Microorganisms in Fermented Mare Milk Products

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Mare milk is consumed by approximatively 30 million people in the world. In countries in Asia and East Europe, mare milk is mainly consumed as source of fermented products, called koumiss, airag or chigee, alcoholic beverages obtained by means of a culture of bacteria and lactose-fermenting yeasts. Recent research concerning mare milk and its derivatives deals mainly with their potential employment for human health.

mare milk koumiss airag lactic acid bacteria yeasts

1. Introduction

The most abundant microorganisms naturally present in milk can be classified in order of their possible roles (i) microorganisms involved in dairy fermentation (e.g., *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Propionibacterium* and fungal populations); (ii) involved in spoilage (e.g., *Pseudomonas*, *Clostridium*, *Bacillus*, and other spore-forming bacteria); (iii) involved in food disease (e.g., *Listeria*, *Salmonella*, *Escherichia coli*, *Campylobacter* and mycotoxin-producing fungi); and (iv) involved in promoting health (e.g., lactobacilli and bifidobacteria) [1]. Human milk from healthy women contains up to 10^9 microbes L^{-1} [2]. These organisms come mainly from the nipple and surrounding skin [2]. Ward et al. studied the complexity of the bacterial community in human milk, finding over 360 prokaryotic genera, mainly belonging to the phyla of Proteobacteria (65%) and Firmicutes (34%), and the *genera* of *Pseudomonas* (61.1%), *Staphylococcus* (33.4%) and *Streptococcus* (0.5%) [3]. Microbial colonization during the first few weeks of life in the gastrointestinal tracts of humans and farm animals is remarkably similar. Bifidobacteria are the predominant lactic acid bacteria (LAB) in infants, whereas lactobacilli are the primary LAB in the tract of new-born foals [4].

The microbial composition of raw mare milk, like that in other similarly less common milk types, has not been studied in depth: nevertheless, it is known to be widely variable, depending on many factors, such as the breed, season, and region [5]. However, many studies regarding the microbiological composition of fermented mare milk are available.

Koumiss, airag and chigee are the main products obtained through mare milk fermentation. Mare milk fermentation is due to lactic acid bacteria (LAB) and yeast interaction. LAB are mainly involved in milk acidification, whereas the yeasts partly modify its titratable acidity due to the production of acidic compounds, such as acetic acid. On the other hand, yeasts produce ethanol that is very important for determining the properties and increasing the stability of fermented milk [6][7].

The interaction between LAB and yeast can be complex and has to be studied in more depth; however, different theories have been suggested. Positive relationships between the two types of microorganisms can occur because lactic acid bacteria are responsible for the lowering of the pH due to the secretion of organic acids, allowing the yeast population to become competitive in the immediate environment, followed by yeast fermentation [8]. Sudun et al. observed a positive correlation regarding glucose and galactose produced by the lactase of LAB, and consumed by yeast for their growth [6].

In addition, the interaction between the two types of microorganisms is reflected in the product. Regarding its safety, the combination of the acidic condition, saturated with carbon dioxide and alcohol, is inhibitory to many spoilage bacteria [8]. Regarding quality, yeast proteolytic and lypolitic metabolism can stimulate LAB growth and play an important role in aroma development [9].

Koumiss is a lactic acid–alcoholic beverage produced traditionally by the fermentation of mare's milk by indigenous organisms or by a starter culture. First, lactic acid is formed, and then alcoholic fermentation of the residual sugar content occurs. Koumiss generally contains about 2% alcohol, 0.5–1.5% lactic acid, 2–4% sugar and 2% fat (Kerr and McHale [10]; quoted by Danova et al. [11]). The alcohol has been known to reach a level of 3.5% and, depending on its content, in Russia there is a differentiation between weak koumiss with 0.7–1% ethanol, normal koumiss with 1–1.75% and strong koumiss with 1.75–2.5% [12].

According to Danova et al., another distinction exists: depending on the lactic acid content, three types of koumiss can be distinguished: 'strong', 'moderate' and 'light' koumiss. Lactic acid bacteria (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus rhamnosus*) acidifying the milk to pH 3.6–3.3 and converting about 80–90% of lactose into lactic acid produce 'strong' koumiss. In 'moderate' koumiss, other *Lactobacillus* (*Lb.*) bacteria (*Lb. acidophilus*, *Lb. plantarum*, *Lb. casei*, and *Lb. fermentum*) with lesser acidification properties and a conversion ratio of about 50% lower the pH to 4.5–3.9 at the end of the process. 'Light' koumiss, produced by *Streptococcus thermophilus* and *Str. cremoris*, is slightly acidic (pH 4.5–5.0) [11]. According to several authors, "moderate" koumiss presents a sweet–sour taste and a yeasty odour and is the most appreciated [11][13]. It was demonstrated that acetaldehyde is the most important substance determining aroma in koumiss and the suitable range of its content is 78.25−257.07 μL L^{-1} [14][15].

