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ORIGINAL ARTICLE

Changes in bacterial populations in refrigerated raw milk collected from a semi-arid area of Algeria

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Abstract Most of the studies on milk microbiota have been performed on cows' milk from animals reared in temperate and humid areas. In this work, changes in the bacterial consortium of refrigerated raw milk collected from cows grazed in a semi-arid area of Algeria were studied during 21 days of refrigerated storage. Twenty bacterial morpho-physiotypes were selected among 150 isolates from milk at different times over storage and identified by partial 16S rRNA gene sequencing. The dominant bacterial populations were characterized by a few species. *Stenotrophomonas rhizophila*, *S. maltophilia* and *Chryseobacterium indologenes* were predominant during the first 7 days, *Lactobacillus pentosus* and *L. plantarum* were isolated only after the 10th day, while *Acinetobacter* spp. was isolated at the end of storage. Compared to the current literature on milk from temperate zones, sluggish and incomplete microbial growth was observed with a long incubation phase ranging from 6.7 to 10.5 days and a maximum growth not exceeding 5.3 log colony-forming units (CFU)·mL^{-1}. The composition of milk microbiota and its evolution over refrigeration suggest a biogeographical characterization due to environmental factors. In particular, the possible presence of antimicrobial molecules coming from plants grazed in the semi-arid zone around the farm may account for the presence of selected microbial species and the extended milk shelf-life. Despite this being a preliminary work, these results encourage the use of arid herbs in animal feed and motivate scientists to focus their efforts on the study of biochemical composition of plants from arid areas and their antimicrobial activity.

Keywords Raw cows' milk · Microbiota · Sluggish growth dynamic · Refrigerated storage · Semi-arid area

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Introduction

The potential content and relative ratios of the basic nutrients, lipids and proteins in milk are genetically determined. However, environmental, nutritional and physiological factors greatly influence milk composition and flavour (Morand-Fehr et al. 2007). As suggested by recent studies, the presence of botanically diverse plant species in pastures can significantly affect the occurrence and content of functional molecules, and namely polyunsaturated fatty acids, in milk (Rubino et al. 2006). Numerous studies have shown the beneficial effects of herbs and spices on animal feed intake, immune functions and health as well as on rumen fermentation and productivity (Frankič et al. 2006). Moreover, it has been reported that the intake of some plants in arid zones results in the important healthy attributes of camel milk and, namely, antidiabetic action (Agrawal et al. 2007).

Raw cows' milk and dairy products are characterized by a wide microbial biodiversity and more than 150 species have been identified by means of the combination of culture dependent and independent methods (Delbès et al. 2007; Vithanage

et al. 2014). Various microbial consortia of raw milk have been studied, particularly in relation to the geographical origin in order to maintain and exploit the microbial diversity characterizing traditional dairy products. Moreover, the influence of animal nutrition and, particularly, of botanically diverse diets as selective agents for milk microbiota and its stability, has been investigated (Delbès et al. 2007). Also, milk treatments and, namely, refrigeration, play an important selective role in inducing a shift in the predominant microbial species, in addition to a selective reduction of their growth rate (Lafarge et al. 2004; Hantsis-Zacharov and Halpern 2007; Franciosi et al. 2011). Recent developments in molecular fingerprinting methods have provided a view of complex microbial ecosystems such as refrigerated raw milk. A significant number of bacterial strains isolated from cooled milk belongs to the genera *Pseudomonas*, *Stenotrophomonas*, *Aeromonas*, *Acinetobacter*, *Flavobacterium*, *Alcaligenes*, *Listeria*, *Bacillus*, *Enterococcus* and *Lactobacillus* (Lafarge et al. 2004; Hantsis-Zacharov and Halpern 2007; Boubendir et al. 2011). Moreover, dynamic changes in the bacterial population in milk after 24 h of refrigeration (Lafarge et al. 2004) and during creaming of milk of different geographical origins (Franciosi et al. 2011) have been characterized. The major part of the studies regarding milk microbial consortia and spoilage patterns during refrigeration have been performed on cows' milk produced by animals fed predominantly with hay or silage, including grasses or herbs of temperate and humid areas. Little information is available on the microbial consortia of milk from cows reared in arid or semi-arid areas such as Algeria, Tunisia and Morocco where cattle and dairy industries are rapidly growing. The milk used in this study came from animals kept in a pasture of herbs and grasses in a semi-arid area of Algeria. The prevalent plants near the animal farm were *Malva sylvestris*, *Cyanodon dactylon*, *Atriplex* spp., *Salsola vermiculata*, *Tamarix* spp. and *Cyperus conglomeratus*. All these herbs are characterized by a demonstrated antimicrobial activity in model systems (Bouaziz et al. 2009; Razavi et al. 2011; Abd El Raheim 2013).

