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Full Length Research Paper

Microflora distribution and assessment of microbiological quality milk from Tunisian collection centres

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Eighty samples of raw milk, collected from eight Tunisian centres, were characterised. All these samples contained approximately 10^{11} cfu/ml of mesophilic aerobic bacteria (MAB), lactic acid bacteria (LAB), yeasts and coliforms dominated the microflora of these samples. They varied from 10^5 to 10^9 cfu/ml. More than 70% of the analysed samples contained 10^6 cfu/ml of *Pseudomonas.* The content of contaminating microflora like *Staphylococcus*, coliforms and mesophilic and thermophilic *Bacillus* ranged from 10^2 to 10^9 cfu/ml. However, mesophilic and thermophilic *Clostridium* were absent in all samples. This study could allow establishing the microflora distribution, revealing the non conformity of these eighty samples with standards, and pointing out eventual microbiological standards values of raw collected milk by comparison with standards.

Key words: Raw milk, milk collect, contamination, milk standards, microbiological references.

INTRODUCTION

The relationship between dairy foods and health effects has been investigated for many years (Heller, 2001). During recent years, numerous studies have been undertaken to obtain scientific evidence for benefit-cial effects of fermented dairy products containing specific probiotic strains (Ouwehand et al., 2002; Tuomola et al., 2002).

Milk is a complete food, containing proteins, fats, carbohydrates, vitamins and mineral salts (Park et al., 2007). Goat's milk is widely used for home consumption world-wide and to produce different cheeses and yoghurts (Pandya and Ghodke, 2007). For this reason, raw milk was considered as an important middle for the microorganism multiplication (Michel, 2001). So, it's imperative to minimise the storage temperature of milk at under 4°C to relent the microorganism growth.

However, in Tunisia, and for 10 years, we have noted an increase of milk consumption and the development of hygienic action for the amelioration of milk quality. So that, many efforts and projects are interested in ameliorating the conditions of milking, transport, storage and the package of milk (Remond, 1992). But, few studies are interested in the problem of milk quality amelioration and microflora milk distribution (Bloquel et al., 1980; Esmasures et al., 1997). This foodstuff presents an important danger in the sanitary plan because it can transmit a lot of pathogen micro organisms like *Staphylococcus*, *Streptococcus, Enterobacteria*, *Bacillus*, *Clostridium, Listeria,* etc., (Parguel, 2004) and pathogenic fungi (Gallo, 1996) like, *Alternaria* causing crisis of asthma to children (Halonen et al., 1997). Furthermore, three factors determine the microorganism growth; (i) the initial number of micro organisms; (ii) the temperature; (iii) the length of storage. At out of teat, when the milk is at an animal temperature (37°C) and in duration of few hours, we can't observe any microorganism multiplication due to the presence of inhibiter substance – lactenines (Cau, 1993) and bacteriocine (Tonnart, 2009) secreted by

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lactic acid bacteria (Allouch et al., 2010). In this state, it's imperative to refrigerate the milk quickly for slow down microorganism proliferation after bacteriostatic phase.

However, under refrigeration at 4°C, it is not sufficient to inhibit the growth of many bacteria and fungi which are able to cause the deterioration of foodstuff quality and make it inedible. For these reasons, we can prevent contamination risks of raw milk from its production by hygienic approach like the cleanness of animal, the teat, the farm of milk production, the milking room, the personnel, the milking materiel and the collect tank and milk transport (Parguel, 2004). Besides, the collection centres represent a big problem of microbiological quality deterioration, essentially due to the negligence of hygienic rules at the milking and carrying of the raw milk (PNTTA, 2006).

In order to evaluate hygienic practices in Tunisian milk collect centres, from milking and collecting approach to industrial package, microflora of samples of raw milk was investigated in this study in the light of its microbiological group distribution to evaluate the collect milk quality with microbiological standards (Table 1). The aim of the current study was therefore, to isolate and identify the microflora of dairy milk.

In addition, molecular characterisation of Lactic Acid Bacteria (LAB) and *Bacillus* was realised in the purpose of studying the antagonism between LAB and *Bacillus* in biofilm of pipelines of milk.

