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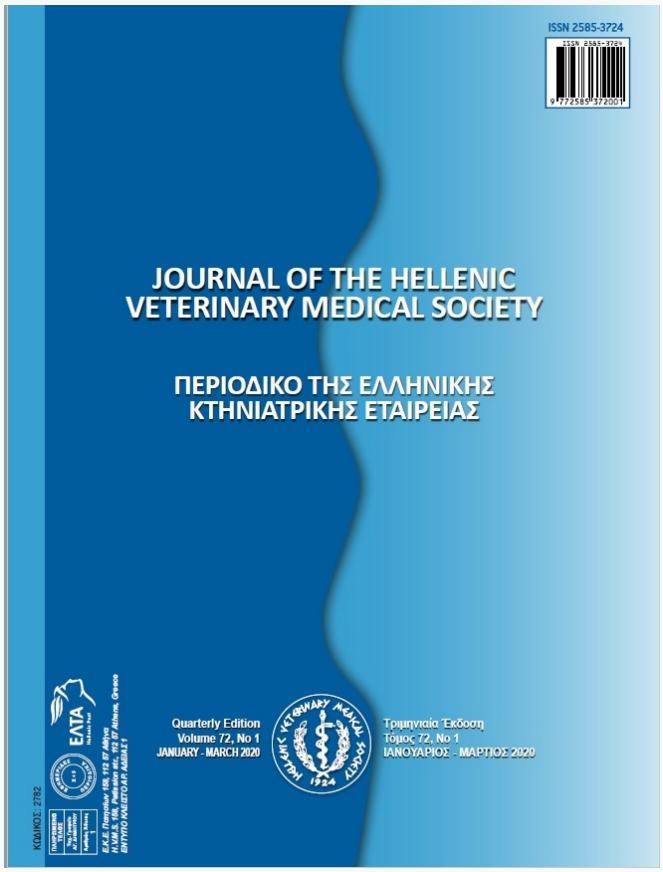

Dynamic changes of psychrotrophic bacterial populations in Algerian refrigerated raw cow milk

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Research article
Ερευνητικό άρθρο

Dynamic changes of psychrotrophic bacterial populations in Algerian refrigerated raw cow milk

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ABSTRACT: The aim of the present study is to evaluate the microbial dynamic changes in raw cow milk during refrigeration. Milk samples were collected from two dairy farms situated in basin and mountain areas in the region of Mila, Algeria. Cold storage of milk samples at 4 °C was used to promote psychrotrophic microbial growth during 10 days. The microbial flora of milk was determined by culture methods and the identification of bacterial isolates was carried using cultural, morphological and biochemical criteria. The variations in total viable count and kinetic parameters of microbial growth were analyzed during refrigeration using Baranyi and Roberts Model. The initial microbial counts ranged from 2.94 ±0.04 to 3.87 ±0.11 log CFU * mL^{-1} and the maximum growth did not exceed 7.00 ±0.12 log CFU * mL^{-1}. The hygienic limit was surpassed after 2 days in the basin farm and later after 6 days in mountain farm. The generation time varied from 1.20 ±0.55 to 4.18 ±0.22 days in the basin farm and mountain farm respectively. The psychrotrophic bacterial populations were identified as *Acinetobacter*, *Pseudomonas*, *Aeromonas*, *Chryseobacterium*, *Enterococcus* and *Lactobacillus*. The notable delay in microbial growth in milk from the mountain farm compared to the one from basin could be related to indoor/outdoor feeding mode adopted in mountain opposed to indoor feeding in the basin farm. This prolonged shelf-life suggests the possible presence of antimicrobial molecules coming from plants grazed around the farm and the selection in the microbiota of some microbial species with antagonist potential.