The aim of this study was to characterize the bacterial population of raw cows' milk from a semi-arid area of Algeria and its evolution over refrigerated storage by using the partial 16S rRNA gene sequencing method. Moreover, microbial growth dynamics over time along refrigeration was evaluated.

Materials and methods

Milk sampling

Raw milk samples were collected in a farm localized in Sidi Okba in the semi-arid region of Algeria (34°51'N/5°43'E). The samples were taken from three healthy cows "Française Frisonne Pie noir" grazing in the zone around the farm. At each sampling time, teat ends were cleaned by wiping with dry paper towels, the first jets were removed and 25-mL samples of raw milk 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.

Microbial analyses

Samples from the 3 cows were analyzed immediately after their collection (time 0) and at different times (2, 5, 7, 12, 16, 21 days) over 21 days of refrigerated storage to evaluate their microbiological quality and safety as well as the presence of selected bacterial pathogens.

The presence/absence of *Listeria monocytogenes* was investigated using conventional qualitative culturing methods according to ISO11290-1:1996/Amd 1:2004. Briefly, milk samples were submitted to primary and secondary enrichment. After incubation, pre-enrichment and enrichment broths were streaked onto Oxford and "Agar Listeria according to Ottaviani & Agosti" (ALOA) agar plates (Oxoid Ltd, Basingstoke, Hampshire, UK). To check for the presence of hemolytic bacteria as indices of possible pathogenic bacteria such as *L. monocytogenes*, *Staphylococcus aureus* and Streptococci, samples were also plated on 5 % (*v*/*v*) horse blood Columbia agar with Cefazolin added at 20 $mg \cdot L^{-1}$ (Sandoz Gmbh, Kundl, Austra). Cefazolin belongs to the first generation of the cephalosporin antibiotic family to which *Listeria* and other bacteria are resistant (Boubendir et al. 2011). Plates were incubated for 48 h at 37 °C.

For microbial enumeration of total viable bacteria, each milk sample was individually serially diluted with sterile saline solution (0.9 % [w/v] NaCl). Samples were then plated 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 Ltd). Plates were incubated at 30 °C for 48 h for total viable counts. Only plates with 10 to 100 colonies 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.

At each sampling time, colonies with distinct morphological differences (color, shape, and size) were selected from SPC agar plates and purified by streaking on the same medium. Cell morphology was determined microscopically after Gram stain preparation. All the isolates were also characterized for catalase (3 % [w/v] H_2O_2) and oxidase (1 % [w/v] tetramethyl p-phenylenediamine dihydrochloride) tests. Twenty morpho-physiotypes were selected among 150 isolates for further identification.

