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ORIGINAL PAPER

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THE EFFECT OF CHIA SEED (*SALVIA HISPANICA*) ADDITION ON THE FATTY ACID PROFILE AND LACTASE ACTIVITY OF FERMENTED MARE'S MILK

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ABSTRACT

Background. Fermented mare's milk beverages supplemented with chia and inoculated with mesophilic LAB with probiotic bacteria were produced. Mare's milk has recently been gaining attention due to its nutraceutical properties. The dominant protein fractions are albumin and globulin. The low fat content is comprised of a distinctive profile. Taking into account the lower casein-to-whey-protein ratio, emulsification and quickly absorbed fat, mare's milk is more desirable for digestion than cow's milk. Fermented mare's milk with chia is free of chemical additives and is a functional dairy product with low fat content and high nutritional value.
Materials and methods. The basic chemical composition was determined using DairySpec FT (Bentley Instruments, Chaska, MN, USA). Mare's milk was pasteurized using a MILKY FJ 15 milk pasteurizer machine. For production beverages with chia, mesophilic LAB with probiotic bacteria BB-12® Probio-Tec™ were used The fatty acid profiles and lactase activity were determined. Additionally, the atherogenic index (AI), thrombogenic index (TI) and hypercholesterolemic fatty acid index (HcFA) of fat milk samples were calculated.
Results. It was found that the lactase enzyme activity of the fermented mare's beverages with chia ranged from 0.17 (in fermented milk with 3 weeks storage) to 0.84 μkat·100 g^{-1} immediately after production. The number of probiotic bacteria remained unchanged in the fermented beverages for the first 2 weeks of storage. After this time, the number of *Bifidobacterium* decreased by 11%. The addition of chia seeds increased the Σ UFA and Σ MUFA respectively by about 12.31% and 6.78%. It could be concluded that fermentation and chia can improve the nutritional value of the product.
Conclusion. The analyses conducted in this study demonstrate that it was possible to produce fermented mare's milk with chia seed (*Salvia hispanica*) addition increasing the Σ UFA and Σ MUFA.

Keywords: mare's milk; fermented mare's milk; chia seeds; natural product, human health, nutritional properties, functional ingredient

INTRODUCTION

Mare's milk is known as a product with lower allergenicity than cow's milk. Compared to its basic chemical composition and content of bioactive components, it differs from cow's milk and is similar to human milk. On average, mare's milk contains 6.5% lactose, 2% protein, 1.0% fat (Barreto et al., 2020; Teichert et al., 2020). The digestive tract of mares lacks the complex hydrogenation processes found in the digestive tract

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of cows. This is the reason for the differences between the composition of mare's and cow's milk (Salimei and Fantuz, 2012). The total amount of protein in mare's milk is 1.85–2.20% and the casein-to-whey-protein ratio (higher proportion of whey proteins) is similar to that found in human milk. The milk in its composition belongs to the albumin group. The milk of other domestic animals (except for the donkeys) refers to the casein group (Kushugulova et al., 2018). In studies in vitro through a two-step simulation using human gastric and duodenal enzymes, it has been shown that the percentage of undigested caseins was almost double for cow caseins compared with horse and human milk. The lower casein-to-whey-protein ratio is more desirable for faster digestion. The development of mares milk as a specialized dairy product can be of interest to the dairy industry, both for infants and also according to scientific findings on its use as a supplement for elderly people (Inglingstad et al., 2010; Jastrzębska et al., 2017; Roy et al., 2020; Tari et al., 2018). Based on the results presented in (Bimbetov et al., 2020) mare's milk can be effective for patients with non-alcoholic steatohepatitis and also in normalizing cholesterol metabolism. From a nutritional point of view, it is also important that mare's milk fat is emulsified and quickly absorbed. Mare's milk is low in fat and cholesterol content and high in polyunsaturated fatty acids (PUFA). The higher amount of linoleic acid in horse's compared to cow's milk helps in lowering the risk of cardiovascular diseases, carcinogenesis, diabetes and modulation of the immune system. The fat contained in mare's milk is almost the best animal fat in nature (Barłowska et al., 2011; Kushugulova et al., 2018; Nayak et al., 2020). It has been observed that the consumption of chia seeds (*Salvia hispanica*) which are gluten-free has increased in recent years. The high nutritive value of chia seeds includes 30.74% total (predominant α-linolenic acid approximately 60% all fatty acids), 42% carbohydrates (including 34.4% total dietary fibre) 16.54% protein, 5.8% moisture, and 4.8% ash (Mohd Ali et al., 2012; Ullah et al., 2015; Valdivia-López and Tecante, 2015). Chia seeds are the richest botanical source of omega-3 α-linolenic of any identified vegetable source, and also contain a higher protein content complete in terms of amino acid than most grains and cereals. Chia seeds (per-gram) contain more α-linolenic acid, protein, calcium and fibre than either flaxseed or salmon (Ullah et al., 2015).