Keywords: raw cow milk, refrigerated storage, microbial growth parameters, psychrotrophic bacterial populations, shelf-life

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INTRODUCTION

In many parts of the world milk and dairy products are indispensable constituents of human nutrition and play an important role in human health and the development of life (FAO, 2013; Quigley et al., 2013). The prolonged storage of milk by refrigeration favors the growth of psychrotrophic bacteria, this category of microorganisms have become a fundamental limitation for dairy products shelf-life (De Jonghe et al., 2011). Initially, they account for less than 10% in refrigerated raw milk, than they evolve as the major microorganisms constituting more than 70% to 90% of the microbiota (Decimo et al., 2014). Particularly, psychrotrophs by their proteases and lipases extracellular enzymes play a leading role in the deterioration of dairy products (Cempírková and Mikulová, 2009).

Numerous bacterial strains were isolated from refrigerated milk, they belong to the genera *Pseudomonas*, *Stenotrophomonas*, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Flavobacterium*, *Bacillus*, *Listeria* and *Enterococcus* (Lafarge et al., 2004; Hantsis-Zacharov and Halpern, 2007a; Boubendir et al., 2011). The majority of psychrotrophic bacteria are known for the spoilage of milk and dairy products. However, some species can be considered as human pathogenic bacteria by producing toxins and / or showing resistance to antibiotics (Samaržija et al., 2012). Dynamic changes of psychrotrophic flora in milk during 24 h of refrigeration (Lafarge et al., 2004) and in creamed milk from different geographical origins (Franciosi et al., 2011) have been investigated. Vyletělova et al. (2000) evaluated microbial growth dynamics of mesophilic and psychrotrophs in cow's raw milk in different times of storage and temperatures. Gargouri et al. (2013) confirmed that the dynamics of psychrotrophic bacteria counts (PBC) in cow's raw milk conserved at 4 °C was linked to storage time and the initial contamination value. Interesting relationships were elucidated between the geographical origin of milk and both genetic diversity and antimicrobial activity, suggesting the profound correlation between the region and some genotypic and metabolic conducts of milk microbiota (Silvetti et al., 2014).

The data on milk microbial dynamics during refrigeration particularly in Algeria is missing, thus the aim of the present study is to evaluate the dynamics of microbial growth during all the time of milk refrigeration in two dairy farms, one in the basin and the other in the mountain of Mila, Algeria.

MATERIALS AND METHODS

Milk sampling

Raw milk samples were collected from two dairy farms located in the region of Mila (Algeria) at the locality of El-Kherba (36° 27'N / 6° 15'E) in the basin and Hamala (36° 34'18 "N / 6° 20'24) in the mountain. In the basin (farm A), the cows feed only inside principally on silage, barley, oats and hay, while in the mountain (farm B), the cows feed both inside and outside, the prevalent plants grazed around this farm were *Thymus*, *Origanum*, *Malva*, *Hibiscus*, *Rosmarinus* and *Olea* leaves (Ouelbani et al., 2016). At each farm, five raw milk samples were collected from healthy cows "Française Frisonne Pie noir". The teat ends were cleaned by wiping using dry paper towels, the preliminary jets were discarded and 25 mL of raw milk sample were directly collected from each of the four teats (i.e., a total of 100 mL per cow) and transferred to the laboratory into individual sterile flasks at 4 °C.