DNA isolation and partial 16S rRNA gene sequencing

Genomic DNA was extracted from pure cultures using an Insta Gene Matrix Kit (Bio-rad, Milan, Italy). For polymerase chain reaction (PCR) analysis and strain identification, two primer pairs (MWG, Germany) were used to amplify the 16S rRNA gene fragment of the bacterial cultures isolated, i.e., LpigF/LpigR (5'-TACGGGAGGCAGCAGTAG-3' and 5'-CATGGTGTGACGGGCGGT-3'), and 16SF/16SR (5'-CAGGCCTAACACATGCAAGTC-3' and 5'-GGGCGGAGTGTACAAGGC-3'). Amplification was performed using a T3000 thermal cycler (Biometra® GmbH, Goettingen, Germany). PCR products were separated by electrophoresis on 1.5 % (w/v) agarose gel stained with ethidium bromide (0.5 $\mu g \cdot mL^{-1}$). The expected amplicons were eluted from gel and purified by the QIAquick PCR Purification Kit (Qiagen). The purified DNAs were sent to BMR Genomics (Padova, Italy) to obtain the sequences. Taxonomic strain identification was performed by comparing the sequences of each isolate with those reported in the "Basic Local Alignment Search Tool" (BLAST) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome).

Nucleotide sequence and accession numbers

A total of 20 bacterial isolates was identified in the present study, and their sequences were deposited in the European Molecular Biology Laboratory (EMBL) Nucleotide Sequence Database under the accession numbers HF678414 to HF678433.

Data analysis

Mean data relative to the three repetitions of total bacterial counts on SPC agar medium for each of the three milk samples during storage at 4 °C were analyzed with the Gompertz equation as modified by Zwietering et al. (1990) in order to obtain the microbial growth parameters, i.e., maximum growth rate (μmax), lag phase length (λ) and maximum cell load attained (A).

Results and discussion

The diversity of raw milk bacterial communities and the effect of refrigeration on their dynamics were studied on cows' milk from a semi-arid area of Algeria.

Mean data of the three repetitions of the three milk samples recorded for total viable bacteria during refrigerated storage at 4 °C were analyzed with Gompertz equation as modified by Zwietering et al. (1990) in order to obtain the microbial growth parameters, i.e., maximum growth rate (μmax), lag phase extension (λ) and maximum growth extent (A). Figure 1 shows growth dynamics of microbiota, evaluated on SPC agar during 21 days of storage at 4 °C, while Table 1 reports the obtained Gompertz parameters. The 3 milk samples were analyzed for 21 days due to a slow microbial growth. The initial counts of milk samples, ranging from 2.5 to 3.5 log $CFU \cdot mL^{-1}$, were similar to those reported in literature and accounted for good farming practices, healthy cow status and a hygienic environment, as confirmed also by the absence of *Listeria* spp. and hemolytic bacteria, which were never detected over 21 days of storage (Lafarge et al. 2004; Rasolofo et al. 2010; Samaržija et al. 2012). On the other hand, the μmax values obtained for the three milk samples were remarkably lower than those reported in literature for *Pseudomonas* spp. under refrigeration conditions. In particular, according to ComBase predictive models the predicted μmax was 1.15 log $CFU \cdot mL^{-1}$ *day (http://www.combase.cc), while Teleken et al. (2009) reported a mean value of 1.8 log CFU mL^{-1} *day. Likewise, the λ values (lag phase length) reported in Table 1 ranged from 6.74 to 10.50 days and the maximum growth level (A value) attained in the stationary phase did not exceed 5.25 log $CFU \cdot mL^{-1}$ (Fig. 1). Also the latter parameters indicate a sluggish and limited microbial growth with respect to the literature (Rasolofo et al. 2010; De Jonghe et al. 2011).

From milk samples analyzed after its collection and over refrigerated storage, 150 bacterial colonies were isolated from SPC agar plates. Twenty different morpho-physiotypes were selected and identified by partial 16S rRNA gene sequencing. In Table 2, families, the closest relative species, the identification (with the percentage of identity), the corresponding accession number and the isolation time during milk storage are reported. The bacteria isolated from refrigerated raw milk were identified

