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ORIGINAL RESEARCH PAPER

COLOSTRUM-DERIVED BIOACTIVE PEPTIDES OBTAINED BY FERMENTATION WITH KEFIR GRAINS ENRICHED WITH SELECTED YEASTS

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The aim of this study was to improve the bovine colostrum biological function through fermentation with kefir grains enhanced with selected yeasts, for developing new nutraceutical and cosmeceutical products. It was found that fermentations with co-culture of 2.5 g% artisanal kefir grains and selected yeast strains (10^6 CFU/100 mL) increased the functional quality of the fermented products compared to the product obtained only with kefir grains. Fresh fermented products obtained with a consortium based on kefir grains and *Candida lipolytica* MIUG D67 demonstrated an increased antioxidant activity of 2.69 mM Trolox Equivalent/g, after 48 h of fermentation. Instead, peptide fractions with MW<10 kDa isolated by membrane filtration from lyophilized fermented products, based on colostrum fermentation with kefir grains enhanced with *Candida lipolytica* MIUG D99 starter, presented markedly increase *in vitro* of ABTS radical scavenging activity, similar to a concentration of 2 nM captopril. These results indicated their possible application in enhance of the quality of the fermented products in order to increase the postbiotic composition with functional impact *in vivo*.

Keywords: colostrum, fermentation, kefir grains, bioactive peptides, ACE inhibition, antioxidant activity, scanning electron microscopy

Introduction

Nowadays, kefir consumption increased among the consumers because of the very high functionality, as well as antimicrobial, antitumor, antihypertensive, antioxidative, anticytotoxic, and hypocholesterolemic properties (Wang *et al*., 2018;

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Yilmaz-Ersan *et al*., 2018). Traditional kefir manufacture involves adding the kefir grains as artisanal starter cultures (Farnworth and Mainville, 2003). Kefir grains represent a unique microbial ecosystem in the nature, consisting of numerous species of lactic acid bacteria (10^8 CFU/g), acetic acid bacteria (10^5 CFU/g), yeasts (10^6-10^7 CFU/g) (Teijeiro *et al*., 2018) and filamentous moulds, associated with polysaccharides (kefiran) (Dertli and Con, 2017) and proteins as matrix (Pogačić *et al*., 2013; de Lima *et al*., 2018; Seo *et al*., 2018).

Colostrum is a rich source of basic nutrients, such as proteins, fat, lactose, vitamins and minerals, and in addition, it plays a fundamental protection role with its antimicrobial substance content, which includes immunoglobulins, lactoferrin, lactoperoxidase, lysozyme and cytokines (Korhonen, 2012; Ayar *et al.*, 2016). In addition, raw colostrum contains valuable bacteria strains (*Lactobacillus* spp. and *Bifidobacterium* spp.), which are known as probiotics (Ayar *et al.*, 2016). In spite of its numerous immunological benefits, colostrum is still under-utilized due to the consumer's resistance and its high perishability.

During kefir manufacture, the bovine colostrum was subjected to proteolysis by enzymes derived from yeast strains in order to obtain bioactive peptides (Korhonen, 2012) that were easily accessible for the kefir grains. *Yarrowia (Candida) lipolytica* yeast is a resourceful tool for wide biotechnological applications (Harzevili, 2014), being present in several food products, mainly dairy products such as cheese, yogurt, and kefir, as well as in sausages, shrimp salads, and soy sauces (Pokora *et al*., 2017). These species are non-pathogenic, being generally recognized as safe (GRAS) (Pokora *et al*., 2017).

