Journal of Food Safety and Food Quality 66, Heft 6 (2015), Seiten 157–184

Arch Lebensmittelhyg 66,
160–166 (2015)
DOI 10.2376/0003-925X-66-160

ISSN 0003-925X

Korrespondenzadresse:
a.hafid.bio@gmail.com

[1]) Department of Natural Sciences and Life, University Centre Abdelhafid BOUSSOUF, Mila, Algeria; [2]) Microbial Biotechnology Laboratory, Sidi Mohamed Ben Abdullah University, Fes, Morocco; [3]) Faculty of Natural Sciences and Life, Mentouri University, Constantine, Algeria

Interactions in milk psychrotrophic bacterial populations

Wechselwirkungen psychrotropher Bakterienpopulationen in Milch

Abdelhafid Boubendir[1]), Soumya Elabed[2]), Mohamed Abdelhafid Hamidechi[3]), Abdelouahab Yahia[1]), Saad Ibnsouda Koraichi[2])

Summary

Raw milk samples (n = 104) were collected from healthy cows at different seasons in the North East part of Algeria. Cold storage of samples at 4 °C was used to promote psychrotrophic microbial growth. The isolates obtained from Sterile Standard Plate Count (SPC) were characterized by phenotypic means using cultural, morphological, biochemical criteria; and genotypicaly using partial 16S rRNA gene sequencing. The psychrotrophic community was diversified and made of 13 bacterial populations, constituted of both spoilage and pathogenic bacteria, with the dominant population *Enterococcus* spp. (19.23 %). Principal Component Analysis was used to study interactions in psychrotrophic bacterial populations. *Enterococcus* registered low correlation values for the majority of the microorganisms detected. *Listeria* was correlated positively with Bacillus (r = 0.564) and negatively with *Enterococcus* (r = –0.468). Factorial Correspondence Analysis was used to study seasonal bacterial incidence. *Listeria* showed an association with winter and spring seasons. The most associated bacteria in time to *Listeria* were *Stenotrophomonas, Pseudomonas* and *Bacillus*. The data obtained allow the understanding of interactions in psychrotrophic bacterial populations, and is useful for preventing the risk of emergence of pathogenic bacteria at different months of the year.

Keywords: Cold storage, psychrotrophic bacteria, spoilage, pathogens

Zusammenfassung

Rohmilch-Proben (n = 104) von gesunden Kühen wurden zu verschiedenen Jahreszeiten im Nordosten Algeriens gesammelt. Die Proben wurden bei 4 °C gelagert, um das Wachstum psychrotopher Mikroorgaismen zu fördern. Isolate von Standard-Keimzahl-Bestimmungen wurden phänotypisch anhand von Kultivierungseigenschaften, morphologischen und biochemischen Eigenschaften sowie genetisch anhand partieller 16S rRNA Gensequenzen charakterisiert. Die psychrotrophe Organismengemeinschaft bestand aus 13 Populationen, von zersetzenden und pathogenen Bakterien, wobei *Enterococcus* spp. (19.23 %) dominierten. Hauptkomponentenanalyse wurde verwendet, um Wechselwirkungen in psychrotrophen Bakterienpopulationen zu untersuchen. Für *Enterococcus* ergaben sich niedrige Korrelationswerte mit der Mehrzahl der detektierten Mikroorganismen. Das Vorkommen von *Listeria* korrelierte positiv mit *Bacillus* (r = 0.564) und negativ mit *Enterococcus* (r = –0.468). Durch faktorielle Korrespondenzanalyse wurde die saisonale Verteilung der Bakterien untersucht. *Listeria* traten vor allem im Winter und Frühling auf und waren vor allem mit *Stenotrophomonas, Pseudomonas* und *Bacillus* assoziiert. Die erhaltenen Daten verbessern unser Verständnis der Wechselwirkungen in psychrotrophen Bakterienpopulationen und tragen zur Vermeidung des Auftretens pathogener Bakterien in verschiedenen Jahreszeiten bei.

Schlüsselwörter: Kühllagerung, psychrotrophe Bakterien, Verderb, Pathogene

Introduction

The consumption of fresh raw milk or naturally fermented milk without any artificial additives or thermal pretreatment is gaining popularity worldwide as it has many advantages including enhanced nutritional value, digestibility, therapeutic benefits and safety against pathogens (Sitohy et al., 2011). However, milk is an excellent culture medium for the growth and reproduction of microorganisms. It is known that raw milk harbor a complex microbial ecosystem encompassing numerous strains. Microorganisms are originate from different sources: air, milking equipment, feed, soil, excrements, grass, water, skin, and hair of the animals, utensils or from the milk handlers (Nocker et al., 2007; Coorevits et al., 2008).

Bacterial spoilage causes significant economic losses for the food industry. Product contamination with psychrotrophic microorganisms is a particular concern for the dairy industry as dairy products are distributed at temperatures permissive for the growth of these organisms. These microbes may account for only a small fraction of the initial flora of processed milk. Bacterial spoilage ensues when growth conditions during refrigerated storage allow psychrotrophic microbes to increase in number and to become the dominant microflora (Dogan et al., 2003; Hantsis-Zacharov and Halpern, 2007). Psychrotrophic bacteria from numerous genera have been isolated from milk, both Gram negative (Pseudomonas, Stenotrophomonas, Aeromonas, Serratia, Acinetobacter, Alcaligenes, Achromobacter, Enterobacter, Proteus, Yersinia, Klebsiella and Flavobacterium) and Gram positive (Bacillus, Listeria, Clostridium, Corynebacterium, Microbacterium, Micrococcus, Enterococcus, Streptococcus, Staphylococcus, and Lactobacillus). Of these, *Pseudomonas* is the most frequently reported psychrotroph in raw milk (Lafarge et al., 2004; Hantsis-Zacharov and Halpern, 2007; Franciosi et al., 2011).