MATERIALS AND METHODS

Sampling method

Eighty samples of raw milk were collected from different collection centres in the cap bon region of Tunisia (Table 1). The collected samples were then kept under refrigeration (4°C) for microbiological analysis

Microflora determination and enumeration

Ten millilitres of each sample have been homogenized with 90 ml of sterile peptone water (1 g/l peptone, 5 g/l NaCl and 2 ml of Tween 80). After serial increasing dilution in sterile peptone-salt solution, milk samples were plated out on selected agar media. Lactic acid bacteria count is performed on M17 and MRS agar medium (De Man et al., 1960) containing 0.5% calcium carbonate to inhibit yeast growth. After 48 h incubation at 37°C, the result is expressed as the number (cfu/ml) colony forming unit. Different colonies were subsequently isolated and purified and enriched in their selective medium MRS, 24 h at 37°C. Identification of lactic acid bacteria was conducted by morphological tests (observation fresh, staining gram-negative and mobility), biochemical and physiological (testing catalase, oxidase, etc.,) and carbohydrate fermentation profiles were determined using specific API 50CHL strips (Api system, Biomerieux, France)

Viable yeasts and fungi were enumerated on Sabouraud agar medium with chloramphenicol (500 µg/ml) to inhibit bacteria growth. After 48 to 72 h incubation at 30°C, isolates were purified and examined through carbohydrates assimilation tests (Api C Aux, Biomerieux).

Table 1. Centres characteristics.

Centres	Capacité (l/an)	Hygiene	Samples
A	1.2 millions	insufficient	10
B	2 millions	insufficient	10
C	1.5 millions	insufficient	10
D	2 millions	insufficient	10
E	1 million	insufficient	10
F	2.5 millions	insufficient	10
G	700000	insufficient	10
H	900000	insufficient	10

Enumeration of *Enterobacteria* was performed on VRBG agar (violet red bile glucose) and incubation was carried out at 37 and 42°C for 48 h. Contaminated microflora such as coliforms was preliminary clustered on the fermentation of lactose with gas production on BLBVB medium (broth lactose bile green) at 37 and 42°C for 24 h. After purification of coliforms on ordinary nutrient agar, a morphological study was conducted and *Enterobacteria* and coliforms were identified using API 20 E strips (Biomérieux).

Enumeration of mesophilic and thermophilic aerobic and anaerobic bacteria such as *Bacillus* and *Clostridium* was performed by plating on appropriate selective agar media (nutrient agar and TSN for the *Bacillus* and *Clostridium* respectively). After heat treatment at 80°C for 10 min, Incubation was conducted at 37 and 42°C for 24 to 48 h. After purification of *Bacillus* strains, a morphological and biochemical study was conducted. The gram-negative, aerobic and catalase + bacteria were identified by API 50 CHB strips (Biomerieux).

Pseudomonas, belonging to the family of the non-enterobacteria, was isolated on Cetrimide selective medium for 48 to 72 h at 30°C and was identified by API 20 NE strips (Biomerieux). *Micrococcaceae,* such as *Staphylococci* counts, were determined by using Chapman medium; after incubation at 37°C for 48 h, isolates were identified by ID 32 Staph API tests (Biomerieux).

Enumeration of mesophilic aerobic bacteria (MAB) was performed on PCA agar (Plat Count Agar) at 30°C for 48 to 72 h. For the purpose of this study, sampling and analysis were done in duplicate and the results presented, are in the range of the found values.

Statistical distribution of the microflora of the milk of collection centres

The statistical analysis of microflora milk in different centres was conducted by averages, determination of variances and gaps type using the ANOVA test: DATASET1.ISD by GraphPad in stats demo version 3.0 Software. Differences were considered statistically significant threshold $p < 0.05$.

Values of microbiological references

Qualitative assessment of raw Milk collection was conducted by a microbiological reference method. Reference values have been established to determine the percentage of 80 samples based on log cfu/ml. These values were compared to the usual normative values required for milk, and believed to determine compliance or none of Tunisian raw milk to the usual standards required.

Tables 2. Microbiological profile of Tunisian collected milk.

Centres	A	B	C	D	E	F	G	H	Standards
MAB	$5\ 10^7$	$6\ 10^8$	10^8	$2\ 10^8$	$6\ 10^7$	310^8	$2\ 10^8$	10^{10}	$5\ 10^5$
Enterobacteria	$4\ 10^6$	$3\ 10^7$	$5\ 10^6$	$6\ 10^8$	10^7	10^8	$6\ 10^7$	$3\ 10^8$	10^3
Total coliform	$3\ 10^5$	$2\ 10^5$	$4\ 10^6$	10^7	$4\ 10^5$	10^6	$3\ 10^7$	$6\ 10^7$	10^3
Fecal coliform	$3\ 10^5$	$4\ 10^5$	$6\ 10^5$	$3\ 10^7$	10^6	610^6	$2\ 10^7$	$3\ 10^7$	10
Staphylococci	$2\ 10^4$	10^5	10^4	$6\ 10^4$	$3\ 10^5$	10^5	$4\ 10^6$	$3\ 10^5$	10
pseudomonas	$2\ 10^4$	$3\ 10^6$	$7\ 10^7$	10^7	$2\ 10^7$	210^6	10^6	10^7	10^3
Mesophilic *Bacillus*	$3\ 10^4$	$2\ 10^4$	$6\ 10^4$	410^5	$6\ 10^4$	$3\ 10^3$	310^3	$4\ 10^4$	Absence
Thermophilic *Bacillus*	$5\ 10^2$	$6\ 10^3$	$2\ 10^4$	310^3	Abs	410^3	610^3	10	Absence
Lactic acid bacteria	$7\ 10^6$	$4\ 10^8$	$2\ 10^8$	$5\ 10^8$	$2\ 10^7$	10^9	$3\ 10^6$	$3\ 10^8$	10^6
Yeasts and fungi	$6\ 10^6$	$2\ 10^7$	310^7	$8\ 10^5$	$2\ 10^6$	10^6	$4\ 10^7$	$6\ 10^7$	10^3
Compliance	None	None	None	None	None	None	None	None	