Food-derived bioactive peptides have a significant potential for bio-applications (Toldra *et al*., 2018). Dairy products are a major source of bioactive peptides. Colostrum, the first diet of mammalian neonates, is richer than milk in bioactive factors (Korhonen and Pihlanto, 2007). A known technology applied to obtain diary product-derived bioactive peptides is fermentation with selected probiotic microorganisms. In the fermented products, there are new bioactive peptides produced by probiotics, besides the naturally occurring ones. Symbiotic consortia of microorganisms from kefir grains were recently used to produce bioactive peptides acting against metabolic syndrome (Ricci-Cabello *et al*., 2012) and a probiotic beverage presenting antioxidant activity and protection to DNA (Fiorda *et al*., 2016). Several studies aimed to separate and identify novel bioactive peptides from milk or colostrum derived from various species, such as bovine, buffalo, donkey, and camel (Dallas *et al*., 2014; Albejo *et al*., 2017; Vincenzetti *et al*., 2017; Ibrahim *et al*., 2018). Dairy bioactive peptides demonstrated microbicide effects against microbial pathogens, cholesterol lowering ability, and blood pressure lowering effects, mainly due to angiotensin conversion enzyme (ACE) inhibition, antithrombotic and antioxidant activities, opioid, cyto- and immuno-modulatory effects (Mohanty *et al*., 2016), which recommend them as potential nutraceuticals or therapeutic products for health supporting. Inhibition of ACE can significantly decrease the blood pressure, reducing the risk of cardiovascular disease (Rahimi *et al*., 2016). Still, there

are limitations on the development of products based on dairy bioactive peptides and further scientific evidences are needed.

This research study was set out to investigate the properties of the fermented functional products obtained from bovine colostrum fermented with wild kefir grains ameliorated with selected yeast strains with lipase and protease activities. Also, the radical scavenging and ACE inhibitory activities of colostrum-derived peptides, in order to select the enhanced kefir grain consortia that could produce optimal bioactive peptides for health applications, was investigated.

Materials and methods

Kefir grains and inoculum preparation

Kefir grains were provided from a manufactural producer, from Republic of Moldova. For reactivation, the kefir grains were transferred five times into pasteurized whole milk (3.5% fat), incubated at 25°C for 24 h, separated from the milk, and washed with sterile distilled water (Dertli and Con, 2017; Yilmaz-Ersan *et al*., 2018). Also, for the co-fermentation, two yeast strains codified as *Candida lipolytica* MIUG D99 and *Candida lipolytica* MIUG D67 were used. The yeast strains are member of Collection of Microorganisms (MIUG) of Bioaliment Research Platform of Faculty of Food Science and Engineering of „Dunărea de Jos" University of Galați, Romania. The stock cultures were preserved in 40% glycerol at -80ºC. Yeast strains were reactivated on Yeast Extract Chloramphenicol Agar medium.

Screening of yeast strains able to produce protease and lipase

Eighteen yeast strains of *Yarrowia lipolytica*, *Candida lipolytica*, *C. krusei* and *C. colliculosa* belonging to the MIUG Collection were tested for their ability to produce lipases and proteases. For highlighting the lipase activity, the following media was used (g%): cow's milk fat 6.3; peptone 0.5; yeast extract 0.25; agar 2.0. Alternately, for identifying the proteolytic strains, the following medium was used (g%): bovine colostrum (Axyar, Belgium) 2.0; agar 2.0; pH 6.7. For protease and lipase activity assay, the radial diffusion method was used. Thus, for lipase activity the specific media was supplemented with 1% Rhodamine B (1 mg/mL) and inoculated „in point" with the yeasts (Lanka and Latha, 2015). After incubation at 28°C for 5 days, around yeast colonies visible upon UV irradiation (λ=350 nm) appeared orange fluorescent halos (Ramnath *et al*., 2017). The colony diameter was measured and expressed in mm. Regarding the protease activity, after incubation at 28°C for 5 days, the active strains were highlighted by the appearance of a clear zone around the colonies, compared to the rest of the medium that is opaque (Cotarlet *et al*., 2008). Hydrolysis index (HI) was determined as the ratio between the diameter of the hydrolysis zone and the diameter of the colony.

Kefir sample production

Bovine colostrum (Axyar, Belgium) was sterilized at 105°C for 10 min., the colostrum (10%, w/v) being inoculated with 2.5% kefir grains and kept for 48 h at 30°C (sample I). For the samples II and III, a concentration of 10^6 CFU/100 mL of

Candida lipolytica MIUG D99 was added in 10% colostrum and then incubated at 150-180 rpm, 30°C for 48 h (sample II) and for 72 h (sample III). After that, the active kefir grains (2.5%, w/v) were added and the samples were incubated in stationary system at 30°C for 48 h (sample II) and for 72 h (sample III). The sample IV was obtained similarly to the sample II, the only difference being the utilization of the strain coded as *Candida lipolytica* MIUG D67. At the end of the fermentation, the kefir grains were separated using a sterilized plastic sieve. Half of the kefir samples were stored at 4°C for further analysis (fresh samples) and half were subjected to lyophilisation with the Martin Christ Alpha 1-4 equipment for peptides extraction.