Microbial interactions are commonly classified based on the effect of the interaction on each population in a binary system. Neutralism occurs when neither population is affected by the presence of the other. Competition refers to an interaction where two populations are competing for a growth-limiting nutrient and which is detrimental to both populations. When one population benefits from the presence or activity of the other while the benefactor is unaffected, the phenomenon is termed commensalism. An interaction where both populations benefit is mutualism, which includes obligatory interactions (symbiosis), facultative interactions (protocooperation), or interactions that result in the enhanced production (or consumption) of a certain product (synergism). Protocooperation involving the mutual exchange of a growth factor or energy source (cross-feeding) is termed syntrophy. Ammensalism refers to an interaction where one population has an indirect (not involving cell cell contact) negative impact on another, such as the production of a bacteriocin by one species that inhibits the growth of another. Although this binary system classification is useful for defining interactions, in natural communities interactions can be complex and include mixed interactions, where more than one type of interaction occurs between two species, as well as interactions involving more than one species (James et al., 1995).

The microbiological profile in raw milk is typically characterized by a multitude of microbial groups, with interactions among not fully understood to date. Moreover, dairy farms and milk industries are increasing throughout Algeria. Factories are producing different kinds of products, including pasteurized milk, cheese, yogurt, etc, and the data about psychrotrophic bacteria still little known. Consequently, the aim of the present study was to explore different interactions in psychrotrophic bacterial populations isolated in raw milk collected in the North East part of Algeria. This research is completed by the study of seasonal bacterial incidence.

SUPPLEMENTAL MATERIAL 1: *Identification results of the presumed bacteria isolated on the Sterile Standard Plate Count (SPC) agar after cold storage of milk samples at 4 °C. Entr: Enterococcus, List: Listeria, Stph: Staphylococcus, Acin: Acinetobacter, Prot: Proteus, Aero: Aeromonas, Psdo: Pseudomonas, Klbs: Klebsiella, Yers: Yersinia, Baci: Bacillus, Flav: Flavobacterium, Alca: Alcaligenes, Sten: Stenotrophomonas.*

	Klbs	Prot	Yers	Acin	Aero	Alca	Flav	Sten	Psdo	Stph	Entr	List	Baci
Gram	–	–	–	–	–	–	–	–	–	+	+	+	+
Mobility	–	+	–	–	+	+	+/–	+	+	–	–	(–)1	+
Oxydase	–	–	–	–	+	+	–	+	+	–	–	–	–
NO_3^-	+	+	+	–		+	–	NR	–	NR	NR	–	+/–
Indole	–	+/–	–	–	+/–	–	+/–	NR	–	NR	NR	–	–
Catalase	+	+	+	+	+	+	+	+	+	+	–	+	+
Lactose	+	–	–	–	+	–	+	+	+/–	+	+	–	–
Mannitol	+	–	+	NR	+	–	–	–	NR	+	+	–	–
Gaz	+	+	–	NR	+	NR		+/–	NR	+	NR	–	–
Saccharose	+	–	+	NR	+	–	+	+/–	+	+/–	NR	+/–	+/–
H_2S	–	+	–	–	+/–	–	NR	–	–	–	–	–	–
Hemolysis	NR	+	NR	NR	+	–	NR	NR	NR	+	+	+	NR
VP	+	–	–	NR	NR	NR	NR	NR	NR	+	+/–	+	–
Esculine	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	+	+	NR
RM	–	+	+	NR	NR	NR	NR	NR	NR	–	+/–	+	+
Urease	+	+	+	–	NR	–	NR	NR	NR	NR	NR	–	–
Citrate	+	+	–	NR	NR	+	NR	NR	NR	NR	+	NR	NR
ONPG	+	–	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
LDC	+	–	–	–	NR	–	NR	NR	NR	NR	NR	NR	NR
ODC	–	+/–	+/–	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR

1: negative mobility at 37 °C, + = positive reaction, – = negative reaction, +/– = variable reaction depending on the strain. NR: no realized

Journal of Food Safety and Food Quality 66, Heft 6 (2015), Seiten 157–184

Materials and methods

Milk sampling

A total of 104 raw milk samples were collected at different seasons in the North East of Algeria in Mila (36°27'N/6°15'E) and Biskra (34°51'N/5°43'E). Samples were obtained, once a month, from six small dairy farms (three farms in Mila and three farms in Biskra), who deliver milk twice a day (once in the morning and once in the afternoon). Joined together, these farms produce approximately 1350 L of raw milk per day. The total number of cows tested was 60, essentially formed by hybrid dairy bovine coming from crossing local dairy cattle with imported cows such "Française Frisonne Pie noir" and "Montbéliard Pie rouge". At each sampling visit, teat-ends are cleaned by wiping with dry paper towels, the first jets are removed and 25 ml of raw milk were directly collected from the four teats of each cow and transferred to the laboratory in individual sterile flasks at 4 °C. The number of samples taken has not been steady all time, because sick and gestate cows were not sampled.