Enumeration of microorganisms

Raw milk samples were processed as soon as they arrived to the laboratory. They were analyzed immediately at the first (time 0) day and at different times (2, 4, 6, 8 and 10 days) over 10 days of refrigerated storage at 4 °C. Serial dilutions (10^{-1}-10^{-5}) were prepared for each sample; 1 ml of each dilution was placed on Sterile Standard Plate Count (SPC) agar, a standard medium corresponding to the American Public Health Association formulation for milk, water, food and dairy products (Oxoid CM0463). Plates were incubated at 37 °C for 48 h, those with the number of colonies 30 to 300 were considered for counting. All the experiments were performed in three replications per milk sample at each time point and results were expressed as mean values. To control the occurrence of hemolytic bacteria as indicators of probable pathogenic bacteria such as *Listeria monocytogenes*, *Bacillus* and *Staphylococcus aureus*, 0.1 ml from the dilution 10^{-1} were also plated on 5% (v/v) horse blood Columbia agar added with Cefazolin at 20 mg * L^{-1} (Sandoz Gmbh, Kundl, Austra), plates were incubated for 48 h at 37 °C (Boubendir et al., 2016). The enumeration of *Lactobacillus* was carried by adding 1 mL of the dilution 10^{-1} to sterile dishes, and liquefied MRS Agar (45°C) is poured into the dish and mixed carefully. Once the medium is solidified, a new layer of uninoculated MRS Agar is poured on the surface to make a layer-plate; the plates were incubated for 48 h at 37 °C.

Identification

At each sampling time (0, 6 and 10 days), colonies with different morphologies (color, shape, and size) were selected and purified by streaking on the same medium. The identification of the presumed bacteria was realized using cultural, morphological and biochemical criteria. The main identification tests used were: Gram staining, mobility (at 25 and 37 °C), catalase presence, oxidase presence, methyl red test (MR test), indole production, Voges-Proskauer reaction (VP test), nitrate reduction, H_2S production (TSI test), urease presence, aesculin hydrolysis and hemolytic activity.

Data analysis

Mean data relative to the repetitions of total bacterial counts on SPC agar medium for milk samples during storage at 4 °C were analyzed using Baranyi and Roberts Model (Baranyi and Roberts, 1995) in order to obtain the microbial growth parameters, i.e., maximum growth rate (μ_{max}), lag phase length (λ) and maximum cell load attained (X_{max}).

RESULTS AND DISCUSSION

Microbial dynamic changes during refrigeration of milk

The dynamics of microbial growth on SPC agar during 10 days of storage at 4 °C are shown in Figure 1, while Table 1 reports the parameters of microbial growth obtained. The initial number of psychrotrophic bacteria in raw milk samples collected from the two farms (A and B) were 3.87 ±0.11 and 2.94 ±0.04 log CFU * mL^{-1} respectively. These values did not exceed the hygienic limit (4.69 log CFU * mL^{-1}) reported by Cempírková and Mikulová (2009) and Gargouri et al. (2013). This denotes the respect of good farming practices, good health state of animals and appreciable hygienic farm environment. The environment where dairy cows are housed and milked influences the microbial contamination of milk by udder preparation techniques, milking machines protocols, the procedures of cleaning and disinfecting milking machines, milk tanks and the hygiene of personnel. Other principal factors are the refrigeration rate of the milk at the required temperature and the time of storage (Rasolofo et al., 2010; Samaržija et al., 2012). The present level of initial microbial contamination of raw milk was almost similar to those reported in other farms globally. The initial contamination of cow's raw milk from mountain farms and foothills areas of Southern and Western Bohemia, registered the mean value of 3.46 log CFU * mL^{-1} at different temperatures (4, 6.5 and 10 °C) (Cempírková and Mikulová, 2009). Another study in Brazil observed an initial PBC of 3.5 log CFU * mL^{-1} in the raw milk sample stored at 7 °C (Machado et al., 2013). In the region of Sfax (Tunisia) the level of PBC in raw milk was 2.89 CFU * mL^{-1} after one day of cold storage at 4 °C (Gargouri et al., 2013). In Ireland the mean value of initial contamination of milk at different temperatures (2, 4 and 6 °C) was 2.87 log CFU * mL^{-1} (O'connell et al., 2016). In the semi-arid area of Algeria (region of Biskra), the initial PBC in raw milk refrigerated at 4 °C ranged from 2.5 to 3.5 log CFU * mL^{-1} (Boubendir et al., 2016).