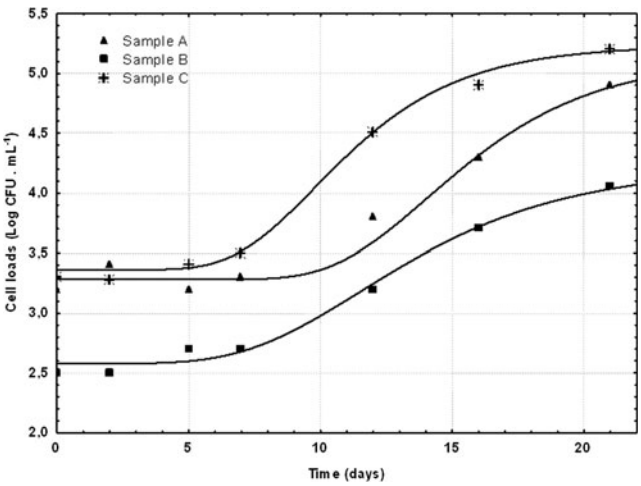

Fig. 1 Growth curves obtained by modeling with the Gompertz equation cell count data of total viable bacteria in milk samples from 3 cows during 21 days of storage at 4 °C

Table 1 Predicted values of Gompertz parameters obtained by modeling mean count data of total viable bacteria over refrigerated storage of three milk samples

	μmax[a]	λ[b]
Sample A	0.19	10.50
Sample B	0.14	7.10
Sample C	0.22	6.74
Mean	0.18	8.11

[a] Maximum growth rate (Δlog CFU·mL^{-1} *day)

[b] Lag phase length (days)

as *Flavobacteriaceae*, *Pseudomonadaceae*, *Moraxellaceae* and *Lactobacillaceae*. Only strains belonging to the species *Chryseobacterium indologenes*, *Stenotrophomonas maltophilia* and *S. rhizophila* were isolated during the first 7 days. Likewise, De Jonghe et al. (2011) isolated *Stenotrophomonas* spp. only at the beginning of the refrigerated storage, suggesting that they either can hardly grow under refrigeration or they are overgrown by better-adapted microbial species. While the mean initial milk microbial counts were found to be similar to those reported in the literature (Rasolofo et al. 2010; Stulova et al. 2010; De Jonghe et al. 2011), the composition of the microbial consortium was more homogeneous when compared with the biodiversity described by Ercolini et al. (2009). The authors detected 35 different random amplified polymorphic DNA (RAPD)-PCR profiles for 66 strains selected among mesophilic and psychrotrophic bacteria isolated from raw milk samples collected from four dairy farms. The identification by 16S rRNA gene sequencing showed that *Pseudomonas* spp. were the most commonly occurring contaminants along with *Enterobacteriaceae,* including *Hafnia alvei*, *Serratia marcescens* and *Citrobacter freundii*, while Gram-positive isolates were mainly represented by the genera *Staphylococcus* and *Lactococcus* and, to a lesser extent, also by *Rhodococcus*, *Bacillus*, *Corynebacterium* and *Carnobacterium*. Despite the limited number of samples and isolates analyzed in our work, which can strongly influence the description of microbial communities in addition to the methods used, thus making comparisons with literature data difficult, our outcomes are in line also with studies based on significantly higher numbers of isolates and milk samples reporting a wide diversity of milk microbiota (Hantsis-Zacharov and Halpern 2007; Samaržija et al. 2012; Vithanage et al. 2014). In fact, *Pseudomonas*, *Acinetobacter*, *Bacillus*, *Staphylococcus*, *Streptococcus*, *Aerococcus*, *Facklamia*, *Corynebacterium*, *Acinetobacter* and *Trichococcus* are the genera usually isolated during the first days of refrigerated storage in untreated milks (Rasolofo et al. 2010; Vithanage et al. 2014). Such a diverse range of microbes identified in raw milk reflects the variety of potential on-farm and transport-related sources (Vissers and Driehuis 2009), milking equipment as well as feeding (Coorevits et al. 2008).