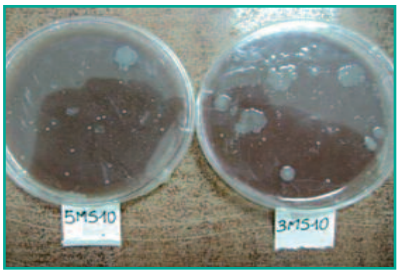
Cultivation

Raw milk samples were kept at 4 °C and cultured after 4, 10, and 21 days to promote psychrotrophic microbiota. After cold storage, samples were 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). The plates were incubated at 30 °C for 48 h. The colonies were selected randomly and purified for identification by streaking on the same medium.

Identification

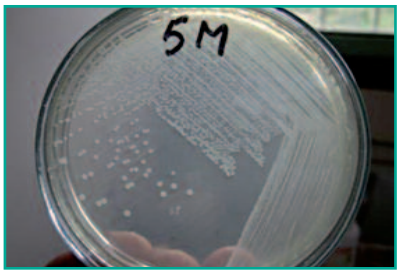
The phenotypic identification of isolates was carried using cultural, morphological and biochemical criteria. The major identification tests employed were: Gram staining, mobility (at 25 and 37 °C), catalase presence, oxydase presence, methyl red test (MR test), Voges-Proskauer reaction (VP test), indol production, esculin hydrolysis, urease presence, nitrates reduction, H_2S production (TSI test), and hemolytic activity. For the genotypic identification, bacterial DNA was extracted from pure cultures using thermal shock. Fragments of 16S rRNA gene were amplified in presence of primers fD1 (5'AGAGTTTGATCCTGGCTCAG3') and Rs16 (5'TACGGCTACCTTGTTACGACTT3') (SIGMA). The size of the amplimer obtained was 700 bp. Polymerase chain reaction was performed with the following protocol: 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min followed by a final extension step of 72 °C for 5 min (Microbial Biotechnology Laboratory, Sidi Mohamed Ben Abdullah University, Fes, Morocco). PCR products were separated by electrophoresis on 1.5 % (w/v) agarose gel (Merck Millipore) stained with ethidium bromide (0.5 µg ml^{-1}). The expected amplicons were eluted from gel and purified by the QIAquick PCR Purification Kit (Qiagen). DNA sequencing was realized at the University Centre of Regional Interface, Fes, Morocco using ABI 3130 sequencer (Appl. Biosystems) according to the manufacturer instructions. GenBank BLASTN tools were used for sequences analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome).

SUPPLEMENTAL MATERIAL 2: *Morphology, macroscopy and microscopy of psychrotrophic bacterial isolates.*

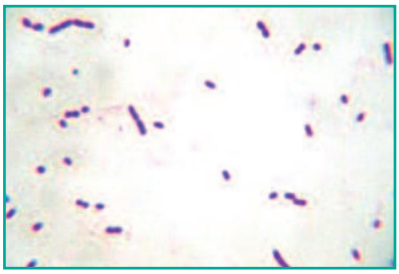


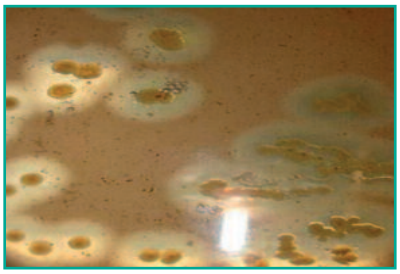
Proteus spp.

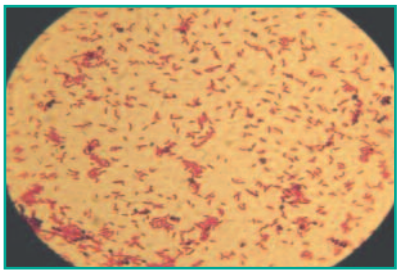
Acinetobacter spp.

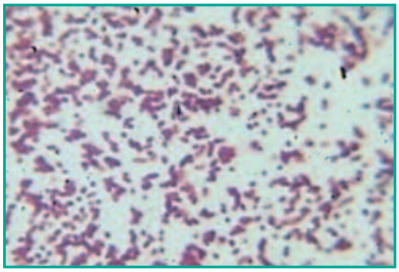


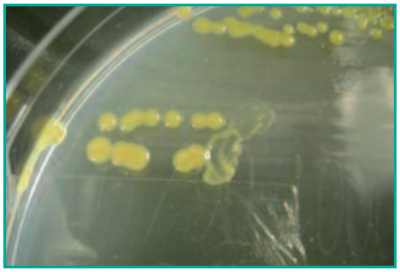
Enterococcus spp.

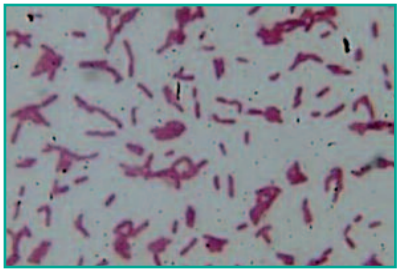

Listeria spp.


Aeromonas spp.


Aeromonas spp.


Staphylococcus spp.


Stenotrophomonas spp.


Klebsiella spp.

Statistical Analysis

The data was tested for normal distribution. The Principal Component Analysis was used to study interactions in psychrotrophic bacterial populations. The matrix of correlation was calculated according to Pearson coefficient. Factorial Correspondence Analysis was used to study seasonal bacterial distributions. The multivariate analyses were conducted with PAST 1.98 software (Hammer et al., 2001).