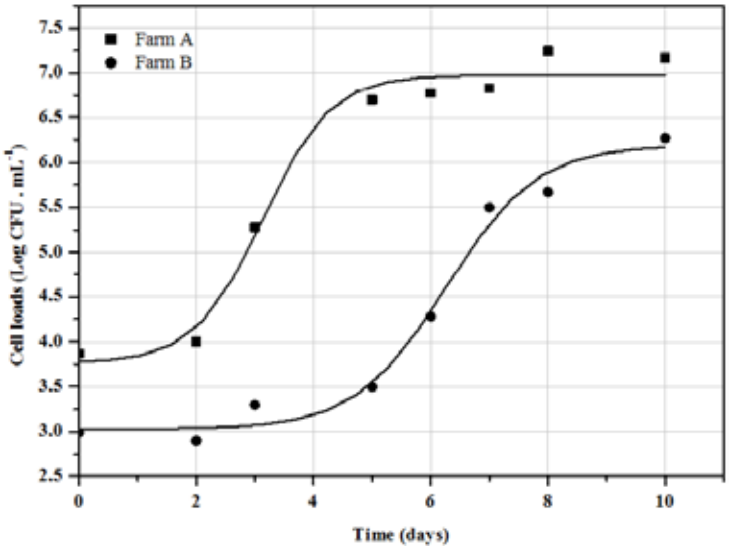

Figure 1. Growth curves of total viable bacteria count obtained by Baranyi and Roberts Model in milk refrigerated during 10 days at 4 °C, from two dairy farms in the basin (A) and mountain (B) in the region of Mila, Algeria

Table 1. Microbial growth parameters obtained by Baranyi and Roberts Model of mean count data of total viable bacteria over refrigerated storage of milk samples, from two dairy farms in the basin (A) and mountain (B) in the region of Mila, Algeria

Farm / Location	Geographical coordinates	Altitude (m)	Initial value[a]	λ^b	$\mu_{max}{}^c$	X_{max}
Farm A (Basin)	36°27'N/6°15'E	598	3.87 ± 0.11	1.20 ± 0.55	0.78 ± 0.16	7.00 ± 0.12
Farm B (Mountain)	36°34'N/6°20'E	829	2.94 ± 0.04	4.18 ± 0.22	0.73 ± 0.05	6.29 ± 0.07

[a] (log CFU * mL^{-1}), [b] Lag phase length (days), [c] Maximum growth rate (Δlog CFU * mL^{-1} *day), X_{max}: Maximum cell load (log CFU * mL^{-1})

The maximum growth levels (X_{max} value) reached during the stationary phase, after 10 days of milk refrigeration, were 7.00 ±0.12 and 6.29 ±0.07 log CFU * mL^{-1} in farm A and B respectively. The hygienic limit of milk was surpassed after 2 days in farm A and later after 6 days in farm B. In Slovenia, the PBC augmented on the second day of collection up 4.61 log CFU * mL^{-1} (Perko, 2011), and in Spain registered 7.04 log CFU * mL^{-1} (De Garnica et al., 2011). In Tunisia, the hygienic limit was surpassed after 2 days of cold storage (Gargouri et al., 2013). In Ireland the PBC level reached was 7.00 log CFU * mL^{-1} (Paludetti et al., 2018). In Brazil, according to Ribeiro Junior et al. (2018) the amount of psychrotrophs augmented to 5.32 log CFU * mL^{-1}. In Algeria at the region of Biskra, the X_{max} level attained in the stationary phase did not exceed 5.25 log CFU * mL^{-1} (Boubendir et al., 2016), while in the region of Djelfa, the level of psychrotrophs attained the threshold of 5.25 log CFU * mL^{-1} at the end of the 5th day of conservation (Yabrir et al., 2018). The difference in maximum growth levels observed in different parts of the world could be due to the variations in the temperature of cooling and storage time of milk.

