicals, which leads to a slowdown in the free radical oxidation of fluorescein. Graphs correlating fluorescence intensity with concentration of solids in analyzed samples were plotted. In compliance with the deduced equations, concentration of the sample IC_{50} corresponding to 50 % inhibition of fluorescence was calculated. Comparative analysis of radical-reducing activity of defatted and fermented colostrum ultrafiltrates is provided in Table 1.

TABLE 1. Characterization of antioxidant properties of defatted and fermented colostrum ultrafiltrates.

Sample name	IC_{50}, μg solids/ml	IC_{50}, μg protein/ml
Ultrafiltrate of defatted colostrum (control)	104.3±5.5	19.1±1.0
Ultrafiltrate of fermented colostrum	52.1±3.9	15.0±1.1

Compared to the control sample, the free radical oxidation of fluorescein was retarded by 2.0 and 1.3 times (based on solids and protein content) when fermented colostrum ultrafiltrate was introduced into the system. The increase in radical-reducing potential of the samples is associated with enrichment of ultrafiltrate with a low molecular mass protein component as a result of colostrum hydrolysis with bacterial proteolytic system (*Lb. acidophilus*).

The previous studies [6] found 5.0 and 7.1 times rise in antiradical properties of colostrum after hydrolysis with purified protease (alcalase) and ultrafiltration, respectively. In general, the growing ratio of peptide fraction in the colostrum samples was accompanied by enhanced antiradical activity.

Antimutagenic Effect

Antimutagenic effect of peptide fractions of defatted and fermented colostrum was studied. As a rule, antimutagenic potential of milk peptides is evaluated in Ames test, based on the frequency of reverse mutations to histidine prototrophy in S. *typhimurium* strains [13]. Antibacterial effect and results of preceding antimutagenic tests of colostrum enzymatic hydrolysates [6] were taken into account when choosing the examined concentration range.

Samples of defatted and fermented colostrum in the studied concentration range did not show a bacteriostatic or bactericidal effect in relation to the test cultures *S. typhimurium* TA 98 and TA 100, which could lead to false positive results. Statistically significant antimutagenic effect was established when the content of defatted and fermented colostrum ultrafiltrates was 0.9–1.8 and 0.43–1.7 mg of solids per plate, respectively (Tables 2 and 3). More pronounced mutation blocking action was observed in experiments with ultrafiltered fermented colostrum, constituting to 9.5–15.3 % for strain *S. typhimurium* TA 98 and 4.6–11.1 % for strain TA 100.

TABLE 2. Statistical evaluation of antimutagenic activity of defatted colostrum ultrafiltrate in Ames test.

Sample amount, mg solids/protein per plate	Decrease of mutation rate, %	
	***S. typhimurium* TA 98**	***S. typhimurium* TA 100**
1.80/0.33	6.9	3.2
0.90/0.17	2.7	0
0.45/0.08	0	0
0.23/0.04	0	0
0.11/0.02	0	0
0	–	–

TABLE 3. Statistical evaluation of antimutagenic activity of fermented colostrum ultrafiltrate in Ames test.

Sample amount, mg solids/protein per plate	Decrease of mutation rate, %	
	***S. typhimurium* TA 98**	***S. typhimurium* TA 100**
1.70/0.49	15.3	11.1
0.85/0.25	13.3	9.0
0.43/0.12	9.5	4.6
0.21/0.06	0	0
0.11/0.03	0	0
0	–	–

Based on the results of peptide profile analysis, fermented colostrum was characterized by a larger content of peptide fraction than defatted first milk. Along with a comparable amount of solids in colostrum samples, increased share of peptide fraction resulted in elevated antimutagenic activity of fermented variant. The reports of Turbay et al. (2012) [14], Sah et al. (2014) [15], our experimental findings [6, 16, 17] and the current research demonstrated promotion of antimutagenic and antioxidant action in fermented and hydrolysed proteins of milk and colostrum.

Antibacterial Action

Antimicrobial properties of peptide fractions of defatted and fermented colostrum were studied in relation to Gram-negative test strain *E. coli* ATCC 8739 and Gram-positive *S. aureus* ATCC 6538. Amino acid composition of antimicrobial peptides, amphipathicity, cationic charge and size determine their ability to bind to anionic cell walls, as well as integrate into membrane bilayers of microorganisms [1, 2]. Growth retardation of test cultures after adding ultrafiltrated samples to cultural medium was estimated. An increase in the detection time index IDT during incubation of test strains on media with peptides was established compared with the IDT parameter in the control.

Weak antimicrobial action was shown for native colostrum sample (I less than 15 %), moderate antibacterial effect was found for fermented variant (I from 15 to 50 %) when cultured with *E. coli* ATCC 8739 (Table 4). The effect of fermented first milk was slightly expressed (6.2 % growth retardation) for *S. aureus* ATCC 6538, while no antibacterial effect was found in experiment with defatted colostrum ultrafiltrate.

In previous studies [7], a weak antibacterial effect (I was 9–19 %) was established with respect to *S. aureus* when colostrum hydrolysate was added to the cultural medium, whereas considerable growth inhibition (I more than

50 %) was shown for *E. coli* ATCC 8739. Thus, low molecular mass fractions of colostrum proved more active against Gram-negative test strain *E. coli* ATCC 8739 than Gram-positive strain *S. aureus* ATCC 6538.

TABLE 4. The level of antibacterial activity of defatted and fermented colostrum ultrafiltrates in relation to test strains.

Sample description	Percentage of inhibition index (I) toward test strains	
	***E. coli* ATCC 8739**	***S. aureus* ATCC 6538**
Ultrafiltrate of defatted colostrum (control)	10 % (weak)	0 % (no antibacterial effect)
Ultrafiltrate of fermented colostrum	38 % (moderate)	6.2 % (weak)

The available literature surveys [1, 2] indicate that antimicrobial peptides characterized to date have been mainly derived by splitting milk proteins with enzymes of gastrointestinal tract or fermentation with probiotic lactic acid bacteria (*Lb. rhamnosus, Lb. helveticus, Lb. delbrueckii ssp. bulgaricus*). The novelty of this research is a comprehensive analysis of antioxidant, antimutagenic and antimicrobial effects of peptide fractions resulting from cleavage of protein component of colostrum with proteolytic system of *Lb. acidophilus*.

CONCLUSION

A study of peptide profile, antioxidant activity, antimutagenic and antimicrobial effects of defatted and fermented colostrum was carried out. Degree of proteolysis of fermented colostrum reached 7.5 %, and the ratio of peptide fraction increased 2.3 times. Fermented colostrum ultrafiltrate was enriched in fraction of proteolysis products with a molecular mass 1200–1300 Da, or peptides consisting of 11–12 amino acid residues. Antioxidant properties of ultrafiltered fermented colostrum increased 2.0 fold compared with defatted sample. Likewise, more pronounced antimutagenic effect was recorded for the ultrafiltrate of fermented colostrum, equalling 9.5–15.3 % for *S. typhimurium* TA 98 and 4.6–11.1 % for strain TA 100. Low molecular mass fractions of first milk proved more active against test strain *E. coli* ATCC 8739 than *S. aureus* ATCC 6538. Colostrum fermentation with *Lb. acidophilus* generated specific peptides with antimicrobial effect. In general, a sample of fermented first milk with confirmed biological activities was produced. It was characterized by enlarged peptide fraction, enhanced antioxidant activity, antimutagenic and antibacterial potential.

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