The pro-health value, digestibility and organoleptic attributes of mare's milk increase as a result of fermentation (Teichert et al., 2020). Complex interactions between bacterial species and the milk matrix they are fermenting have been observed. The major bacteria used in food fermentations are lactic acid bacteria (LAB). Many functional characteristics of *Lactobacillus* and *Streptococcus* bacteria are responsible for their use in food production. Using milk fermentation microorganisms *Streptococcus thermophilus* and *Lactobacillus bulgaricus* causes the transformation of lactose into lactic acid. This is an important fact, especially for people who have a reduced ability to digest lactose due to low levels of lactase enzyme activity. Lactase deficiency may lead to lactose intolerance. In addition, the consumption of fermented milk is very important for the health of the gut microbiota (Facioni et al., 2020; Hill et al., 2017; Oak and Jha, 2018; Sansanwal et al., 2017).

Taking into account numerous scientific reports about the importance of fermented milk as part of a balanced and healthy diet, we hypothesized that mare's milk with its functional properties can be matrices compatible with chia seeds. In our preliminary studies (unpublished data), we demonstrated the suitability of chia seeds for the production of fermented milk. From a technological point of view, chia seeds are introduced into milk as a non-dairy ingredient before inoculation and fermentation. Fermentation results in the digestion of approximately 0.91% lactic acid and a simultaneous reduction in lactose content of 26.7%. The fermented mare's milk with chia can even be dedicated to people with lactose intolerance. Therefore, the aim of our study was to investigate the effect of chia seed addition on the fatty acid content and lactase activity of fermented mare's milk.

As a result, we wanted to obtain novel beverages with health benefits different from those already existing.

MATERIALS AND METHODS

Mare raw milk samples

Mare's milk was collected from 9 Polish Coldblood mares reared on an equine dairy farm in the Wielkopolska region (Western Poland). Mares were between 5 to 8 years of age with live weights between 602 and 820 kg.

The mares were mechanically milked from both teats of the udder once a day in the evening after 3 h of physical separation from their foals for 12 days. Bulk mare's milk from successive days ($n = 6$) was collected according to ISO 707.

Compositional and physicochemical analysis of milk

The basic chemical composition was determined using DairySpec FT (Bentley Instruments, Chaska, MN, USA). The pH acidity of milk was analyzed using a pH-meter model CP-505 (Elmetron, Zabrze, Poland) equipped with an electrode EPS-1 (Elmetron, Zabrze, Poland).