The minor richness of the milk microbiota examined in this work could suggest a biogeographic characterization

Table 2 Families, closest relative species, identity percentage, accession number and time of isolation of 20 biotypes isolated in raw milk refrigerated at 4 °C during 21 days of storage

Family	Closest relative species	Identity (%)	Accession number	Time of isolation (days)
Pseudomonadaceae	*Stenotrophomonas rhizophila*	99	HF678432	7
	Stenotrophomonas maltophilia	99	HF678416	7
	Stenotrophomonas maltophilia	99	HF678417	7
	Stenotrophomonas rhizophila	100	HF678420	7
	Stenotrophomonas rhizophila	100	HF678425	7
	Stenotrophomonas maltophilia	100	HF678419	7
Flavobacteriaceae	*Chryseobacterium indologenes*	99	HF678415	7
	Chryseobacterium indologenes	99	HF678423	7
	Chryseobacterium indologenes	99	HF678414	10
	Chryseobacterium spp.	95	HF678433	16
	Chryseobacterium indologenes	98	HF678427	21
	Chryseobacterium indologenes	99	HF678418	21
Lactobacillaceae	*Lactobacillus* spp.	80	HF678430	10
	Lactobacillus pentosus	100	HF678422	16
	Lactobacillus plantarum	100	HF678429	16
	Lactobacillus plantarum	99	HF678421	21
	Lactobacillus pentosus	99	HF678431	21
Moraxellaceae	*Acinetobacter guillouiae*	99	HF678424	16
	Acinetobacter guillouiae	98	HF678426	21
	Acinetobacter spp.	93	HF678428	21

depending on specific environmental factors. On the other hand, variations in the composition of milk microflora have been reported following changes in the cow feeding environment, i.e., from inside to outside grazing (Hagi et al. 2010).

Although *Chryseobacterium indologenes* has been found in refrigerated raw milk (Hugo et al. 2003; Shimomura et al. 2005; Bekker 2011), this species is more generally regarded as an usual inhabitant of soils and plants (Bekker 2011). This is in agreement with outdoor grazing of the cows employed in this study. Moreover, this microbial species is known for its ability to degrade toxic plant compounds, including polyphenols (Lopez et al. 2003; Bekker 2011). Soil and the rhyzosphere around plants roots are also the main environmental reservoirs of *Stenotrophomonas maltophilia* and *S. rhizophila*. In general, *Stenotrophomonas* spp. are also known to show resistance against plant antimicrobial metabolites (Ryan et al. 2009; Alavi et al. 2014). Moreover, interestingly, *S. rhizophila* possesses unique genes essential for high salinity tolerance and is a plant growth promoter in highly salinated soils (Alavi et al. 2014) which are similar to the semi-arid ones around the subject farm of this investigation. Several *Chryseobacterium* species have shown an ability to grow at 5 °C. However, their growth rate under refrigeration is significantly lower than that of *Pseudomonas* spp. (Bekker 2011). The prevalence of *C. indologenes* up to the 21st day could account for the prolonged lag phase reported in Table 1 and exceeding 7 days. Moreover, the persistence of this species and the limited number of microbial species found in the milk suggest that it could play an antagonistic role. In fact, it has been reported to produce antimicrobial pigments and to be able to antagonize plant pathogens (Hugo et al. 2003; Bekker 2011; Yang et al. 2012).

While the above-described microbial species were the dominant ones up to 10–16 days of storage, *Lactobacillus* spp. were isolated on the 10th day, while *L. plantarum*, *L. pentosus* and *Acinetobacter guillouiae* were isolated at the 16th and 21st days. After 21 days, the spoilage bacteria *A. guillouiae* and *Chryseobacterium indologenes* cohabitated with *L. plantarum* and *L. pentosus*. The late isolation of *Acinetobacter* spp., which are regarded as usual components of refrigerated milk microbiota, could account for a possible antagonistic effect of the antimicrobial molecules released by *C. indologenes* (Yang et al. 2012; Liu et al. 2014) or for the presence in the milk of natural antimicrobials derived from plants grazed by cows, e.g., *Malva sylvestris*, *Cyanodon dactylon*, *Atriplex* spp., *Tamarix* spp. and *Cyperus conglomeratus* (Bouaziz et al. 2009; Chaudhari et al. 2011; Razavi et al. 2011; Abd El Raheim 2013).

