

Quality of fresh and stored mares' milk

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Abstract

Mares' milk is characterised by unique nutritional profile. In this study, the microbiological analysis of mares' milk was performed. The presence of total bacteria, total lactic bacteria, *Lactobacillus* spp., *Streptococcus lactis*, *Salmonella* spp. and coliforms was investigated. Moreover, the influence of refrigerated and frozen storage on the total bacteria count, vitamin C, acidity and colour of milk was examined. Pathogenic *Salmonella* spp. and coliforms were not detected in the raw milk. It was revealed that mares' milk can be stored for 72 hours under refrigeration at a temperature of +4 °C without reducing its microbiological quality. Most of the physicochemical properties remained unchanged, while colour measurements demonstrated a change in b* value after 48 h of refrigerated storage. Vitamin C content remained relatively stable during a week-long storage. Freezing of milk improved its microbiological status and caused significant changes in all colour components (L*, a*, b*). The obtained results demonstrated that mares' milk had a high microbiological quality, favourable chemical composition and high vitamin C content which make this product a valuable potential component of functional foods.

Key words: mares' milk, refrigeration, freezing, shelf life, microbiology

Introduction

Mares' milk has unique health benefits associated with its favourable chemical composition (Uniacke - Lowe et al., 2010; Salimei and Fantuz, 2012; Čagalj et al., 2014; Pieszka et al., 2016). As such it has been proposed for the treatment of skin, respiratory and gastrointestinal disorders (Chiofalo et al., 2006; Foekel et al., 2009; Zava et al., 2009). The consumption of raw milk poses a risk for consumers, due to its possible contamination with pathogenic microorganisms. Even though mares' milk contains a very low number of

total bacteria, consumers should be aware that it might be a source of pathogenic microorganisms (Verraes et al., 2014).

Pasteurization of milk guarantees a microbial safety and a shelf-life extension. However, in the case of mares' milk freezing and refrigeration are the most common methods of storage and conservation (Claeys et al., 2014). These processes slow down the microbial growth and chemical changes in milk but do not guarantee the inactivation of all vegetative pathogens (Robinson, 2002; Verraes et al., 2014)

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In the scientific literature, there are few reports on microbiota present in mares' milk (Hazeleger and Beumer, 2016), but up to now physicochemical changes occurring during the storage have not been studied comprehensively. Most of the results available in this area were conducted on human or cows' milk. Therefore, the goal of this research was to examine raw mares' milk samples for the presence of lactic acid bacteria and human pathogenic bacteria, such as *Salmonella* spp. and coliforms. Qualitative changes occurring in mares' milk during refrigerated and freezing storage were also analysed.

Materials and methods

Animals and milk collection

The experiment was carried out on milk obtained from 10 multiparous mares of the *Polish Coldblood horse* breed between the 150 and 200 days of lactation. Milk was collected from the mares twice in a three weeks' interval; the first time for microbiological analysis, and the second time for analyses related to changes occurring during the storage. Milk obtained from each mare, for the second time, was collected mechanically according to the procedure described by Markiewicz-Keszycka et al., (2013). After milking it was divided into three parts; one part was analysed as soon as possible after milking, the second part was refrigerated at 4 °C and the third part was frozen and stored at -30 °C for 3 weeks. Samples were then thawed in a water bath at 40 °C for a total bacterial count, acidity, and colour analyses. Milk samples for microbiological analysis were hand-milked from each mare, from both udder halves, according to the National Mastitis Council (1990) procedures. Sample collection was preceded by fore-stripping, teat cleaning and pre-dipping with 1 % iodophor. Then each teat was thoroughly dried to remove the pre-dip with a single dry paper towel per mare and scrubbed with gauze pad moistened with 70 % ethyl alcohol. 100 mL of milk was collected to sterile test tube as rapidly as possible. After obtaining the sample, test tubes were immediately placed on ice and kept refrigerated until delivered to the microbiological laboratory.