Preparation of peptide fractions

Samples of lyophilized fermented colostrum with kefir grains were dissolved in water in a concentration of 1% (w/w), incubated overnight and then, the solutions were centrifuged at 8900 rpm. Collected supernatants were fractionated by centrifugal ultrafiltration using membrane filter units (AmiconUltracel YM-10, Millipore, cutoff at 10 kDa) and supernatants, containing peptides with MW below 10 kDa (P1-P4), were preserved at 4ºC for further analysis. The concentration of peptides in each sample was determined using bicinchoninic acid assay kit (Thermo Fisher).

Determination of pH and acidity

During the fermentation, the pH was measured with the MP 2000 pH meter (Mettler Toledo, Switzerland). For the total titratable acidity assay (TTA) a quantity of 4 g of kefir was transferred into a 50 mL Erlenmeyer flask and filled up with distilled water. After mixing, 10 mL of diluted sample was titrated under shaking with 0.1 N NaOH by using a solution of 1% phenolphthalein (in 70% ethanol) as indicator (Tiţa and Bârcă, 2017). The TTA was expressed in Thörner degrees (ºTh).

Antimicrobial activity

Testing the antimicrobial activity against two indicator microorganisms considered the most frequent in food microbiota (*Aspergillus niger* MIUG M5 and *Bacillus subtilis* MIUG B1), was performed using the well-radial diffusion method (Chifiriuc *et al*., 2011). The indicator microorganisms were grown on specific agar media, such as: Yeast Extract Chloramphenicol Agar for mould and Plate Count Agar for bacteria, and incubated at 25°C for 3-5 days (for moulds) and at 37°C for 24 hours (for bacteria).

The inoculum was obtained by transferring 3-5 colonies using the sterile loops (10 µL) and suspending them in Malt extract broth (for moulds) and Nutrient broth (for bacteria) followed by overnight incubation at 25°C (for moulds) and 37°C (for bacteria). To test the antimicrobial effect, the specific culture medium was poured into sterile Petri dishes and homogenized with 1 mL of each inoculum. After the medium solidification, wells with a diameter of 5 mm were made. Then, 100 µL of the colostrum kefir samples were putted into the wells. After that, the plates were incubated at specific temperatures for each microorganism. The test was positive

when the inhibition zone around the well was highlighted. The diameter of the inhibition zone was measured and expressed in mm.

Antioxidant capacity

The antioxidant potential of the fresh kefir samples was determined by using DPPH (2,2-diphenyl-1-picrylhydrazyl, Fluka Chemie) in methanol solution (0.1 M), after 30 min of reaction (Yuan *et al*., 2013). The antioxidant activity was expressed as µM Trolox/g sample using a Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) calibration curve.

Radical scavenging activity of peptide samples was determined towards the cationic radical 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS), as previously described by Crăciunescu *et al*. (2012). Briefly, 7 mM ABTS solution reacted with 2.45 mM potassium persulfate (1:1, v/v) by incubation in the dark at room temperature for 12-16 h to produce ABTS radical. For experiments, 1 mL ABTS radical solution was mixed with 100 µL peptide samples and allowed to react for 6 min. The optical density was read at wavelength of 734 nm using an UV/VIS spectrophotometer (Jasco V 650, Japan). Colostrum and fermented kefir samples were analysed in similar conditions for comparison. A calibration curve was obtained using different concentrations of Trolox (0-500 µM) as standard antioxidant. The radical scavenging activity was expressed as µM Trolox/mg protein.

ACE-inhibitory activity assay

Inhibition of ACE was measured using an experimental model *in vitro*, as previously described by Papadimitriou *et al*. (2012). Briefly, 50 µL of peptide solution or distilled water (control) were incubated with 180 µL of hippuryl-L-histidyl-L-leucine solution (5mM) in sodium borate (100 mM, pH 8.3) and 20 µL of ACE solution, at 37ºC for 90 min. Colostrum and fermented kefir samples were similarly treated, for comparison. Captopril, a known drug for lowering blood pressure was used as standard in the concentration range of 1-25 nM (Ahmad *et al*., 2017). The resulted hippuric acid was extracted in ethyl acetate, heat evaporated, dissolved in distilled water and the OD was measured at wavelength of 228 nm, using an UV/VIS spectrophotometer (Jasco V 650, Japan). The ACE-inhibitory activity of peptide samples was calculated according to the equation 1:

$$\text{ACE-inhibitory activity (\%)} = (1 - OD_{sample}/ OD_{control}) \times 100 \quad (1)$$