Results and discussion

Psychrotrophic bacterial populations

Psychrotrophic bacterial populations dominate milk microbiota, were diversified and made of 13 populations (Tab. 1.). This result agrees with the literature and confirms once more that cold storage of milk promotes psychrotrophic microbial growth (Lafarge et al., 2004; Hantsis-Zacharov and Halpern, 2007; Franciosi et al., 2011).

Enterococcus spp. was the most dominant population of bacterial community (19.23 %). Enterococci constitute a large proportion of the autochthonous bacteria associated with the mammalian gastrointestinal tract. However, although it is generally believed that the primary habitat of enterococci is the intestinal contents of warm-blooded animals, the gastro-intestinal contents of cold-blooded animals, including insects and birds, constitute other important habitats as well. Thanks to their psychrotrophic nature and their adaptability to different substrates and growth conditions they are also able to survive during milk refrigeration (Giraffa, 2003; Mannu et al., 2003). Giannino et al. (2009) confirmed the presence of *E. faecium, E. faecalis* and *Streptococcus thermophilus* in raw milk, proving its importance as source of the typical fermenting microflora.

The percentage of positive samples contaminated by *Listeria* spp. was 5.77 % (Tab. 1.). Studies have shown different levels of contamination with Listeria in raw milk. The incidence of Listeria was 6 % in Turkey (Vardar-Ünlü et al., 1998) and 2.2 % in Iran (Moshtaghi et al., 2007). In Algeria, few studies have been done to estimate the incidence of Listeria in raw bovine milk. A study realized by Hamdi et al. (2007) revealed 2.61 % of Listeria in 153 samples collected in the region of Algiers and Blida. Farm animals and their environment may present an important source of milk contamination. *Listeria* spp. are shed in the faeces of asymptomatic animal carriers. Therefore, contamination of milk is normally due to faecal contamination during the milking (Jemmi and Stephan, 2006).

Enterobacteriaceae isolated from raw milk were Proteus, Klebsiella and Yersinia. This microbial group is of technological interest, because some psychrotrophic Enterobacteriaceae species can produce proteolytic and lipolytic enzymes that negatively affect the organoleptic characteristics of dairy products; moreover, they can be pathogenic (Lafarge et al., 2004; Franciosi et al., 2011). There presence in milk is due to direct contact with contaminated sources in dairy farms environment and mammalian secretion of infected animals (Oliver et al., 2005). Marco et al. (2008) reported that several human pathogens have been detected in raw milk including *Escherichia coli, Salmonella typhimurium* and *Yersinia enterocolitica.* These pathogens have been linked to livestock, feed, and storage environment.

Furthermore, Gram-negative bacteria such as *Pseudomonas* spp., *Stenotrophomonas* spp., *Flavobacterium* spp., and *Alcaligenes* spp. were found. These genera are frequently isolated from refrigerated raw milk and dairy products environments. Indeed, during cold storage after milk collection, psychrotrophic bacterial populations dominate the microflora, and their extracellular enzymes, mainly proteases and lipases, contribute to the spoilage of dairy products (Hantsis-Zacharov and Halpern, 2007).

Psychrotrophic bacterial interactions

The data was normally distributed. The Principal Component Analysis provides a matrix of correlation, calculated according to Pearson coefficient between the whole of bacteria isolated (Tab. 2.). Bacterial contribution in the construction of the two principal components is the result of their correlation with these axes. *Bacillus* spp. had the strongest correlation (r = 0.828) with the first principal component (PC1), while *Stenotrophomonas* spp. produced the most important correlation (r = 0.757) with the second principal component (PC2). For this reason, the first principal component (PC1) was considered representative of Gram positive community and the second principal component (PC2) was considered representative of Gram negative community (Fig. 1). The first and the second principal components explained 53.231 % of the bacterial community variation.

The most negative correlation of Listeria was observed with Enterococcus (r = –0.468). Consequently, an antagonistic effect of Enterococcus against Listeria can be suggested. Indeed, the works of Elotmani et al. (2002), Laukova and Marekova (2002), confirmed the inhibitory effect of bacteriocins produced by Enterococcus strains against Listeria. Interestingly, Enterococcus registered low correlation values for the majority of the microorganisms detected except for *Aeromonas.* Indeed, almost all of enterococci are strongly active against the food spoilers and food-borne pathogens such as *L. monocytogenes, Clostridium* spp., *Staphylococcus aureus,* and *Bacillus* spp. Probably, the antagonistic interaction phenomena is caused by

TABLE 1: *Distribution of different psychrotrophic bacteria isolated from raw milk samples (n = 104) in the North East of Algeria*

Organism	Number of positive samples/Number of samples collected	% Positive
Enterococcus spp.	20/104	19.23
Acinetobacter spp.	9/104	8.65
Aeromonas spp.	8/104	7.69
Staphylococcus spp.	6/104	5.77
Listeria spp.	6/104	5.77
Pseudomonas spp.	5/104	4.81
Bacillus spp.	4/104	3.85
Stenotrophomonas spp.	3/104	2.88
Proteus spp.	3/104	2.88
Yersinia spp.	2/104	1.92
Klebsiella spp.	2/104	1.92
Flavobacterium spp.	1/104	0.96
Alcaligenes spp.	1/104	0.96

Journal of Food Safety and Food Quality 66, Heft 6 (2015), Seiten 157–184

TABLE 2: *Matrix of correlation (Pearson (n)) of 13 psychrotrophic bacterial populations isolated in the North East of Algeria. Entr: Enterococcus, List: Listeria, Stph: Staphylococcus, Acin: Acinetobacter, Prot: Proteus, Aero: Aeromonas, Psdo: Pseudomonas, Klbs: Klebsiella, Yers: Yersinia, Baci: Bacillus, Flav: Flavobacterium, Alca: Alcaligenes, Sten: Stenotrophomonas*