Airag, which is called also tsege in Inner Mongolia, contains *Lb. helveticus*, *Lactobacillus fermentum*, and *Saccharomyces cerevisiae* [16]. The traditional technique of production in a cowhide vessel of this product in Mongolia was included in 2019 in the Representative List of the Intangible Cultural Heritage of Humanity.

In an attempt to identify which properties are related to the most appreciated sensory characteristics of airag, it was found that only its electrical conductivity has a statistically significant relationship with its taste score: higher electrical conductivity values are associated with lower taste scores; even though the mean pH values were not different, calcium and phosphorus concentrations were lower in the airag samples rated with high scores, taking in account 51 different airag samples exhibited in a competition in Mongolia [17].

Many mesophilic LAB and yeasts have been detected in koumiss. According to tradition, fresh milk is inoculated with a small quantity of already-fermented milk as natural starter, but many other different substances could be used as well [18]. The use of raw milk and natural undefined starter cultures causes a strong variability in the microbial composition. Many authors have observed a wide variability in species and strains [19][20]. It has been evidenced that milk derivatives from each family have their particular microbiota [21].

A few studies have investigated the relationship between koumiss microbiota and the production of volatile flavour compounds or organic acids, and their effects on taste. Despite the wide variability of microbes and yeasts involved, a “core” microbiota was identified, represented by four bacterial *genera* (*Lactobacillus*, *Acetobacter*, *Lactococcus*, and *Pseudomonas*), and two yeast *genera* (*Kazachstania* and *Candida*), and it was observed that notwithstanding the differences depending on the region and production techniques, the basic volatile flavours in traditional koumiss are similar [20][22].

Since this product is administered in hospitals, in the former USSR guidelines were started in 1969 to regulate its production according to a standard: in this case, a pure culture should be used as starter (with *L. bulgaricus* and *S. lactis*, which have antibacterial properties). Regardless, according to soviet authors, afterwards old koumiss was commonly used as starter [18].

Like in other fermented dairy products, the genus *Lactobacillus* (*Lb.*) plays an important role in affecting the aroma, texture, and acidity of koumiss. Recently, much interest has been focused on the usage and safety of these strains, as the properties of probiotics are more known and appreciated. For this reason, special attention has been paid to the accurate identification and characterization of a potential probiotic microorganism to use as the selected starter. Regarding probiotics selection, it is necessary to assess properties affecting specific health benefits, such as the modulation of the immune system, survival and persistence in the host, and proven safety and stability. Regarding LAB identification, the comparison of molecular sequences, mainly 16S rRNA-encoding genes, is commonly used even if it is not always effective to identify genetically close species. It was stated that if the 16S rRNA gene sequence identity shared by two microorganism is lower than 97%, at the genomic level they are considered to belong to different species. If the shared identity values are higher than 97% or the sequences are identical, the organisms appear closely related and total DNA–DNA hybridization data or more discriminative analyses are needed for species identification [23].

Regarding LAB characterization, several techniques can be used, from the more traditional phenotypic approach like whole-cell protein and cell wall composition analysis, and other morphological, physiological, and biochemical analyses [24] to more recent molecular and genomic characterization [25]. Methods for characterizing probiotics were recommended in the advisory report of the Working Group “8651 Probiotics” of the Belgian Superior Health Council (SHC) by Huys et al. [26].

2. Lactic Acid Bacteria

Studies regarding the isolation, identification, and characterization of lactic acid bacteria (LAB) in fermented mare milk have been carried out mainly by Asian research groups in the regions where this production has been a tradition for centuries, namely, Mongolia and Inner Mongolia and the autonomic Region of China.

These kinds of studies began with a notable frequency in the early years of the 2000s, and are still actively going on, probably with the intent to find the most suitable combination of LAB for the industrial production of horse milk derivatives.