The generation times (λ values) obtained in the present study ranged from 1.20 ±0.55 to 4.18 ±0.22 days in farm (A) and (B) respectively. Few studies evaluated the microbial parameters of growth during milk refrigeration. Cempírková and Mikulová (2009) in Southern and Western Bohemia registered a lag phase length of 2 days; Vithanage et al. (2017) in Australia have observed that the lag phase of psychrotrophs continues to 3 days. In Algeria, Boubendir et al. (2016) remarked an extended lag phase ranging from 6.74 to 10.50 days, while Yabrir et al. (2018) observed a lag phase of 3 days. On the other hand, the value of lag phase length obtained in milk from mountain was higher than those calculated according to ComBase predictive models (http://www.combase.cc) at the temperature of 4°C. The predicted lag time for *Listeria monocytogenes / innocua* was 4.15 days, *Aeromonas hydrophila* (3.36 days), *Pseudomonas* (1.68 days) and *Yersinia enterocolitica* (1.09 days).

The notable delay in microbial growth registered in milk collected from the mountain farm compared to the one from basin suggests the influence of specific environmental factors in dairy farms. The shelf-life of milk samples collected from the mountain adopting inside/outside feeding mode was elongated compared to the one of basin adopting only inside feeding mode. Indeed, the variations of milk microflora composition have been linked to changes in cow feeding environment. The microbial composition of milk and its activity is influenced by grazing from inside to outside and the nature of forage (Sanz Sampelayo et al., 2007). The variation in durations of lag phase in refrigerated raw milk collected from different geographies could be explained by the presence of antimicrobial natural molecules in milk. Numerous bioactive molecules of plants grazed by cows have a fundamental role for rumen health and display an antimicrobial activity. Furthermore, the concentration in milk of a variety of volatile compounds, such as terpenes is enhanced when cows are grazing in herb-rich pasture (Michel et al., 2001; Larsen et al., 2012).

The delay in microbial growth observed in the mountain farm could be related to the possible presence of antimicrobial molecules coming from the plants grazed around the farm. A variety of bioactive molecules including thymol, carvacrol, eugenol derived from plants, demonstrated antibacterial against both gram-positive and gram-negative pathogens (Ananda Baskaran et al., 2009). The antagonist effect of carvacrol, eugenol, and thymol against the main bacterial mastitis pathogens in milk was demonstrated, particularly against *S. aureus, Escherichia coli, Streptococcus agalactiae* and *S. dysgalactiae* (Gaysinsky et al., 2007; Ananda Baskaran et al., 2009).

According to Darsanaki et al. (2012), the cow

can graze olives leaves with a confirmed inhibitory effect against pathogenic bacteria. Olive leaves aqueous extract exhibited antimicrobial activity against *P. aeruginosa, S. aureus, E. coli, K. pneumoniae* and *B. subtilis*. Also, *Malva* plant with *M. sylvestris* and *M. parviflora* extracts, have demonstrated antimicrobial activity against a large variety of pathogenic bacteria such as *P. aeruginosa*, *E. coli* and *S. aureus* (Sharifi-Rad et al., 2019). In addition, *Hibiscus* extracts have shown a good antimicrobial activity in microbiological medium and in milk, especially against *S. aureus* and *E. coli* (Higginbotham et al., 2013). *Rosmarinus officinalis* essential oil has demonstrated antibacterial activity on milk microorganisms against *Staphylococcus spp., Bacillus cereus* and *Aeromonas hydrophila* (Paşca et al., 2015).