	Entr	List	Stph	Acin	Prot	Aero	Psdo	Klbs	Yers	Baci	Flav	Alca	Sten
Entr	1												
List	–0,468	1											
Stph	0,068	0,218	1										
Acin	–0,272	0,491	0,444	1									
Prot	0,000	–0,189	–0,289	–0,289	1								
Aero	0,612	0,055	0,389	0,111	–0,289	1							
Psdo	0,000	0,378	0,289	0,866	–0,500	0,289	1						
Klbs	–0,167	–0,200	–0,068	–0,068	0,354	–0,612	–0,354	1					
Yers	0,196	0,367	0,080	0,480	–0,277	0,320	0,555	–0,196	1				
Baci	0,302	**0,564**	0,431	0,431	–0,426	0,492	0,533	–0,302	0,650	1			
Flav	0,196	0,367	0,080	0,480	–0,277	0,320	0,555	–0,196	1,000	0,650	1		
Alca	0,196	–0,419	–0,320	–0,320	0,139	–0,480	–0,277	0,784	–0,154	–0,237	–0,154	1	
Sten	–0,075	0,564	0,123	–0,185	–0,107	0,185	–0,107	–0,302	–0,237	0,318	–0,237	–0,237	1
	Entr	List	Stph	Acin	Prot	Aero	Psdo	Klbs	Yers	Baci	Flav	Alca	Sten

a complex combined effect of production of antimicrobials and competition or depletion of specific nutrients like vitamins, minerals, trace elements or peptides (Leroy et al., 2003; Garcia et al., 2004).

The correlation between Staphylococcus and Listeria was weakly positive (r = 0.218). In contrast, Leriche and Carpentier (2000) indicated that the percentage of adherent cells of two strains of *L. monocytogenes* isolated from dairy and meat environments was reduced by the presence of *Staphylococcus sciuri.* The mechanisms involved in these interactions remain unknown but the data suggest that they are complex. Rieu et al. (2008) reported that the effect of *L. monocytogenes* EGD-e on the population of *S. aureus* was strain dependent: *S. aureus* population either increased or decreased or was not affected in the presence of *L. monocytogenes* EGD-e in dual species biofilms.

A positive correlation is observed between Listeria and Bacillus (r = 0.564). The most likely explanation for this phenomenon is the common soil origin of the two Gram positive bacteria (Gandhi and Chikindas, 2007; Zahran et al., 2008). According to Coorevits et al. (2010), *B. cereus* is a common soil organism, and soiling of teats is thus most probably the major contamination source of raw milk with this pathogen. It is the most important pathogen for the dairy industry due to production of toxins causing food illnesses, production of deteriorative enzymes resulting in decreased milk quality and its ability to grow at storage temperature (4–7 °C).

A slight correlation was observed between Aeromonas and Listeria (r = 0.055). Messi et al. (2003) demonstrated the antibacterial activity of *A. hydrophila* isolated from water against *Listeria* spp. (*Listeria seeligeri, Listeria welshimeri* and *Listeria ivanovii*) by producing bacteriocin-like substance (BLS). Also, they showed that *A. hydrophila* has an antibacterial activity against Gram-positive and Gram-negative bacteria, including food-borne pathogens, but the activity emerged only with non-phylogenetically related genera or species. Furthermore, Bacteriocins and BLS seem to be important in the regulation of population dynamics in bacterial ecosystems, favoring the microorganism in the competition for the colonization of environmental, food and human microbial habitats. Lafarge et al. (2004) confirmed the emergence of psychrotrophic bacteria such as *Listeria* spp. and *Aeromonas hydrophila* in the bacterial population in milk associated with refrigeration. Also, Martins et al. (2006) isolated *Aeromonas hydrophila* from cooled raw milk stored at 4 °C.

A positive correlation was observed between Flavobacterium and Listeria (r = 0.367). This result is in agreement with Bremer et al. (2001) who confirmed the positive interaction in dual species bacterial biofilm when *L. monocytogenes* was cocultured with *Flavobacterium* spp.; the number of *L. monocytogenes* cells attaching to stainless