Fermentation

Mare's milk was pasteurized (72°C, 15 s) using a MILKY FJ 15 milk pasteurizer machine (Franz Janschitz GmbH, Althofen, Austria). In the beginning, chia seeds (commercial product from local marked in Poland) 4 kg was mixed thoroughly with a 10 l of milk to prevent the formation of lumps. For production beverages, mesophilic LAB with probiotic bacteria BB-12® Probio-Tec™ identified as *Bifidobacterium animalis* subsp. *lactis* from Chr. Hansen (Hørsholm, Denmark) was used. Fermentation at 43°C lasted 20 min and after cooling to 22°C, a culture starter no 75106 Abiasa Inc. was added (Quebec, Canada) at 15 IU to 50 L of milk. The strains were: *Lactocococcus lactis* subsp. *lactis*, *Lactocococcus lactis* subsp. *cremoris*, *Lactocococcus lactis* subsp. *lactis biovar diacetylactis*, *Leuconostoc mesenterroides* subsp. *cremoris*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus casei* and yeasts: *Kluyveromyces fragilis*. Fermentation continued at 22°C until a pH of 4.5 was obtained. Fermented milk was poured into polystyrene (PS) containers (capacity for 150 g of the product), then cooled to 5 ±1°C. The product was tested 48 h after the end of the fermentation process on a pilot plant scale ($n = 6$).

Lactase activity

Lactase activity was determined using the method proposed by (Passerat et al., 1995). The results were given in microkatals per one hundred grams (µkat/100 g) of the sample. One unit of the enzyme was defined as the amount of catalyst that converted 1 mol of the substrate within 1 s under the specified reaction conditions.

Fatty acid profile

The fatty acid profile was determined using a gas chromatograph (Varian Star CP 3800) fitted with a flame ionization detector and a 100 m fused-silica capillary column (0.25 mm i.d.), coated with 0.2 µm CP-Sil 88 (Chrompack, Varian) according to the methodology described by Cais-Sokolińska et al. (2015). Atherogenic index (AI; eq. 1), thrombogenic index (TI; eq. 2), hypercholesterolemic fatty acid index (HcFA; eq. 3) of fat milk samples were calculated using the following formulas (Kara, 2020).

$$AI = (C12{:}0 + 4 \times C14{:}0 + C16{:}0) / UFA \quad (1)$$

$$TI = (C14{:}0 + C16{:}0 + C18{:}0) / [(0.5 \times MUFA) + (3 \times n\text{-}3) + (0.5 \times n\text{-}6) + (n\text{-}3/n\text{-}6)] \quad (2)$$

$$HcFA = C14{:}0 + C16{:}0 \quad (3)$$

where:
UFA – unsaturated fatty acids,
MUFA – monounsaturated fatty acids.

Additionally, the content of fatty acids with an hypocholesterolemic effect (DFA) and hypercholesterolemic fatty acids (OFA) were calculated.

Statistical evaluation

Verification of the statistical hypotheses was accomplished by adopting an $\alpha = 0.05$ level of significance using the Statistica data analysis software, version 13 (TIBCO Software Inc., Palo Alto, California, USA). The influence of fermentation on the milk was evaluated by one-way ANOVA followed by Tukey's HSD post hoc test for multiple comparisons.

RESULTS

Characteristics of mare's milk composition

The gross composition of the mare's milk is presented in Table 1. The mean content of total solids was 95.6 g/kg (P5-P95 from 94.5 to 96.7 g/kg). It has been shown in the literature that the fat content of mare's milk is lower than other species. The fat content (14.5 ±0.59) is similar to that reported by other authors for raw mare's milk (Cais-Sokolińska et al., 2018; Claeys et al., 2014; Salimei and Fantuz, 2012; Sansanwal et al., 2017). In our study, the lactose content 64.6 ±1.01 g/kg is comparable to that indicated by Potočnik et al. (2011) for the same breed. Moreover, the content

Table 1. Gross composition of milk of Polish Coldblood mares

Components g/kg	Mean	SD	P5-P95
Solids-not-fat	95.6	0.77	94.5–96.7
Fat	14.5	0.59	13.8–15.3
Lactose	64.6	1.04	63.2–66.1
Casein	12.4	0.29	11.8–12.9
Whey protein	11.3	0.76	10.5–12.1
Ash	5.7	0.28	5.4–6.1

of lactose is lower in fermented milk. As can be seen from Table 1, the casein content is 12.4 g/kg (P5-P95 from 11.8 to 12.9 g/kg), and the whey protein content is 11.3 ±0.76 g/kg. The obtained results with ash content P5-P95 from 5.4 to 6.1 g/kg) are similar to those reported by Fantuz et al. (2016) and Miraglia et al. (2020).