16S rDNA amplification and sequencing of LAB and *Bacillus*

Genomic DNA from MRS and GN agar cultures were extracted by phenol extraction method as reported by Vaquero et al. (2004) where PCR-mediated amplification of the complete 16S rDNA was carried out in a Gradient Master Thermocycler (Bio Rad). All reagents, if not indicated otherwise, were purchased from Biogène, United Kingdom. The amplification conditions were as follows: 1 µl genomic DNA, 20 Mm reaction buffer, 200 µm each of the four deoxynucleotides, 1 U Taq polymerase, 25 mM of each primer (Biogène):

F: S-D-Bact-0008-a-S-20 AGAGTTTGATCCTGGCTCAG,
R: S-D-Bact-1495-a-S-20 CTACGGCTACCTTGTTACGA

to a final volume of 25 µl. The PCR conditions were: (94°C/4 min) 1×, (94°C/45 s, 55°C/1 min, 72°C/2 min) 30×, (72°C/7 min) 1×.

Analysis of PCR product

The PCR products were electrophoresed on 1.5% agarose gel and visualized, followed by BET staining. PCR product was also sequenced in order to confirm the sequence and then the results from PCR cross-section analysis were compared with the available cross-sections by using Blast software.

RESULTS AND DISCUSSION

Enumeration of different genera

In this study, the microflora of eighty samples of raw milk collection was studied. The samples were examined to achieve a distribution microflora of the milk in time and try to determine the origin of the contamination. The microbiological analysis focused on the number of mesophilic aerobic bacteria, *Enterobacteria,* total and faecal coliforms, lactic acid bacteria, yeasts and fungi, *Pseudomonas*, *Staphylococcus, Bacillus and Clostridium*. The results of counting microflora milk in 8 centres of collection, showing the noncompliance of milk collected in the usual values required by the standards of raw milk collection (Tunisian standards 02- 1982 and AFNOR: 1997 release days June 2001) is shown in Table 2.

The analysis of the evolution of the average rate of the seeds of 8 collection centres (Figure 1), led to sizeable rates of mesophilic aerobic bacteria (10^{10} cfu/ml) which is far in excess, relative to the required standards ($5\ 10^5$ cfu/ml). In addition, we have observed a fairly low variance of thermo resistant spores (10^3 to 10^4 cfu/ml), but it remains away from standards, requiring the absence of spores, because they have sugar and protein breakdown, thus, constituting a cause of milk corruption because of their resistance to pasteurization, and possibly to sterilization; thanks to their spores. Milk collection also contains lactic acid bacteria, *Enterobacteria*, yeasts and fungi with an average of 10^6 to 10^7 cfu/ml ($p > 0.05$). The most dominant microorganisms in 8 collection centres are lactic bacteria and coliform with significantly higher rates (10^6 to 10^8 cfu/ ml) and our results were similar to those reported by Makovec et al. (2003).

Enterobacteria consists mainly of coliforms which have very high rates (10^6 cfu/ ml), exceeding the standards required (10^3 cfu/ml) and they may be indirect consequence of unsafe state hall of trafficking and especially, cattle (teats) hardware not satisfying the required standards of dairy.