Observation of kefir grains and kefir samples by SEM

Kefir grains were sliced in order to obtain samples for scanning electron microscopy (SEM). The samples collected from the surface and center part of the kefir grain were prepared for SEM analysis according to Guzel-Seydim *et al*. (2005) method with few modifications. The kefir grains were fixed in 25% glutaraldehyde in water at pH 7.0 for 4 h at 25°C. The samples were washed with three changes of 0.2 M phosphate buffer, pH 7.0. Then, the samples were dehydrated in 15%, 30%, 50% and 70% ethanol for 10 min each 85% and 95% ethanol, for 15 min each, and 100% ethanol, for 1.5 h. After dehydrating, samples were critical-point dried and mounted

on aluminum stubs and coated with gold using the SPI-Module Quartz Crystal Thickness Monitor, SPI Supplies (USA). The preparations were observed using a Quanta 200 FEI scanning electron microscope (Hitachi Instruments, Inc., San Jose, CA, USA).

Statistical analysis

The experiments were performed in triplicate. The results were expressed as mean values ± standard deviation (SD). Statistical analysis was performed using one-tailed paired Student t-test on pairs of interest, and the differences were considered significant at $p<0.05$.

Results and discussion

Screening of yeast strains able to produce protease and lipase

Eighteen yeast strains belonging to the MIUG Collection were tested based on their potential to metabolise the bovine colostrum 2% (w/v) and 5% (w/v) fat from caw milk as substrates. Two strains, coded *Candida lipolytica* MIUG D99 and *Candida lipolytica* MIUG D67 were selected as active producers of lipase and protease (Table 1).

Table 1. Selection of yeast strains able to produce lipases and proteases

No.	Strain code	Protease assay Hydrolysis ratio, 28ºC, 5 days	Lipase assay Colony diameter, 28ºC, 5 days (mm)
1	*Yarrowia lipolytica* MIUG D5	1.26	17.00
2	*Yarrowia lipolytica* MIUG D6	1.87	22.00
3	*Yarrowia lipolytica* MIUG D7	1.33	28.00
4	*Candida lipolytica* MIUGD106	-*	20.50
5	*Candida lipolytica* MIUG D67	2.00	25.50
6	*Candida lipolytica* MIUG D69	2.37	26.50
7	*Candida lipolytica* MIUG D96	-	31.50
8	*Candida lipolytica* MIUG D98	-	19.50
9	*Candida lipolytica* MIUG D99	2.22	32.00
10	*Candida lipolytica* MIUG 100	-	19.00
11	*Candida lipolytica* MIUGD101	-	19.00
12	*Candida lipolytica* MIUGD111	-	31.00
13	*Candida lipolytica* MIUG D73	-	10.00
14	*Candida colliculosa* MIUGD108	-	7.00
15	*Candida krusei* MIUG D97	-	9.50
16	*Candida colliculosa* MIUG D102	-	6.00
17	*Candida colliculosa* MIUG D115	-	6.50
18	*Candida krusei* MIUG D74	-	8.00

-*developed colony, without clear zone

In this research, the most active strain regarding lipolytic activity was *C. lipolytica* MIUG D99 strain with a colony growth diameter (on medium with fat from caw milk) of 32.0 mm. Our data are in agreement with the literature that specifies a high capability to produce extracellular lipases and proteases by the non-conventional yeast *Yarrowia (Candida) lipolytica* strains (Pokora *et al*., 2017). Gdula *et al*. (2003)

reported that yeast strains *Y. lipolytica* A-101 and W29 produced high level of lipase after 54 h of incubation at 25ºC (2.3 mm) on Spirit blue agar medium, by agar diffusion method. Furthermore, these strains were able to grow in butter fat.

pH and acidity kinetics of the colostrum kefir samples

It is known that the initial concentration of the kefir grains, agitation and time of the fermentation, and temperature influence the pH, viscosity, final lactose concentration and the microbiological profile of the final product (Leite *et al*., 2013).