Microbiological analysis

Determination and enumeration of the total lactic bacteria was performed by the serial dilution method according to Koch (Kunicki-Goldfinger, 2007) and expressed in colony-forming units (cfu/mL). M17 agar was used for isolating and enumerating of *Streptococcus lactis*. MRS culture media were used for culturing and isolation of *Lactobacillus* spp. (Ashraf and Shah, 2011). For the isolation and identification of *Salmonella* spp. XLT4 agar medium was used (Miller and Tate, 1990). In order to ensure that isolated bacteria belong to the *Salmonella* spp. procedure by Mossel et al., (1963) was applied. Chromocult® Coliform Agar (Merck) was used to determine a total coliform number (Manafi and Kneifel, 1989). All microbiological analyses were performed on fresh milk in five replications.

The basic chemical composition, as well as density and freezing point of milk, were determined by a Milkoscan FT 120 instrument (Foss Electric, Hillerod, Denmark) on fresh milk.

Total bacteria count (TBC) and somatic cells count (SCC) were evaluated using a Bacto Count IBCm (Bentley, Minnesota, USA) according to the ISO 21187 (2004) standard. The analysis of total bacterial count was conducted on fresh milk as well as on milk stored under refrigeration (4 °C) in 24 h intervals for 7 days (d). Analysis of SCC was performed on fresh milk. A Bacto Count apparatus was calibrated according to the ISO 4833 (2003) standard (Bentley Polska Sp. o. o.).

Acidity was analysed in fresh and frozen milk after 3 weeks of storage. It was measured at room temperature using a Handylab 2 pH Meter. Titratable acidity analysis was carried out according to the Soxhlet-Henkel method (PN-68/A-86122).

Vitamin C analysis was conducted according to the method proposed by Omaye et al. (1979), modified and described by Markiewicz-Keszycka et al. (2014). The content of vitamin C was determined in fresh milk and after 2, 4, and 7 d of storage.

The colour of milk samples was measured in triplicates in the CIE (Commission Internationale d'Éclairage) L*a*b* system, where L* represents lightness and ranges from 0 for black to 100 for white, a* represents the colour's position between

green ($-a^*$) and red ($+a^*$), and b^* represents the colour's position between blue ($-b^*$) and yellow ($+b$). The Minolta CM 5 Colorimeter (Minolta Corp., Osaka, Japan) with an illuminant C and a 2° observer was used. The measurements of the colour were performed on fresh milk, milk stored under refrigeration (4°C) in 24h intervals for a period of 7 d as well as on frozen milk (after 3 weeks of freezing storage).

Statistical analysis

Analysis of the results was conducted using the SAS 9.4 software (2014). The influence of refrigerated storage on SCC, vitamin C content and colour of milk was analysed with one-way ANOVA, and the means were compared using Duncan multiple range test; in the case of TBC the Kruskal-Wallis non-parametric test was applied. Student's t-test was used when comparing the averages of fresh and frozen milk. The data relating TBC in the milk was subjected to logarithmic transformation before statistical verification (Ali and Shook, 1980).

Results and discussion

Milk characteristics

The analysed mares' milk was characterised by a dry matter content of $9.33\% \pm 0.60$. An interesting aspect was the low fat content of the examined milk ($0.17\% \pm 0.14$). The literature indicates that the fat content of mares' milk depends on the breed as well as on the lactation stage, and it ranges from 0.4-2.2% (Caroprese et al., 2007; Pikul and Wójtowski, 2008). In the conducted research, the content of protein and lactose was $1.56\% \pm 0.14$ and $6.58\% \pm 0.20$, respectively. The high content of lactose indicates that mares' milk is a valuable material for the production of fermented drinks and allows a good growth of the intestinal microbiota (Küçükçetin et al., 2003; Pieszka et al., 2016). The freezing point of examined milk was -0.560°C , while its density was 1.0370 g mL^{-1} .

In this experiment, the average pH value of fresh milk was 7.31 and was similar to the values obtained by Pagliarini et al., (1993) but slightly higher than in the study by Küçükçetin (2003). The titratable acidity of fresh mares' milk was 2.23°SH , similarly to average values achieved by Čagalj et al., (2014).

SCC in milk is the main indicator of the mammary gland health and milk quality. The analysed fresh mares' milk exhibited low SCC content at the average level of $2.0 \times 10^3\text{ mL}^{-1} \pm 1.22$. Similar results were achieved by Markiewicz-Keszycka et al., (2013) and slightly higher SCC content was noted by Danków et al., (2006). According to Kulisa et al. (2010) and Cieślak et al. (2015) the differences in the SCC are associated with mares' breed, age, consecutive lactation and lactation stage. In general, mares' milk in comparison to ruminants' milk is characterised by low SCC content (Danków et al., 2006; Hamed et al., 2012). This may result from a low capacity of mares' udder, the high concentration of antibacterial factors in milk, as well as from the presence of favourable milk microbiota (Ogundele, 2002).