Lactase activity

The lactase enzyme activity of the fermented mare's beverages with chia (Table 2) ranged from 0.17 (in fermented milk with 3 weeks storage) to 0.84 μkat·100 g^{-1} immediately after production). The next hours of fermentation significantly increased lactase enzyme activity. The exception was the last 18–24 h of incubation ($P > 0.05$). There were no differences between samples after the end of fermentation ($P > 0.05$). After 3 weeks, the lactase enzyme activity of fermented mare's milk was 0.12 μkat·100 g^{-1}. The values between the samples during the entire storage did differ significantly ($P < 0.05$). With the passage of storage time, the activity of lactase contained in the samples was significantly lower than immediately after their production. This direction of change in lactase activity was demonstrated regardless of the type of samples tested. After 3 weeks, the lactase activity decreased by 3.5 times.

The results of correlating the number and type of characteristic microflora with the lactase activity produced by them were presented using a distance-weighted least-squares smoothing procedure (Fig. 1). The segmentation and type of contour lines provide interpretable information on the phenomenon studied for each type of sample separately, irrespective of its fermentation time and subsequent storage.

Table 2. Changes in lactase enzymatic activity in mare's milk and mare's milk with chia during fermentation and further storage, μkat·100 g^{-1}

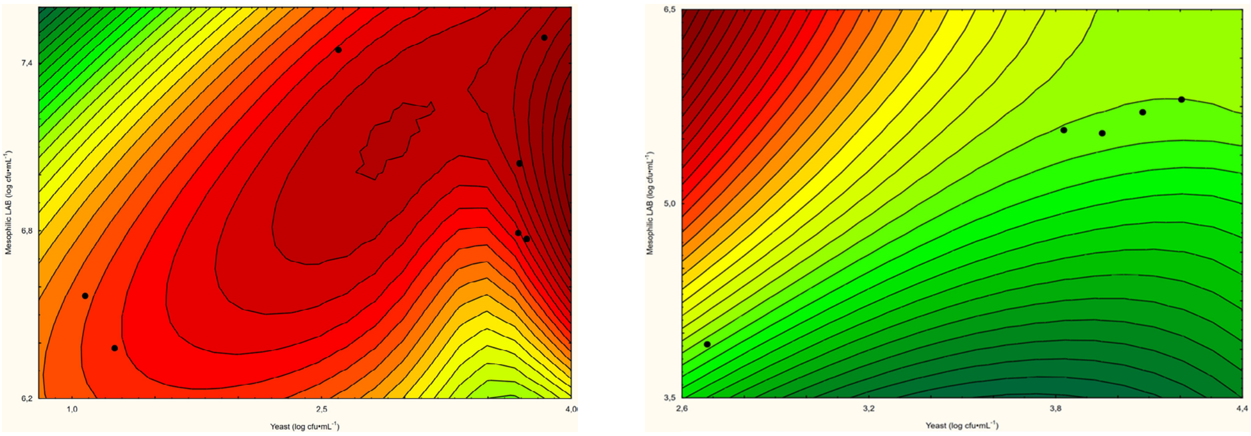
	Fermented mare's milk	
	without chia	with chia
Fermentation, h		
6	0.40 ^{aA}	0.39 ^{aA}
12	0.53 ^{aB}	0.51 ^{aB}
18	0.72 ^{aC}	0.74 ^{aC}
24	0.79 ^{aC}	0.77 ^{aC}
Storage, weeks		
0	0.79 ^{aD}	0.84 ^{bD}
1	0.35 ^{aC}	0.42 ^{bC}
2	0.20 ^{aB}	0.28 ^{bB}
3	0.12 ^{aA}	0.17 ^{bA}

a–b, A–D – different small letters with mean values in the rows and capital letters with mean values in columns separately for fermentation and storage indicate statistically significant differences, at the level of $\alpha = 0.05$.