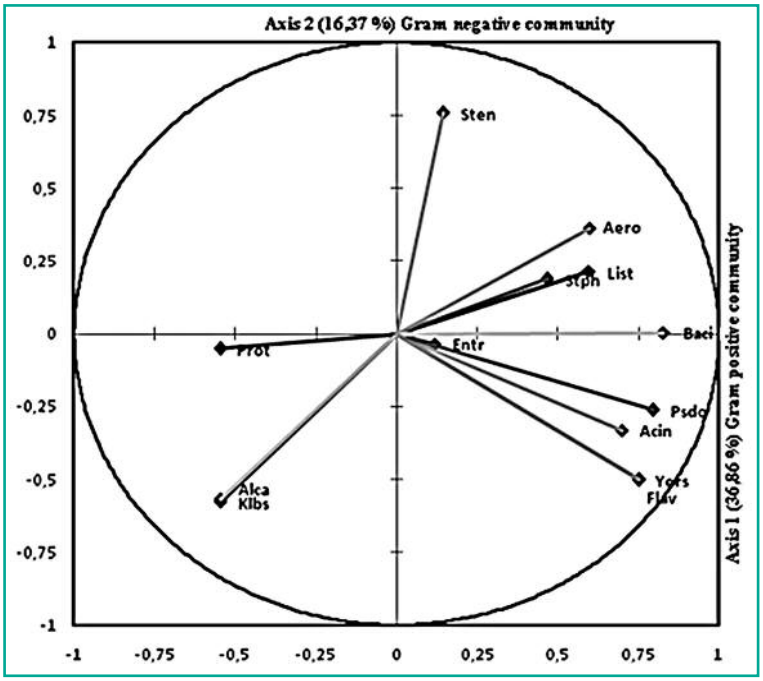


FIGURE 1: *Principal Component Analysis of interactions in psychrotrophic bacterial populations in raw milk in the North East of Algeria. Axis 1 indicates the first principal component, and axis 2 the second principal component. Entr: Enterococcus, List: Listeria, Stph: Staphylococcus, Acin: Acinetobacter, Prot: Proteus, Aero: Aeromonas, Psdo: Pseudomonas, Klbs: Klebsiella, Yers: Yersinia, Baci: Bacillus, Flav: Flavobacterium, Alca: Alcaligenes, Sten: Stenotrophomonas.*

steel increased significantly compared to *L. monocytogenes* single culture biofilm. Psychrotrophic bacteria such us Chryseobacterium and Flavobacterium occur frequently in dairy products (Hantsis-Zacharov and Halpern, 2007; Giannino et al., 2009).

There is a positive correlation between Listeria and Pseudomonas (r = 0.378). In contrast, Norwood and Gilmour (2001) observed a negative effect on the growth of *L. monocytogenes* when grown in the presence of *Pseudomonas fragi.* This result could be due to the different geographic origins of bacterial strains. *Pseudomonas* spp. is one of the dominant components of the microbiota of refrigerated raw milk. It's presence in refrigerated raw milk is quite common (Arcuri et al., 2008; Franciosi et al., 2011).

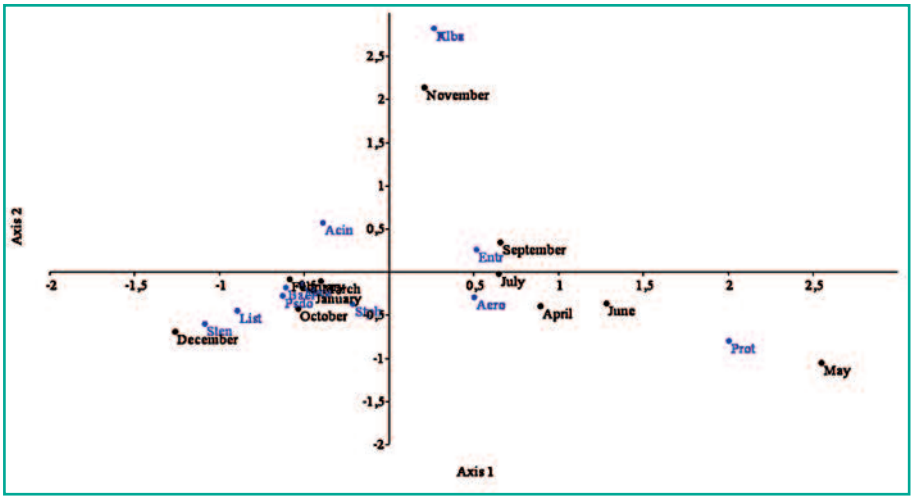


FIGURE 2: *Factorial Correspondence Analysis of seasonal psychrotrophic bacterial incidence in raw milk in the North East of Algeria. Entr: Enterococcus, List: Listeria, Stph: Staphylococcus, Acin: Acinetobacter, Prot: Proteus, Aero: Aeromonas, Psdo: Pseudomonas, Klbs: Klebsiella, Yers: Yersinia, Baci: Bacillus, Flav: Flavobacterium, Alca: Alcaligenes, Sten: Stenotrophomonas.*

A strong positive correlation was registered between Acinetobacter and Pseudomonas (r = 0.866). Pseudomonas and Acinetobacter are the dominant genera in psychrotrophic bacterial communities in raw milk (Hantsis-Zacharov and Halpern, 2007; Franciosi et al., 2011).

Seasonal psychrotrophic bacterial incidence

The most important gathering is located in the west part of the graphic (Fig. 2). It includes *Stenotrophomonas* spp., *Listeria* spp., *Pseudomonas* spp., *Bacillus* spp., *Flavobacterium* spp., *Yersinia* spp. and *Staphylococcus* spp. This clustering is explained by the similar bacterial behavior compared with the factor time. However, *Proteus* spp. seems more associated to May and June months in the east part of the graphic. *Klebsiella* spp. and *Alcaligenes* spp. emerge together only in the month of November and are represented in the north part of the graphic.

Listeria presented a clear association with winter season in the months of December and January, and also was associated to spring in March. The most associated bacteria in time to Listeria were Stenotrophomonas, Pseudomonas and Bacillus (Fig. 2). Few studies described the effect of seasons on the incidence of *Listeria* spp. and other psychrotrophic bacteria in raw milk. Contamination of raw milk with Listeria is usually more common in winter, most likely because silage feeding in many parts of the world is more common in this season (Waak et al., 2002; Broseta et al., 2003). Kalac (2011) confirmed that silage is a rich source of contamination by undesirable bacteria such *L. monocytogenes, Bacillus cereus* and *Clostridium tyrobutyricum.* He added that poor silage quality is caused by slight acidity resulting from inadequate lactic fermentation, or aerobic spoilage. Wagner and McLauchlin (2008) considered that cow listeriosis reach the peak in spring. They mentioned also that *Listeria* spp. can tolerate seasonal stress variations from extreme temperatures near freezing in winter to high temperatures in summer. *Staphylococcus* was more associated with winter months in January and February. This result is in agreement with Rea et al. (1992) who isolated *Staphylococcus* spp. in winter in Ireland. *Staphylococcus* spp. belongs to mastitis-causing organisms, and can be implicated as causative agent of foodborne illness.