The way of pH decrease is an essential parameter to estimate the fermentation capacity of the starter microorganisms used in fermented dairy products. The activity and growth rate of starter cultures are known to vary with lactose and protein contents of colostrum, and the fermentation conditions (Rogelj and Perko, 1998).

The initial pH of the medium based on bovine colostrum was 6.7 units. Firstly, the bovine colostrum was subjected to proteolysis by enzymes derived from selected yeast strains in order to obtain bioactive peptides (Korhonen, 2012). In fact, the production of different bioactive peptides from milk and colostrum proteins through microbial proteolysis is well known (Korhonen, 2009). This could explain the gradually increase of the pH values of the kefir samples, reaching values from 6.8 up to 7.8, after 72 h of incubation (sample III). After kefir grains were added, the pH values decreased mainly during 24 h and reached similar values at the end of the fermentation processes (Table 2).

Table 2. The pH kinetics of the colostrum kefir samples

Samples	Time of fermentation, h					
	Colostrum hydrolyses by the yeast strains			Fermentation with kefir grains		
	24	48	72	24	48	72
Sample I	-	-	-	5.2±0.01	4.7±0.03	-
Sample II	6.8±0.01	7.4±0.04	-	4.7±0.03	4.6±0.02	-
Sample III	6.8±0.03	7.3±0.01	7.8±0.01	4.9±0.02	4.7±0.04	4.7±0.02
Sample IV	6.8±0.02	7.3±0.02	-	4.9±0.01	4.8±0.01	-

The values are a mean of three replicates ± standard deviation

During incubation, the microbial consortia from the kefir grains converts lactose from the colostrum to obtain energy for their maintenance and growth, and release metabolites, primarily lactic acid, that decrease the pH values of the kefir samples (Suriasih *et al*., 2012; Ayar *et al.*, 2016). Ideally, the pH of kefir product is between 4.0 and 4.4 units (Teijeiro *et al*., 2018) for proper conservation, and the measured values of the colostrum kefir samples were in this spectrum. The data proved that the kefir grains were well adapted to the colostrum media. In addition, the literature underlines that the drop in pH during fermentation was greater when the concentration of added grains increased, and that the ratio of grains to milk/colostrum affected the viscosity of the final fermented product (Farnworth and Mainville, 2003). Ayar *et al.* (2016) investigated the effect of colostrum on microbial populations of yogurt and kefir. The authors reported pH values ranging from 4.67 to 4.73 for all fermented products.

Regarding the titratable acidity among all samples, especially at the colostrum hydrolysis, no significantly differences were detected in samples fermented for 48 h, at 30ºC, in comparison to the sample incubated for 72 h at the same temperature (Table 3). After, the kefir grains were added the titratable acidity values of the colostrum kefir samples were found in the range of 43.75 to 200°Th. Also, it can be observed that colostrum kefir sample fermented only with kefir grains (sample I) have the lowest acidity 62.5°Th, after 48 h of incubation at 30ºC. In the case of samples II and III was showed that the time of the fermentation have no positively influenced on the sample's acidity. Instead, for sample IV, when was used an enhanced kefir grains with *Candida lipolytica* MIUG D67, higher acidity was obtained (200°Th, after 48 h).

Table 3. The acidity kinetics (ºTh) of the colostrum kefir samples

Samples	Time of fermentation, h					
	Colostrum hydrolyses by the yeast strains			Fermentation with kefir grains		
	24	48	72	24	48	72
Sample I	-	-	-	43.75±0.18	62.5±0.22	-
Sample II	25±0.17	18.75±0.11	-	110.5±0.22	125.0±0.17	-
Sample III	25±0.18	25±0.25	25±0.17	125±0.17	137.5±0.28	137.5±0.28
Sample IV	25±0.17	25±0.24	-	143.5±0.01	200±0.01	-

The values are a mean of three replicates ± standard deviation

Tiţa and Bârcă (2017) reported that the control sample for fermented milk products enriched with vegetables ingredients (*Cantharella cibarius*) indicated values of acidity between 85-110ºTh, after 1-6 days of storage at 4ºC. Nacheva *et al*. (2017) reported that the pasteurized goat milk was inoculated with 2% kefir grains and incubated statically at 25°C for 24 h, in order to obtain fermented product with pH 4.6-4.8 and 90-100ºTh.