The number of probiotic bacteria remained unchanged in the fermented beverages for the first 2 weeks of storage. After this time, the number of *Bifidobacterium* decreased by 11%. The analysis of matched surface contour plots using the direct imaging method allowed us to determine the convergence of the number of probiotic bacteria with the remaining characteristic microflora in the samples immediately after their formation and throughout the storage period (Fig. 2). The number of characteristic microflora formed a triangle in three-dimensional space. The relative proportions of each type of microflora were scaled to add to 1 within each case. Thus, the vertex coordinates were given a value of 1 for a given microflora type and 0 for the other two types.

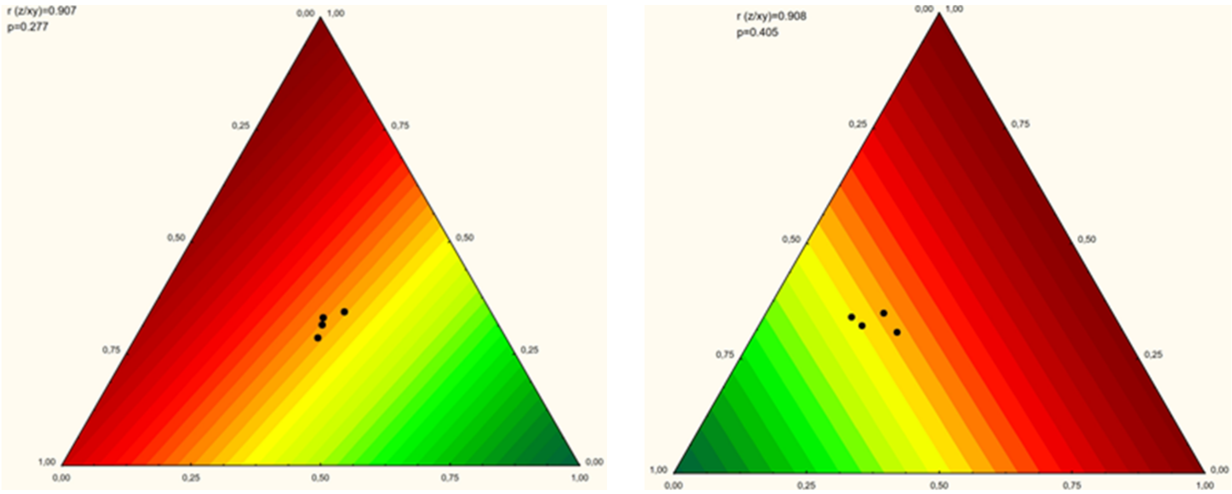
Fatty acid profile

The analysis of fatty acid profiles showed statistically significant differences in the concentration of SFA which is 18.27% lower in fermented milk than those of the unfermented mare's milk (Table 3). The addition of chia seeds increased the Σ UFA and Σ MUFA



Δ – lactase activity, µkat·100 g^{-1}, cross section: >0.900 <0.875 <0.775 <0.675 <0.575 <0.475 <0.375 <0.275 <0.175

Fig. 1. Interdependence of lactase enzymatic activity on counts of characteristic microflora in mare's milk without chia and mare's milk with chia during their fermentation and further storage, df = 11



Δ – goodness of fit, axes – component values in a 0–1 scale: Z – *Bifidobacterium*, log cfu·mL^{-1}, X – mesophilic LAB, log cfu·mL^{-1}, Y – yeast, log cfu·mL^{-1}, cross section: >16 <16 <12 <8 <4 <0 <–4 <–8

Fig. 2. A dependence of probiotic bacteria count on the counts of other characteristic microflora in fermented mare's milk without chia and mare's milk with chia after their fermentation and further storage, df = 11

respectively by about 12.31% and 6.78%. The *n*-6/*n*-3 values ratio in unfermented and fermented mare's milk were about 1:1. This proportion is following the recommended value of 4:1 and a balanced ratio of *n*-3 and *n*-6 PUFAs in the diet is necessary for health. According to (Simopoulos, 2016), it is a very important determinant for reducing the risk of many chronic diseases. The Σ 1.04 Atherogenic index (AI) was lower in mare's milk than in fermented mare's milk, and the thrombogenic index (TI) take into account the