Microbiota of mares' milk

Our study confirms the high microbiological quality of mares' milk. The pathogens, such as coliforms and *Salmonella* spp. have not been detected. According to the cited literature these types of bacteria have not been found in mares' milk so far (Salimei and Fantuz, 2012; Verraes et al., 2014; Hazeleger and Beumer, 2016). However, it needs to be stressed that *Salmonella* spp. can originate in milk from the animals and the environment and it is one of the most common causes of dairy-related illness in the United States and Western Europe (Robinson, 2002; Claeys et al., 2013).

Coliforms are Gram-negative rods able to ferment lactose and are responsible for spoilage of milk. Typically, this group is represented by the genera *Escherichia* and *Enterobacter*. The presence of these bacteria suggests that the other organisms of faecal origin, including pathogens, may also be present. These types of bacteria can lead to an inappropriate fermentation process in which gases such as CO_2 and H_2 are formed resulting in a deterioration of taste, smell and overall product quality (Todaro et al., 2017).

The comprehensive microbiological analysis presented in the review of Colavita et al., (2016) shows that the number of pathogens such as *Escherichia coli* O157, *Salmonella* spp., *Campylobacter* spp., *Yersinia enterocolitica*, *Brucella* spp., *Mycobacterium* spp., *Bacillus cereus*, *Cronobacter sakazakii*, *Streptococcus equi* subsp. *zoepidemicus*, *Rhodococcus equi*, *Streptococcus dysgalactiae* subsp. *equisimilis*, *Clostridium difficile* and *Burkholderia*

mallei in equine milk is very low. However, it should be highlighted, that mares' milk, despite of its high microbiological quality, should be subjected to the thermal processing before consumption.

Another group of microorganism present in milk are Gram-positive mesophilic bacteria such as lactic acid bacteria (LAB). The number of LAB present in the examined mares' milk is shown in Table 1. The study by Hazeleger and Beumer (2016) also demonstrated that LAB are common in mares' milk. The number of LAB present in mares' milk recorded by the above authors were at the same level as in the present study (2-4 log). Several of these microorganisms are employed in the production of fermented foods such as kumis - a dairy alcoholic drink (Wouters et al., 2002; Bornaz et al., 2010). Their presence determines the shelf-life, safety and organoleptic characteristics of dairy products (Widyastuti and Febrisiantosa, 2014). LAB show inhibitory activity for many pathogenic microorganisms and are of a big industrial importance as they are associated with the healthy microbiota of human mucosal surfaces (Nuraida, 2015). However, the appearance of these bacteria in a large number ($>10^6$ cfu/mL) can cause defects of milk associated with fermentation by-products (Robinson, 2002).

Influence of refrigeration storage on total bacteria count (TBC)

The changes in TBC during 7 d refrigerated storage are shown in Table 2. When analysing the logarithmic phase of bacterial growth, it was revealed that significant growth only occurs after 96 hours of storage ($p \leq 0.01$). During the first 72 hours of storage, milk was exhibiting high microbiological quality, which was undoubtedly associated with the initial low TBC in milk. The high microbiological quality of mares' milk was also confirmed in the previous studies by Markiewicz-Keszycka et al., (2013).

Table 1. Counts of different microbial groups in fresh mares' milk (cfu $\times 10^3$ mL⁻¹)

Bacteria	Mean	SD
Total lactic bacteria	3.69	0.32
<i>Lactobacillus</i> spp.	0.61	0.23
<i>Streptococcus lactis</i>	2.67	0.26
<i>Salmonella</i> spp.	N.D.	
Coliforms	N.D.	

SD - standard deviation; N.D. - not detected

It needs to be emphasized that the storage of milk at 4 °C does not stop the growth of microorganisms, but slows it down significantly (Guinot-Thomas et al., 1995). According to Murphy et al., (1983), milk possesses several antimicrobial systems such as the antibacterial proteins which remain active for 24-48 hours at a temperature of 4 °C. Mares' milk seems to have stronger antibacterial properties than cows' milk, because of higher content of bacteriostatic ingredients such as lactoferrin, lysozyme, immunoglobulins and lactoperoxidase (Zhang et al., 2008).