On the contrary, Proteus showed a different temporal bacterial behavior. Compared with other populations, it was associated with hot months of April, May and June situated in the east part of the graphic (Fig. 2). This member of Enterobacteriaceae family is stimulated by high temperatures and may be also an indicator of milk contamination by cow excrements. A minority of bacteria were associated with summer and spring months located in the east part of the graphic, suggesting that feeding with fresh silage available in these seasons may reduce milk contamination.

This work confirms once more that raw milk stored at refrigeration temperature encourage the emergence of psychrotrophic bacteria. Bacterial psychrotrophic populations isolated were diversified and constituted by both spoilage and pathogenic microorganisms. Enterococcus registered low correlation values for the majority of the microorganisms detected, suggesting a large antagonistic effect. Therefore, Enterococcus can offer opportunities as biopreservative and improve the safety of raw milk and dairy products by inhibiting the growth of contaminating. Our data confirm that microbial consortium influences the way bacterial populations proliferate in refrigerated raw milk. The presence of some bacterial populations could facilitate or impede the colonization and persistency of others in the microbial ecosystem. The mechanisms involved in the interactions remain little known but our data suggest that they are complex. It will be interesting to characterize the mechanisms involved in the interactions. Few studies described the effect of season on the incidence of psychrotrophic bacteria in raw milk. Our results have an important impact on animal and human health protection. Monitoring seasonal incidence of psychrotrophic populations reinforce the prevention of pathogenic bacteria in high risk periods. Finally, the exploitation of microbial diversity and interactions could be a new strategy to fight against pathogenic and spoilage microorganisms in raw milk stored at low temperatures.

Conflict of interest

The authors state that no conflict of interest exists.

Journal of Food Safety and Food Quality 66, Heft 6 (2015), Seiten 157–184

References

Arcuri EF, Lima DA, Silva PD, Vasconcelos Paiva Britos MA, Feitosa Brito JR, Lange CC, Dos Anjos Maglhães MM (2008): Contagem, isolamento e caracterização de bactérias psicrotróficas contaminantes de leite cru refrigerado. Cienc Rural 38: 2250–2255.

Bremer PJ, Monk I, Osborne CM (2001): Survival of *Listeria monocytogenes* attached to stainless steel surfaces in the presence or absence of *Flavobacterium* spp. J Food Prot 64: 1369–1376.

Broseta SM, Diot A, Bastian S, Riviere J, Cerf O (2003): Estimation of low bacterial concentration: *Listeria monocytogenes* in raw milk. Int J Food Microbiol 80: 1–15.

Coorevits A, De Jonghe V, Vandroemme J, Reekmans R, Heyrman J, Messens W, De Vos P, Heyndrickx M (2008): Comparative analysis of the diversity of aerobic-spore-forming bacteria in raw milk from organic and conventional dairy farms. Syst Appl Microbiol 31: 126–140.

Coorevits A, De Jonghe V, Vandroemme J, Van Landschoot A, Heyndrickx M, De Vos P (2010): How can the type of dairy farming influence the bacterial flora in milk?. In: Organic Farming and Peanut Crops. Editors Grossman DC, Barrios TL, pp. 123–136, Nova Science Publishers, Inc.

Dogan B, Boor KJ (2003): Genetic Diversity and Spoilage Potentials among *Pseudomonas* spp. Isolated from Fluid Milk Products and Dairy Processing Plants. Appl Environ Microbiol 69: 130–138.

Elotmani F, Revol-Junelles AM, Assobhei O, Milliere JB (2002): Characterization of Anti-*Listeria monocytogenes* Bacteriocins from *Enterococcus faecalis, Enterococcus faecium* and *Lactococcus lactis* strains isolated from Raib, a Moroccan Traditional Fermented Milk. Curr Microbiol 44: 10–17.

Franciosi E, De Sabbata G, Gardini F, Cavazza A, Poznanski E (2011): Changes in psychrotrophic microbial populations during milk creaming to produce Grana Trentino cheese. Food Microbiol 28: 43–51.

Gandhi M, Chikindas ML (2007): Review. Listeria: A foodborne pathogen that knows how to survive. Int J Food Microbiol 113: 1–15.

Garcia MT, Canamero MM, Lucas R, Ben Omar N, Pulido RP, Galvez A (2004): Inhibition of *Listeria monocytogenes* by enterocin EJ97 produced by *Enterococcus faecalis* EJ97. Int J Food Microbiol 90(2): 161–170.

Giannino ML, Marzotto M, Dellaglio F, Feligini M (2009): Study of microbial diversity in raw milk and fresh curd used for Fontina cheese production by culture-independent methods. Int J Food Microbiol 130: 188–195.

Giraffa G (2003): Functionality of Enterococci in dairy products. Int J Food Microbiol 88 (2–3): 215–222.