Table 3. Composition of fatty acids of mare's milk and fermented mare's milk, % of total lipids

MFA	Mare's milk	Fermented mares milk	
		without chia	with chia
Σ SFA	47.71 [b]	38.99 [a]	39.89 [a]
Σ UFA	51.83 [c]	42.96 [a]	48.25 [b]
Σ MUFA	27.61 [a]	27.28 [a]	29.13 [a]
Σ *n*-3 FA	10.59 [a]	10.47 [a]	12.71 [b]
Σ *n*-6 FA	9.80 [a]	9.52 [a]	9.96 [a]
n-3/*n*-6	1.08 [a]	1.10 [a]	1.32 [b]
HcFA	25.33 [b]	24.23 [ab]	24.69 [ab]
AI	1.04 [a]	1.22 [c]	1.25 [c]
TI	0.54 [a]	0.51 [a]	0.47 [a]
DFA	53.92 [b]	44.52 [a]	45.19 [a]
OFA	45.62 [b]	37.43 [a]	38.11 [a]
DFA/OFA	1.18 [a]	1.18 [a]	1.19 [a]

Σ SFA – sum of C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C14:0 *iso*, C15:0, C15:0 *iso*, C15:0 *anteiso*, C16:0, C16:0 *iso*, C17:0, C17:0 *iso*, C17:0 *anteiso*, C18:0, C18:0 *iso*, C20:0, C22:0, and C24:0.
Σ UFA – sum of MUFA (C10:1, C12:1, C14:1 *cis*-9, C16:1 *cis*-9, C16:1 *trans*-9, C17:1 *cis*-9, C18:1 *cis*-9, C18:1 *cis*-11, C18:1 *cis*-12, C18:1 *trans*-9, C18:1 *trans*-11, and C20:1 *cis*-11) and PUFA (C18:2 *cis*-9, *trans*-11, C18:2 *cis*-9, *cis*-12, C18:3 *cis*-6, *cis*-9, *cis*-12, C18:3 *cis*-9, *cis*-12, *cis*-15, C20: 2*n*-6, C20: 3*n*-6, C20: 4*n*-6, C20: 5*n*-3, and C22: 5*n*-3).
Σ *n*-3 FA – sum of C18: 3*n*-3, C20: 5*n*-3, and C22: 5*n*-3.
Σ *n*-6 FA – sum of C18: 2*n*-6, C18: 3*n*-6, C20: 2*n*-6, C20: 3*n*-6, and C20: 4*n*-6.
HcFA – hypercholesterolemic fatty acid index.
AI – atherogenic index.
TI – thrombogenic index.
DFA – UFA + C18:0.
OFA – SFA – C18:0.

relationship between the pro-thrombogenic (saturated) and anti-thrombogenic fatty acids (unsaturated), indicative of the potential accumulation of blood flakes in the blood vessels (Becskei et al., 2020). The TI value (0.54) was higher in raw milk than in fermented milk. The values of fatty acids with a hypocholesterolemic effect (DFA) and hypercholesterolemic fatty acids (OFA) decreased in fermented milk ($p < 0.05$).