Influence of refrigeration storage on vitamin C content in mares' milk

Vitamin C analysis did not reveal any significant changes during week-long storage, however, a downward trend was visible (Table 2). During 7 d storage, the decrease in vitamin C content in milk was approximately 11 %, after four days the losses were 7 %, while after two days 1.5 %. Romeu-Nadal et al. (2008) reported a 60 % drop in vitamin C content during the storage of human milk at +4 °C for 96 hours. Similar results were achieved by Buss et al. (2001). Thus, it should be suspected that vitamin C in mares' milk is more stable than in the case of human milk.

Table 2. Changes of total bacteria count and vitamin C in mares' milk during refrigerated storage

Time of storage Hours/days	Item	
	Mean	SD
	Total bacteria count (log ₁₀)	
Fresh milk/0 d	3.70 ^A	0.10
24 h/1 d	3.68 ^A	0.13
48 h/2 d	3.71 ^A	0.14
72 h/3 d	4.01 ^A	0.29
96 h/4 d	4.90 ^B	0.14
120 h/5 d	5.46 ^C	0.16
144 h/6d	5.83 ^D	0.05
168 h/7d	5.85 ^D	0.07
	Vitamin C (mg 100 mL ⁻¹)	
Fresh milk /0 d	11.38	2.01
48 h/2 d	11.21	2.02
96 h/4 d	10.59	1.95
168 h/7 d	10.15	1.82

A-D - averages in column with different superscripts are statistically different ($p \leq 0.01$), SD - standard deviation

Vitamin C is very sensitive to the numerous factors such as a type of packing, exposure to the sunlight, heat treatment as well as time and temperature of storage (Gliguem and Birlouez-Aragon, 2005; Abramovich et al., 2013). Research by Romeu-Nadal et al. (2008) on human milk indicated that in order to avoid vitamin C oxidation and losses, it is recommended to store it in a refrigerator for up to 3 hours, frozen at $-20\text{ }^{\circ}\text{C}$ for max. 5 months, or at $-80\text{ }^{\circ}\text{C}$ for 8 months.

Influence of the refrigeration storage on milk colour

The colour of milk is the parameter of the sensory characteristics, strongly influencing consumers' acceptability. The obtained results for L^* value (83.61) in fresh milk were lower than those recorded by Uniacke-Lowe (2011) which can be attributed to the low fat concentration in the milk examined in this study. The above author reported that mares' milk was whiter in comparison to cows' milk and that skimming increased the participation of luminance in its colour (L^*). The white colour of milk is related mainly to the scattering of light by the dispersed phase of casein micelles and fat globules (Pandya and Ghodke, 2007; Uniacke-Lowe, 2011).

In the current research, the parameter L^* and a^* value did not change significantly during 7 d storage. However, after 48 h storage the b^* value changed from negative -0.59 (in the fresh milk) to the positive value of 0.79 ($p \leq 0.05$). This indicates the change of colour from blue to yellow. The information in the scientific literature regarding the changes of the colour occurring in mares' milk during the storage is

limited. The only available research was conducted on heat treated cows' milk (Gaucher et al., 2008). Storage conditions and technological processes such as homogenisation can also change a physical structure of milk resulting in colour changes (Kneifel et al., 1992; Kristensen et al., 2001; Hassan et al., 2009). Moreover, the pigments contained in milk, mainly carotene and riboflavin, influence its hue (Nozière et al., 2006). The colour of milk may also be affected by the diet of animals, disease processes, as well as the physiological factors associated with milk production (Argüello et al., 2004; Calderón et al., 2007).

Influence of freezing on TBC, acidity and colour of milk.

The changes of selected qualitative parameters caused by freezing at $-30\text{ }^{\circ}\text{C}$ for 3 weeks are shown in Table 3. Freezing caused an over a three-fold reduction in the TBC ($p \leq 0.01$). The improvement of the microbiological quality of milk after freezing was reported in many studies (Sanchez-Macias et al., 2010; Ahrabi et al., 2016). However, it needs to be stressed that freezing even at very low temperatures does not destroy bacteria completely. Particular attention should be paid to the pathogenic bacteria as they can survive the freezing and reactivate after thawing.