Hamdi TM, Naïm M, Martin P, Jacquet C (2007): Identification and molecular characterization of *Listeria monocytogenes* isolated in raw milk in the region of Algiers (Algeria). Int J Food Microbiol 116: 190–193.

Hammer DAT, Harper PD, Ryan Pas T (2001): Paleontological statistics software package for education and data analysis. Palaeont Electron http://palaeoelectronica.org/2001_1/past/issue1_01.htm. 4 (9).

Hantsis-Zacharov E, Halpern M (2007): Culturable Psychrotrophic Bacterial Communities in Raw Milk and Their Proteolytic and Lipolytic Traits. Appl Environ Microbiol 73: 7162–7168.

James GA, Beaudette L, Costerton JW (1995): Interspecies bacterial interactions in biofilms. J Ind Microbiol 15: 257–262.

Jemmi T, Stephan R (2006): *Listeria monocytogenes:* food-borne pathogen and hygiene indicator. Revue Scientifique et technique. Office international des epizooties 25: 571–580.

Kalac P (2011): The Effects of Silage Feeding on Some Sensory and Health Attributes of Cow's Milk: A Review Food Chem 125: 307–317.

Lafarge V, Ogier JC, Girard V, Maladen V, Leveau JY, Gruss A, Delacroix-Buchet A (2004): Raw Cow Milk Bacterial Population Shifts Attributable to Refrigeration. Appl Environ Microbiol 70: 5644–5650.

Laukova A, Marekova M (2002): A bacteriocin-mediated antagonism by *Enterococcus faecium* EK13 against *Listeria innocua* in tofu. Arch Lebensmittelhyg 53: 28–30.

Leriche V, Carpentier B (2000): Limitation of adhesion and growth of *Listeria monocytogenes* on stainless steel surfaces by *Staphylococcus sciuri* biofilms. J Appl Microbiol 88: 594–605.

Leroy F, Foulquie Moreno MR, De Vuyst L (2003): *Enterococcus faecium* RZS C5, an interesting bacteriocin producer to be used as a co-culture in food fermentation. Int J Food Microbiol 88 (2–3): 235–240.

Mannu L, Paba A, Daga E, Comunian R, Zanetti S, Dupre I, Sechi LA (2003): Comparison of the incidence of virulence determinants and antibiotic resistance between *Enterococcus faecium* strains of dairy, animal and clinical origin. Int J Food Microbiol 88 (2–3): 291–304.

Marco ML, Wells-Bennik MHJ (2008): Impact of bacterial genomics on determining quality and safety in the dairy production chain. Int Dairy J 18: 486-495.

Martins ML, Pinto CLO, Rocha RB, De Araújo EF, Vanetti MCD (2006): Genetic diversity of Gram-negative, proteolytic, psychrotrophic bacteria isolated from refrigerated raw milk. Int J Food Microbiol 111: 144–148.

Messi P, Guerrieri E, Bondi M (2003): Bacteriocin-like substance (BLS) production in *Aeromonas hydrophila* water isolates. Fems Microbiol Lett 220: 121–125.

Moshtaghi H, Mohamadpour AA (2007): Incidence of *Listeria* spp. in Raw Milk in Shahrekord, Iran. Foodborne Pathog Dis 4 (1): 107–110.

Nocker A, Burr M, Camper AK (2007): Genotypic microbial community profiling: a critical technical review. Microb Ecol 54: 276–289.

Norwood DE, Gilmour A (2001): The differential adherence capabilities of two *Listeria monocytogenes* strains in monoculture and multispecies biofilms as a function of temperature. Lett Appl Microbiol 33: 320–324.

Oliver SP, Jayarao BM, Almeida RA (2005): Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. Foodborne Pathog Dis 2: 115–129.

Rea MC, Cogan TM, Tobin S (1992): Incidence of pathogenic bacteria in raw milk in Ireland. J Appl Bacteriol 73: 331–336.

Rieu A, Lemaître JP, Guzzo J, Piveteau P (2008): Interactions in dual species biofilms between *Listeria monocytogenes* EGD-e and several strains of *Staphylococcus aureus.* Int J Food Microbiol 126: 76–82.

Sitohy M, Mahgoub S, Osman A (2011): Controlling psychrotrophic bacteria in raw buffalo milk preserved at 4 °C with esterified legume proteins. LWT-Food Sci Technol 1–6.

Vardar-Ünlü G, Ünlü M, Bakici MZ (1998): Incidence of *Listeria* spp. from Raw Milk in Sivas. Turk J Med Sci 28: 389–392.

Waak E, Tham W, Danielsson-Tham ML (2002): Prevalence and fingerprinting of *Listeria monocytogenes* strains isolated from raw whole milk in farm bulk tanks and in dairy plant receiving tanks. Appl Environ Microbiol 68: 3366–3370.

Wagner M, McLauchlin J (2008): Handbook of *Listeria monocytogenes.* Ed. Dougyou Liu. London, New York: Boca Raton. 554p. ISBN: 978-1-4200-5140-7.

Zahran DA, Hendy BA, El Hifnawi HN (2008): Incidence and Radiation Sensitivity of *Bacillus cereus, Listeria monocytogenes* and Their Toxins in Some Chicken Products. World Appl Sci J 5(2): 182–188.

Address of corresponding author:
Dr. Abdelhafid Boubendir
Département des Sciences de la Nature et de la Vie
Institut des Sciences et de la Technologie
Centre Universitaire Abdelhafid BOUSSOUF de Mila
RP.26
Mila 43000
ALGERIE
a.hafid.bio@gmail.com