DISCUSSION

There are many arguments in the literature on the preventive and therapeutic effects of consuming fermented cow's milk, but there is still limited information about fermented mare's milk. The daily consumption of certain foods and beverages results in specific changes that occur in the organism. Mare's milk is known for its high value of nutritionally important substances, which have beneficial properties. According to Miraglia et al. (2020), mare's milk is characterized by a large quantity of lactose (63 g/kg), protein (16.8 g/kg) and a reduced fat content (1.03 g/kg). Fotschki et al. (2016) argue that the lactose concentration of mare's milk is relatively high in comparison to milk from cows, goats, and sheep. It is recognized that about 70% of the adult population has limited expression of lactase enzyme with a wide variation among different regions and countries. Lactase deficiency may lead to lactose intolerance (Facioni et al., 2020). The amount of ingested lactose and the insufficient activity of the lactase might cause numerous gastrointestinal symptoms (e.g., bloating, nausea, abdominal pain. Sometimes people with lactose malabsorption suffer from various disorders e.g., headache, skin lesions, eczema, cognitive dysfunction. Nowadays, the avoidance of dairy products in people with lactose intolerance is not recommended (Barreto et al., 2020; Misselwitz et al., 2019). In addition, the consumption of fermented milk plays an important role in the gut microbiota, due to their content in probiotics (Hertzler et al., 2017; Oak and Jha, 2018). According to Shaukat et al. (2010), probiotic bacteria may vary in their ability to improve lactose digestion and reduce maldigestion symptoms. Most lactose-intolerant people can eat fermented dairy products without exhibiting typical symptoms (Fassio et al., 2018).

Lactose in fermented beverages with live bacteria is better tolerated by people (who cannot digest lactose) than lactose in unfermented milk. Usually, they haven't exhibited typical symptoms (Fassio et al., 2018). In our study, we measured the lactase enzyme activity. The fermented mare's beverages with chia ranged from 0.17 (in fermented milk with 3 weeks storage) to 0.84 $\mu kat \cdot 100\ g^{-1}$ immediately after production. After 3 weeks, the lactase activity decreased by 3.5 times. Guo et al. (2019) have shown that the lactose contents in Chigee from mare's milk (traditionally fermented in Inner Mongolia) was 4.2 ±1.6% and was lower compared to those reported in fresh mare milk. The explanation is that during the process of spontaneous dairy fermentation, the lactic acid bacteria (LAB) use lactose as a source of carbon and produce lactic acid, while yeasts enhance the alcohol content (Guo et al., 2019; Sun et al., 2014).

None of the studies reported the comparative examination of the fatty acid composition of fermented mare's milk with chia. Results obtained by Derewiaka et al. (2019) shows that the addition of 2% chia seed oil to natural yogurt resulted in higher amounts of linoleic and α-linolenic acid. In another study carried out by Eker and Karakaya (2020) the addition of chia seeds caused the detection of α-linolenic and linoleic acids in yogurt containing chia seeds. The profile of fatty acids largely determines the health quality of milk. According to Pilarczyk et al. (2015), AI and TI values of >1.0 are required in milk, and they have beneficial effects on the cardiovascular system. According to Fantuz et al. (2016) and Miraglia et al. (2020), calculations of fatty acid composition, atherogenic (AI) and thrombogenic index (TI) suggest that mare's milk can be a good alternative for people with allergic or inflammatory conditions. In our research, the AI index value for fermented mare's milk was 1.04 and averaged 1.2 for the fermented mare's milk. The unique fatty acid composition of mare's milk could also help it become a valuable food for elderly consumers (Salimei and Fantuz, 2012).

CONCLUSIONS

The present study followed the influence of mare's milk fermentation and storage on lactase activity. We observed that after 3 weeks, the lactase activity decreased by 3.5 times. The number of probiotic bacteria remained unchanged in the fermented beverages for the first 2 weeks of storage. After this time, the number of *Bifidobacterium* decreased by 11%. Microflora used in the production of fermented beverages can improve lactose digestion and reduce maldigestion symptoms (Kok and Hutkins, 2018). It seems particularly important for older people who may have problems with the digestion of lactose. The results showed that the addition of chia seeds increased the Σ UFA and Σ MUFA respectively by about 12.31% and 6.78%. The present study gives an idea regarding the composition of chia seeds that have a positive effect on the nutritional properties of fermented mare's milk. We are planning to further extend our research by adding powdered chia seeds, which should have an even better effect on increasing the fatty acid content of fermented mare's milk.

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