The process of freezing increased titratable acidity ($p \leq 0.05$) and slightly decreased pH of mares' milk, but no significant changes in pH were observed. Similarly, the study conducted by Katsiari et al. (2002) on ewe milk did not show any influence of freezing on acidity changes.

Table 3. The influence of freezing on total bacteria count, titratable acidity and color of mare' milk

Item	Fresh milk		Frozen milk	
	Mean	SD	Mean	SD
Total bacteria count ($\times 10^3\text{mL}^{-1}$)	5.12 ^C	1.25	1.50 ^D	0.83
Total bacteria count (\log_{10})	3.70 ^C	0.10	3.18 ^D	0.16
Titratable acidity ($^{\circ}\text{SH}$)	2.23 ^c	0.46	3.11 ^d	0.93
Color according to CIE				
L^* (lightness)	83.61 ^C	1.70	86.87 ^D	1.51
a^* (redness)	-2.04 ^C	0.28	-2.53 ^D	0.40
b^* (yellowness)	-0.59 ^C	1.16	2.10 ^D	1.40

C-D, c-d - averages within a row with different superscripts are statistically different; capital letters ($p \leq 0.01$), small letters ($p \leq 0.05$), SD - standard deviation

The results of this study demonstrated that freezing had a significant impact on the colour of milk in all components of the spectrum (L^* , a^* , b^* ; $p \leq 0.01$). The colour of the thawed milk became brighter with the higher value of b^* and lower value of a^* compared to the fresh milk. These changes are probably due to the destabilisation of casein micelles by salting-out (Morr, 1975). Lack of information in the literature on the qualitative changes in frozen mares' milk, justify the need for further research in this area.

Conclusions

Milk of the *Polish Cold-blooded* mares is characterised by high microbiological and cytological quality. Pathogenic *Salmonella* spp. and coliforms were not detected. The experiment revealed that mares' milk might be stored for at least 72 hours at a temperature of + 4 °C without reducing its microbiological quality. Vitamin C content remained on a relatively stable level during week-long storage. Freezing the milk at -30 °C for a period of 3 weeks improves its microbiological condition and, at the same time, causes significant changes in all colour components and leads to an increase in titratable acidity. Despite its high microbiological quality, it is suggested to pasteurise raw mares' milk before consumption, to ensure the safe product for the consumers.

Kvaliteta svježeg i skladištenog kobiljeg mlijeka

Sažetak

Mlijeko kobile odlikuje se izuzetnom hranjivom vrijednošću. U ovom istraživanju ispitivana je mikrobiološka kvaliteta kobiljeg mlijeka pri čemu je utvrđen ukupan broj bakterija mliječne kiseline, *Lactobacillus* spp., *Streptococcus lactis*, *Salmonella* spp. i koliformne bakterije. Također je utvrđivan i utjecaj hladnog skladištenja ohlađenog i zamrznutog mlijeka na ukupan broj bakterija, količinu vitamina C, kiselost i boju mlijeka. U sirovom mlijeku nije utvrđena prisutnost patogenih mikroorganizama roda *Salmonella* spp. kao niti koliformnih bakterija.

Dokazano je kako se mlijeko kobile može skladištiti 72 sata u hladnjaku na temperaturi +4 °C bez umajenja njegove mikrobiološke kvalitete. Osim toga, većina fizikalno-kemijskih svojstava mlijeka ostala je nepromijenjena, a jedino je došlo do promjene vrijednosti parametra b^* nakon 48 sati čuvanja u hladnjaku. Količina C vitamina ostala je relativno stabilna tijekom sedmodnevnog čuvanja. Zamrzavanje mlijeka utjecalo je na poboljšanje mikrobiološke kvalitete mlijeka te je istovremeno uzrokovalo znatne promjene u svim parametrima boje (L^* , a^* , b^*). Rezultati ovog istraživanja dokazuju da kobilje mlijeko ima visoku mikrobiološku vrijednost, poželjan kemijski sastav te visoke količine vitamina C, što ovaj proizvod čini vrijednim potencijalnim prehrambenim proizvodom.

Cljučne riječi: kobilje mlijeko, hlađenje, zamrzavanje, trajnost, mikrobiologija

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