The evolution of psychrotrophic bacterial populations in mountain milk

The evolution of psychrotrophic bacterial populations in mountain milk stored at 4 °C during 10 days is reported in Figure 2. In the first day (time 0), before refrigeration, the bacterial populations were initially detected at low levels in fresh raw milk, they were identified as *Acinetobacter*, *Pseudomonas*, *Aeromonas*, *Chryseobacterium*, *Enterococcus* and *Lactobacillus*, the initial population microbial load varied from 1.00 to 2.51 log CFU * mL^{-1}. After 6 days of refrigeration, a rise in the number of the totality psychrotrophic bacterial populations is remarked except for *Chryseobacterium*, the maximum microbial growth reached 3.31 log CFU * mL^{-1} with *Enterococcus* being the dominant population in the microbiota. At the end of refrigeration after 10 days, *Enterococcus* and *Lactobacillus* populations surmount largely in number the others psychrotrophs with 4.41 and 4.00 log CFU * mL^{-1} respectively. The *Chryseobacterium* regains growth and attained 2.14 log CFU * mL^{-1}, while the growth of *Acinetobacter*, *Pseudomonas* and *Aeromonas* seems to be reduced. However, the absence of *Listeria*, *Staphylococcus*, *Bacillus* and other hemolytic pathogenic bacteria is remarked during all the time of refrigeration.

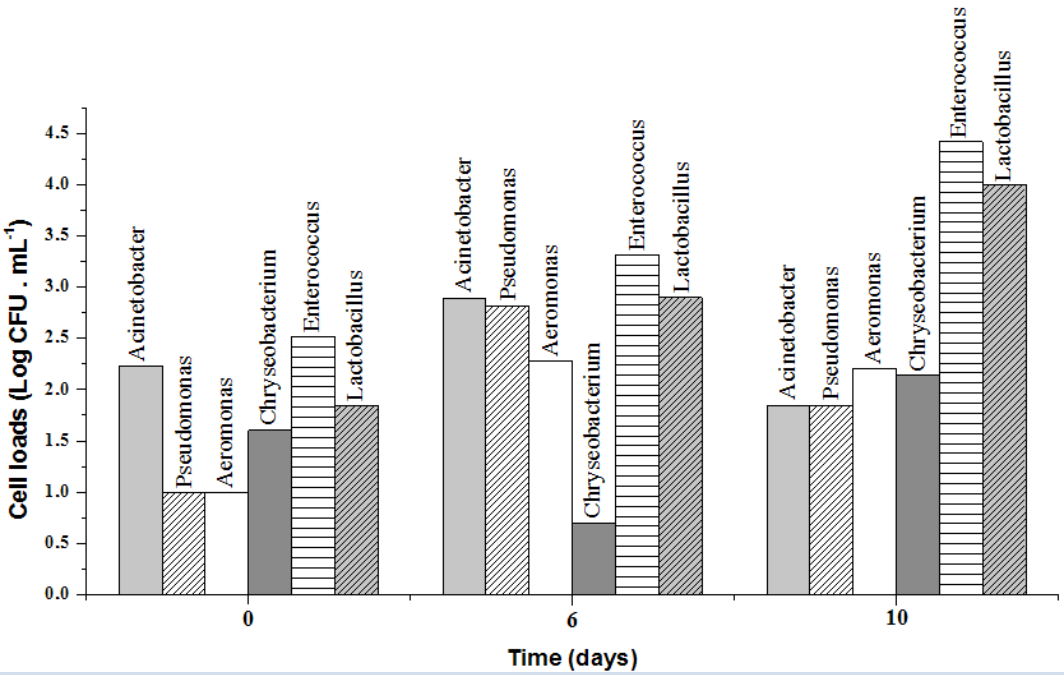

Figure 2. The evolution of psychrotrophic bacterial populations in mountain milk stored at 4°C during 10 days in the region of Mila, Algeria

Psychrotrophic bacteria were frequently isolated in cooled milk and dairy products environments, their origin can be from forage, feed, bedding material, faeces and soil, dust; when in contact with cow's teat skin the milk may be contaminated (Gleeson et al., 2013). Usually in untreated milks, *Pseudomonas* and *Acinetobacter* genera are isolated during the first days of cold storage (Rasolofo et al., 2010; Vithanage et al., 2014), and both exhibit essentially lipolytic activity (Hantsis-Zacharov and Halpern, 2007a). According to Gislene et al. (2015), the genus *Pseudomonas* is detected in raw milk during the first day at a low level,

however the numbers of *Pseudomonas* increase after 6 days of refrigeration. The genera *Acinetobacter* and *Pseudomonas* are recognized as the major psychrotrophic groups in refrigerated raw milk with high spoilage potential (Machado et al., 2015; Yuan et al., 2018).