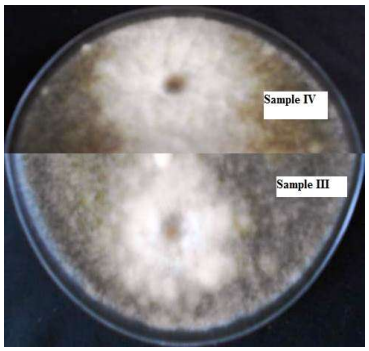
Antimicrobial activities

The inhibitory activity of fermented products based on colostrum fermentation with wild kefir grains ameliorated with selected yeast strains was tested against different spoilage microorganisms by measuring the zone of growth inhibition (Figure 1). The strongest antimicrobial spectrum was obtained against *Bacillus subtilis* MIUG B1. All colostrum kefir samples showed inhibition zone ranging between 3.0 and 5.0 mm, higher antibacterial activity highlighting in case of sample IV. Instead, for *Aspergillus niger* MIUG M64, it was observed the spore inhibition for all tested samples.

The antimicrobial activity of the colostrum fermented samples was correlated with the titratable acidity of the samples that means the organic acids produced by the lactic bacteria strains could be responsible for the growth inhibition of the spoilage microorganisms. In fact, the literature underlines that the antimicrobial potential of the fermented products could be linked to the production of organic acids, peptides (bacteriocins), carbon dioxide, hydrogen peroxide, ethanol and diacetyl (Muhialdin

et al., 2011). These compounds could be implicated in the reduction of food borne pathogens and also in the treatment and prevention of gastroenteritis and vaginal infections (Leite *et al*., 2013). The antimicrobial activity of four kefirs types, fermented for 24-72 h against eight food-borne pathogens (*Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Enterococcus faecalis*, *Escherichia coli*, *Salmonella enteritidis*, *Pseudomonas aeruginosa* and *Cronobacter sakazakii)* was revealed by spot method (Kim *et al*., 2016). The strongest antimicrobial spectrum was obtained after at least 36-48 h of fermentation for all kefirs.

Chifiriuc *et al.* (2011) observed that all kefir samples possessed antimicrobial activity against *B. subtilis*, *S. aureus*, *E. coli*, *E. faecalis* and *S. enterica*, but did not inhibit *P. aeruginosa* and *C. albicans* strains.

a) 

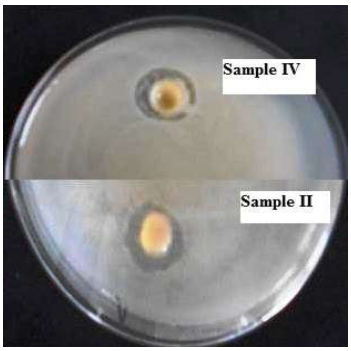
b) 

Figure 1. Antimicrobial activity of the colostrum kefir samples against *A. niger* (a) and *B. subtilis* (b)

Antioxidant activity of fermented samples

Antioxidants are usually used in nutraceutical and pharmaceutic products recommended in oxidative stress-related conditions, to prevent oxidative damage through free radical mediated reactions. The antioxidant activity of the fresh colostrum kefir sample was evaluated by DPPH method. It was found that fermentations with co-culture of 2.5 g% kefir grains and selected yeast strains (10^6 CFU/100 mL) increased the functional quality of the fermented products, comparing to product obtained only with kefir grains fermentation (Table 4).

Fermented products obtained with consortium based on kefir grains and *Candida lipolytica* MIUG D99 (sample II) and *Candida lipolytica* MIUG D67 (sample IV) demonstrated an increased antioxidant potential (2.69 mM and 2.38 mM TE/g), after 48 h of fermentation, in comparison to the product obtained only with kefir grains fermentation (sample I) (Table 4).

Fiorda *et al*. (2016) concluded that kefir grains fermentation improved the antioxidant activity of both substrates (honey and soybean hydrolyzed extract) used. For fermented products SMKB-soybean-based kefir beverage and HKB-honey-based kefir beverage values of 1071.00 and 1213.95 µM Trolox/g were obtained.