Most of the knowledge of LAB in fermented mare milk has been gained through culture-dependent methods, and the subsequent isolation and identification of these microorganisms. The number of isolated and identified strains is strongly variable in the manuscripts, depending on the aim of each piece of research, varying from 2 for koumiss [27] to 258 for chigee [28]. the results of 18 studies carried out with several methods are shown. Briefly summarizing the species identified with these approaches, it is possible to conclude that 45 different species have been identified, but the most common were *Lb. plantarum*, identified in 12 of the 18 studies; *Lb. casei*, identified in 10 studies; *Lb. helveticus,* found in 9 studies; *Lb. kefiranofaciens,* found in 7 studies; and *Lb. paracasei,* found in 6 studies. Less frequent were the species *Lb. fermentum*, *Lb. kefiri*, and *Leuconostoc mesenteroides,* found in 5 studies; *Lb. diolivorans,* found in 4 studies; *Lc. lactis* subsp. *lactis*, *Enterococcus faecium* and *S. thermophilus,* found in 3 studies; and *Lactococcus lactis* subsp. *cremoris*, *Lb. acidophilus*, *Lb. coryniformis*, *Lb. curvatus*, *Lb. farciminis*, *Lb. pentosus*, *Weissella kandleri* and *Lb. delbrueckii,* identified in two studies. The other species were found only one time.

Recently, considerable efforts have been made to develop more rapid, culture-independent methods. One study was carried out with this approach and in particular by means of denaturing gradient gel electrophoresis (DGGE) [29]. The authors concluded that the biodiversity of ten samples of collected koumiss, made by nomadic families in one region of China, was high. In particular, the dominant species identified by DGGE were *Lb. acidophilus*, *Lb. helveticus*, *Lb. fermentum*, and *Lb. kefiranofaciens*. Less frequent were *Enterococcus faecalis*, *Lactococcus lactis*, *Lb. paracasei*, *Lb. kitasatonis*, and *Lb. kefiri*. *Leuconostoc mesenteroides*, *Streptococcus thermophilus*, *Lb. buchneri*, and *Lb. jensenii* were occasionally found [29].

Comparing all the data, it is possible to highlight that the only species found with high frequency using the two approaches was *Lb. helveticus*, a thermophilic, homofermentative, proteolytic species traditionally used both for the manufacture of Swiss-type cheeses and long-ripened Italian cheeses or in the production of fermented beverages in north Europe [30]. However, due to its high proteolytic activity, *Lb. helveticus* is very effective in the production of bioactive peptides such as angiotensin-converting enzyme (ACE) inhibitory peptides [31]. The strain heterogeneity of this species isolated from koumiss was evidenced by an intra-species genotypic and phenotypic characterization [32].

The other three dominant species for DGGE have also been isolated and identified by culture-dependent research, but surprisingly, the species isolated in 12 of the 15 culture-dependent studies, *Lb. plantarum*, has not been recognized in the samples of koumiss analysed with DGGE. Even if this aspect suggests some considerations about the advantages and disadvantages of both approaches, other culture-independent research should be conducted to discuss this comparison.

More recently, new next-generation sequencing technologies have been applied to explore genes implicated in microbial metabolism: shotgun metagenomic analyses provide taxonomic and functional data about complex microbial communities, with culture-independent methods [33]. In particular, a study investigated bacterial function during several phases of koumiss fermentation by metagenomics. It was observed that the microbial composition of koumiss changes mostly in the first 36 h of fermentation, and the predominant species is *Lb. helveticus* [33].

The predominance of this species was observed in another study, which analysed the metagenomes of 23 koumiss samples collected in several regions of China: sequences representing 216 different species were found and *Lb. helveticus* comprised 78.9% of the total sequences in koumiss, followed by *Lactobacillus kefiranofaciens* (6.0%) and *Lactococcus lactis* (4.2%) [34].

Analysing the bacterial metagenomes of koumiss from Mongolia and Inner Mongolia by the single-cell genomics technique allowed the identification of rare bacterial species never detected in koumiss before, such as *Lb. otakiensis* and *Streptococcus macedonicus*, both present in other fermented foods or dairy products [35].

Since new genetic methods provide more precise metrics to classify bacteria, the genus *Lactobacillus* was recently re-evaluated and new genus and species names were recently adopted [36]. Therefore, in the more recent studies, LAB names are in accordance with the new taxonomy rules [37][38].