In this study *Aeromonas* grow well at low temperature, this finding is in accordance with the results of Lafarge et al. (2004). Also, Mankai et al. (2012) isolated *Aeromonas hydrophila* from refrigerated raw milk conserved at 4 °C. This genus can contaminate milk by infecting the udder tissues and multiplying in mammary tissues (Nahla, 2006). Similarly, *Chryseobacterium* strains are part of the psychrotolerant and proteolytic microflora that causes numerous defects in food products (Hantsis-Zacharov and Halpern, 2007b; Machado et al., 2017). This genus is commonly regarded as a usual resident of soils and plants and is known for its aptitude to degrade toxic plant composites, comprising polyphenols (Lopez et al., 2004; Bekker, 2011). The members of the genus *Chryseobacterium* demonstrated antagonistic activities against human pathogenic bacteria such as *P. aeruginosa*, *L. monocytogenes*, *Salmonella typhi* and *S. aureus* (Lim et al., 2011).

Enterococcus was the dominant bacterial population during all the period of refrigeration; this finding is in accordance with the study of Mcauley et al. (2015). In Turkey, Citak et al. (2005) showed that *E. faecalis* was the major *Enterococcus* in raw milk. Giannino et al. (2009) registered the presence of *E. faecalis*, *E. faecium* in raw milk, establishing their essential role as origin of the typical fermenting microflora. Furthermore, enterococci are used to prolong the shelf-life and enhance the hygienic safety of food since they produce antimicrobial substances for example lactic acid, bacteriocins (enterocins) and hydrogen peroxide. Nevertheless, the presence of virulence factors and the emergence of multiple resistance to antibiotics among enterococci in food isolates require a careful safety evaluation before biotechnical use (Franz et al., 2007). Bacteriocins incite a great attention being commonly active against numerous gram-positive food borne pathogens such as *Clostridium botulinum*, *L. monocytogenes* and *S. aureus* (Morandi et al., 2012).

Lactobacillus population manifests here an unusual physiological adaptation to cold environment manifesting a psychrotrophic behavior at low temperature and was present all the period of cold storage. Usually, *Lactobacillus* grows as mesophilic and shows a maximum growth at 37 °C (Toqeer et al., 2006). Mami et al. (2012) demonstrated that *Lb. plantarum* can inhibit the growth of *L. monocytogenes*, *S. aureus* and other populations of undesirable bacteria in milk. This inhibition could result from the production of organic acids, especially lactic acid, which reduce pH environment, hydrogenous peroxide (H_2O_2), diacetyl (2, 3-butanediona), reuterin, bacteriocins and carbon dioxide (CO_2) (Gutiérrez-Cortés et al., 2017).

CONCLUSION

There is a notable delay in microbial growth in milk collected from the mountain farm adopting inside/outside feeding mode compared to the one from basin adopting only inside feeding. This prolonged shelf-life suggests the possible presence of antimicrobial bioactive molecules from plants grazed by cows outside the farm and the selection in the microbiota of some microorganisms with antagonist potential. The dynamic changes of bacterial populations habiting refrigerated milk could be in favor of long shelf-life and safety against pathogenic bacteria and spoilage agents. Taking into consideration the reinforcement of the data for the implementation a predictive model, the period of time will be prolonged and other measurements will be achieved in future works. Also, the exploration of other bacteria inhabiting refrigerated milk, the typing of the isolates using molecular tools and their kinetic study during all the time of refrigeration could be further developed.

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CONFLICT OF INTEREST

None of the authors have any conflicts of interest to declare regarding this work.

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