Table 4. Antioxidant activity of the colostrum fermented products (mM TE/g sample)

Samples	Colostrum hydrolyses by the yeast strains, 48-72 h	Fermentation with kefir grains, 48-72 h
Sample I	-	0.14±0.05
Sample II	1.22±0.10	2.38±0.27
Sample III	0.00	2.11±0.01
Sample IV	2.19±0.33	2.69±0.33

The values are a mean of three replicates ± standard deviation

Instead, the bioactive peptides from kefir samples obtained by different fermentation processes of bovine colostrum and corresponding fractions with MW lower than 10 kDa were analysed for antioxidant activity using ABTS assay. The results showed that both fermented colostrum (sample I) and the peptide fractions had negligible antioxidant activity, lower than that of colostrum (Table 5). Kefir samples II, III and IV presented significantly ($p<0.05$) higher ABTS radical scavenging activity, compared to colostrum sample (Table 4). Kefir sample III (0.99 µM Trolox/mg protein) was 1.67 times more active as radical scavenger than colostrum (0.59 µM Trolox/mg protein).

Bioactive peptides separated by ultrafiltration from kefir samples II-IV presented a higher antioxidant potential than corresponding fermented samples and colostrum (Table 5).

Table 5. Radical scavenging activity of bovine unfermented colostrum, fermented colostrum with kefir samples and peptide fractions

Samples	Radical scavenging activity of fermented colostrum with kefir (µM Trolox/mg protein)	Radical scavenging activity of peptide fractions (µM Trolox/mg protein)
Sample I	0.49 ± 0.02*	0.12 ± 0.01*
Sample II	0.76 ± 0.04*	1.10 ± 0.05*
Sample III	0.99 ± 0.05*	2.78 ± 0.12*
Sample IV	0.81 ± 0.04*	1.09 ± 0.04*
Unfermented colostrum	0.59 ± 0.03	-

*$p<0.05$, compared to unfermented colostrum

The peptide fraction resulted from sample III was the most active in inhibiting ABTS radical (2.78 µM Trolox/mg protein) from all, presenting a 2.8 and 4.7 times higher activity than the corresponding fermented colostrum sample (0.99 µM Trolox/mg protein) and colostrum (0.59 µM Trolox/mg protein), respectively.

Antihypertensive activity

In this study, ACE-inhibitory activity of fermented kefir samples and their corresponding peptide fractions was determined using a known substrate. The results showed that fermented kefir samples I and II presented higher inhibitory activities,

compared to samples obtained from variants III and IV (Table 6). The values (8.98%, 9.32%) were 2.2-2.3 times higher than that of colostrum (2.80%). Among fermented colostrum samples, that from sample II had the highest inhibitory potential, but not significantly different ($p>0.05$) from sample I. A steep increase in ACE inhibition was observed for all peptide fractions with MW lower than 10 kDa. The values varied between 28.77% for sample III peptide and 51.12% for sample II peptide (Table 6). Similar to fermented kefir samples, peptide fractions from samples I and II presented the highest inhibitory activity (40.63% and 51.12%, respectively). They were 14.5 and 18.25 times more active than bovine colostrum (2.80%), indicating a high potential in lowering the blood tension. Moreover, similar inhibition percentages of 31.70% and 64.42% were obtained using 1 nM and 2 nM captopril, respectively.

Table 6. ACE-inhibitory activities of bovine unfermented colostrum, fermented colostrum with kefir samples and peptide fractions

Samples	ACE-inhibitory activity of fermented colostrum with kefir (%)	ACE-inhibitory activity of peptide fractions (%)	ACE-inhibitory activity of captopril (%)	
			Concentration (nM)	(%)
Sample I	8.98 ± 0.41*	40.63 ± 1.98*	1	31.70
Sample II	9.32 ± 0.47*	51.12 ± 2.45*	2	64.42
Sample III	5.62 ± 0.28*	28.77 ± 1.43*	5	73.76
Sample IV	3.97 ± 0.19*	36.37 ± 1.77*	25	81.04
Unfermented colostrum	2.80 ± 0.14	-	-	-

*$p<0.05$, compared to unfermented colostrum

Previous studies on bovine milk fermented with kefir grains showed a huge number of peptides (1591 peptides), some of which were naturally occurring in milk, while some were released by native milk enzymes and 38.3% were newly released during fermentation (Dallas *et al*., 2016). Database identification matched with 29 of these peptides, from which 15 peptides with antihypertensive activity and two with strong antioxidant activity (Dallas *et al*., 2016).