The families *Flavobacteriaceae*, *Pseudomonadaceae*, *Moraxellaceae* and *Lactobacillaceae* include psychrotrophic genera frequently isolated from refrigerated raw milk and dairy products (Lafarge et al. 2004; Hantsis-Zacharov and Halpern 2007; Boubendir et al. 2011; Franciosi et al. 2011). The majority of the identified species in refrigerated raw milk were Gram-negative bacteria. These results are in accordance with those reported by Lafarge et al. (2004) regarding the effect of milk cooling on microbial shift. Gram-negative bacteria usually account for more than 90 % of the microbial population in cold-stored raw milk. On the other hand, interestingly, *Lactobacillus pentosus* and *L. plantarum* showed a psychrotrophic behavior. Their ability to grow at low temperatures could be positive for further dairy manufacture. Indeed, Franciosi et al. (2011) reported that several strains belonging to *Streptococcaceae* showed psychrotrophic aptitude in milk.

Despite the limited number of samples analyzed in this preliminary work, the prevalence of *Stenotrophomonas* spp. and *Chryseobacterium indologenes* during the first 7 days in combination with the suggested presence of antimicrobial plant metabolites in milk could account for the above-described delayed and sluggish microbial growth. As observed by Rochfort et al. (2008), many bioactive molecules of plants grazed by cow also in temperate zones play an important role for rumen health and are endowed with antimicrobial activity. Moreover, grazing herb-rich pasture has been shown to increase the concentration in milk of a range of volatile compounds, such as terpenes (Larsen et al. 2012), and is also shown to modify the milk fatty acids profiles (Rubino et al. 2006). In agreement with our hypothesis, the antimicrobial activity of essential oils of plants growing in the semi-arid zone of this study, such as *Tamarix* spp., *Atriplex nummularia*, *Malva sylvestris*, *Cyperus* spp. and *Cyanodon dactylon* on Gram-positive and Gram-negative bacteria, has been shown by several authors (Bouaziz et al. 2009; Chaudhari et al. 2011; Razavi et al. 2011; Abd El Raheim 2013).

The antimicrobial compounds in milk, presumably, does not directly reflect the composition of the feed, most likely because some terpenes, fatty acids or polyphenols are metabolized by the cow (Rubino et al. 2006). However, recent studies showed promising results regarding the use of phytochemicals not only as growth and production promoters (Frankič et al. 2006), but also to improve the safety and shelf-life of milk (Cava et al. 2007; Gutierrez et al. 2009; Shobharani and Agrawal 2010), as suggested by this preliminary paper. Nevertheless, the possible contribution of a specific plants' antimicrobials from this area to the organoleptic characteristics of milk and derived dairy products should be investigated. In fact, differences in volatiles and sensory properties of cheeses in relation to feeding and grazing systems have been reported (Coppa et al. 2011). Furthermore, specific changes in milk composition in relation to the herbs and plants used for animal feeding, and the action mechanism of the plants included in the diets on milk microbiota and its dynamics during refrigeration need to be evaluated.

In this study, the bacterial population of refrigerated raw milk collected from a semi-arid area of Algeria was

characterized. Limited and sluggish bacterial growth was observed over 21 days of refrigerated storage. Moreover, the microbial consortium was characterized by the presence of a limited number of species compared to literature data which usually refer to milk samples from temperate zones. The extended shelf-life over refrigerated storage is supposedly due to the possible presence of plant metabolites with antimicrobial activity growing in the arid pasture. Although preliminary, the outcomes of this study indicate arid herbs in animal feed may be exploited due to their bioactivity to produce milk with enhanced safety characteristics and an improved shelf-life.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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