Technological properties and probiotic aptitudes have been considered for strains isolated from koumiss and airag. In particular, probiotic aptitude, such as bile tolerance and other preliminary tests, has been evaluated for strains of *Lb. casei*, *Lb. helveticus* and *Lb. plantarum* [39] and for different strains of *Lb. acidophilus* [40]. Different technological properties have been investigated in several species. The fermentation properties of four *Lb. casei* strains were studied by Xu et al. [41], and in *Lb. rhamnosus* and *Lb. paracasei* by Zuo et al. [42]. The latter authors were also able to verify the performances of those strains in cheese and yogurt manufacturing [42]. The effects of distinctive proteolytic activity on casein degradation have been studied for six *Lb. helveticus* strains isolated from home-made airag samples [43].

In airag collected in Mongolia, Watanabe et al. isolated two novel microorganisms belonging to the genus *Bifidobacterium*. Their phenotypic and genotypic characteristics demonstrated that the strains can be considered a single *Bifidobacterium* species never observed before, and its proposed name was *Bifidobacterium mongoliense* sp. nov. [44].

3. Yeasts

The yeasts in koumiss are the main aspect responsible for the presence of ethanol and carbon dioxide. The amount of ethanol in koumiss is slightly higher than in kefir because the amount of lactose in mare milk is higher than in cow milk [45]. However, not only are lactose-fermenting yeast species, belonging to the genus *Kluyveromices* (*K.*), mainly *K. marxianus* and *K. fragilis*, and *Candida* (*C.*) *kefir,* common in koumiss, but also non-lactose-fermenting species, such as *Saccharomyces unisporus* (*S.*), are found [46]. A study which explored the correlation between microflora and volatile flavour substances demonstrated a correlation with the *genus Candida* and ethanol, considered the most important alcohol flavour compound [22].

In the manufacture of koumiss, a considerable amount of free amino acids are produced by yeasts, ranging around 470–490 mg kg^{-1} [12][15].

Few studies are available about the yeast composition of fermented mare milk. To the authors' knowledge, six studies have been conducted on koumiss [9][22][46][47][48][49], one on chigee [50], one on airag [21] and one on hurunge, which is a the starter culture for fermented traditional dairy products such as chigee [51]. To briefly summarise, 11 genera and 24 species were isolated but only non-lactose-fermenting species *S. cerevisiae* was isolated in all the studies, and lactose-fermenting *K. marxianus* was found in seven of the nine studies. Lactose-fermenting species *C. krusei* and non-lactose-fermenting yeasts such as *Pichia* (*P.*) *membranaefaciens*, *C. kefyr*, *C. valida*, *Dekkera anomala*, *Kazachstania unispora*, and *Issatchenkia orientalis* were found in two studies, and the other species, *C. buinensis*, *C. pararugosa*, *Geotrichum* sp., *K. wickerham*, *P. cactophila*, *P. deserticola*, *P. fermentans*, *P. manshurica*, *P. membranaefaciens*, *S. dairensis*, *S. servazzii*, *S. unisporus*, *Trichosporum asaii*, *Penicillium carneum*, *Clavispora lusitaniae* and *Torulaspora delbrueckii,* were found in only one study.

Interestingly, a polyphasic approach was used in a complete and complex study, using a culture-independent and also culture-dependent method, to study yeasts present in koumiss sampled from three representative regions of China, Mongolia, Xin Jiang and Qing Hai [9]. Using 96 samples, 655 isolates and also DGGE, the authors were able to show how the yeast community in koumiss is complex and rich in different species [9].

Another research article showed that the prevalent yeast found at high altitudes in Kazakhstan was *S. unisporus*, which was different from lower zones, where lactose-fermenting yeasts are more common, mostly belonging to the *Kluyveromyces genus* [46]. The authors hypothesized that a high altitude could affect the LAB population composition during the preparation of koumiss, with a selection of LAB not metabolizing galactose, causing therefore an enrichment of this sugar and the prevalence of *C. buinensis,* which ferments galactose but not lactose, and of the *Saccharomyces genus* compared with *Kluyveromyces* [46].

In traditional koumiss from Inner Mongolia, Guo et al. identified 57 fungal species, also, among them the genera *Penicillium*, *Cladosporium* and *Aspergillus* were detected in all samples. These are filamentous fungi considered the cause of spoilage in dairy products. Therefore, the production process of traditional koumiss requires sanitation measures and a better control of environmental hygiene [48].

In conclusion, it could be observed that in comparison with fermented dairy products from other species, in mare milk the higher lactose content (6–7% compared to 4–5% of cow, yak, goat and camel's milk) usually determines the prevalence of lactose-fermenting yeast strains, but this trait could be modified by the altitude effect, which, when operating in a selection of LAB, can increase the galactose content, causing therefore the major presence of galactose-fermenting yeast strains.

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