Korhonen (2009) concluded that a wide diversity of naturally formed bioactive peptides were identified in fermented dairy products. Many factors, such as starter cultures used, type of fermented product, time of fermentation and also storage conditions could influence the occurrence, specific activity and number of bioactive peptides in fermented products. It is remarkable that these calcium-binding, antihypertensive, antioxidative, immunomodulatory and antimicrobial peptides can be found at the same time in the fermented products (Korhonen, 2012).

Distribution of the microbiota in the kefir grains and fermented products as observed using SEM

Seen by the naked eye, the outside surfaces of the kefir grains looked smooth and shiny. However, under SEM, the grain surfaces were very rough. The surface roughness may be the effect of the kefir grains preparation for electron microscopy. In the inner portion of the kefir grains and in the fermented colostrum sample small craters were revealed. Lactobacilli, yeast and fibrillary material were observed at ×10 000 on the exterior portion of the grain (Figure 2b). In all probability, the granular material observed in the fermented product linked to particles of coagulated colostrum (Figure 2a) and the fibrillary material to the polysaccharide kefiran, as noted in other studies (Leite *et al*., 2013). Two different types of lactobacilli (long and curved) were observed in the inner part of the kefir grain along with yeast cells (Figures 2a). However, in this study, short lactobacilli and yeasts were observed in the fermented product. These high populations of yeast and *Lactobacillus* spp. observed were in agreement with Guzel-Seydim *et al.* (2005).

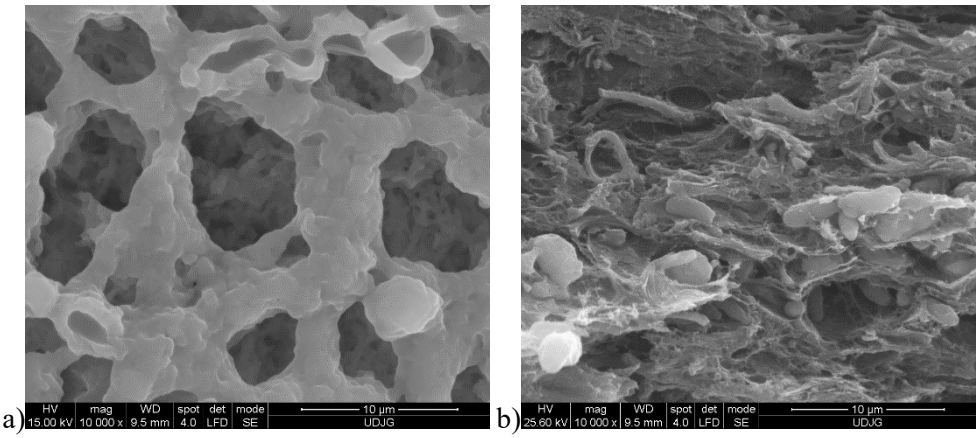


Figure 2. Scanning electron micrographs of fermented colostrum with enhanced kefir grains (sample IV) (a) and inner part of kefir grains (b)

Conclusions

The results of the present study demonstrated improved functional characteristics of the bovine colostrum by fermentation with kefir grains enhanced with selected yeasts with both the protease and lipase activity. The literature does not mention any information concerning this process achieved in two phases, such as colostrum hydrolyses by the yeast strains and then, fermentation with kefir grains. In summary, the results allowed the selection of optimal fermentation process of bovine colostrum using kefir grains enhanced with selected yeasts that could have the different functional properties. Thus, the fermented products obtained by co-cultivation of yeast strain *C. lipolytica* MIUG 99 and artisanal kefir grain (samples II and III) contained the bioactive peptides with ACE inhibitory properties and antioxidant activity, respectively. Both fermented colostrum samples and corresponding peptide fractions

presented higher bioactivity than unfermented colostrum. The colostrum fermented by co-cultivation of yeast *C. lipolytica* MIUG 67 strain and artisanal kefir grain showed antimicrobial activity against spoilage microorganisms. The colostrum kefir samples revealed characteristics of nutritionally complete products possessing active compounds with antimicrobial and antioxidant activities. These results offer new perspective in order to obtain multifunctional three-biotic fermented products (having a pre-, pro- and post-biotic effects) used in further development of nutraceuticals and cosmeceuticals.

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