The evolution graph of germs (Figure 1) also shows psychrophilic, such as *Pseudomonas* bacteria, which have a high rate of 10^7 cfu/ml, exceeding the required standard (10^3 cfu/ml). Actually, at a dose of 10^4 cfu/ml, the multiplication of the *Pseudomonas* is accompanied by a significant metabolic activity (Smith, 2003) and also among the lipolytique and/or proteolytique activity responsible for defects and deterioration of unpleasant milk flavours (Richard and Gaillard-Martinie, 1992). It was also reported by Bloquel et al. (1980) that after storage at 4°C during 4 days, a bacterial selection follows, which favours psychrophilic gram negative bacteria initially present and a significant proliferation was shown in milk. In addition, the non-refrigerated tanks of the merchants and the long distances separating the

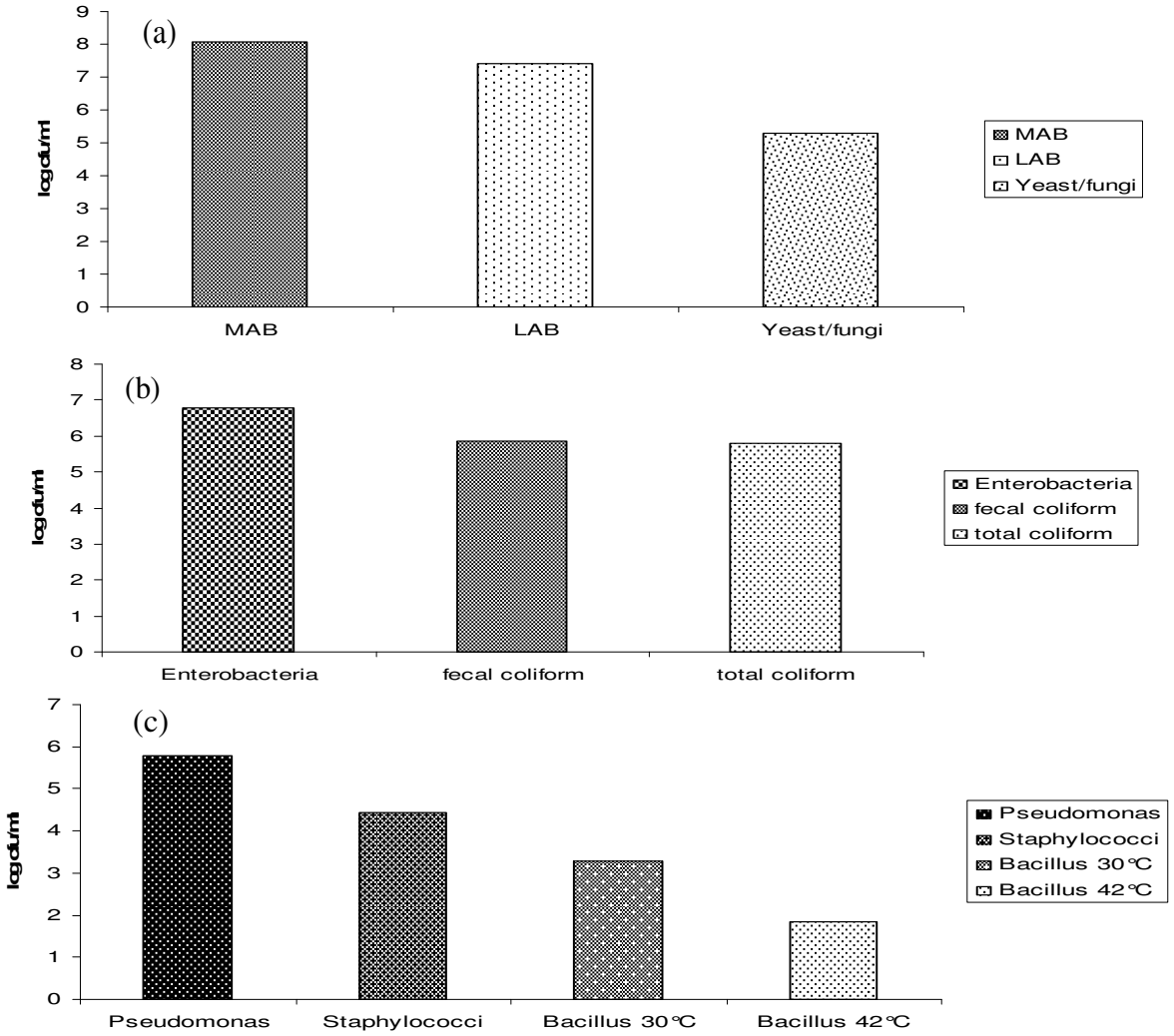

Figure 1. Microflora evolution in collection centres; (a); mesophilic aerobic bacteria (MAB), lactic acid bacteria (LAB). yeasts and fungi; (b); enterobacteria, total and fecal coliform; (c); *Pseudomonas, Staphylococci,* mesophilic *Bacillus* and thermophilic *Bacillus*.

collection centre farms are likely to vary the temperature of the milk (4 to 12°C) which determines the increase of its acidity and lowering the pH, thus provoking lactic flora and the psychrophilic growth (Mafart, 1996; Michel, 2001; Perez et al., 1999).

Staphylococci occur at very high rates (10^5 cfu /ml), far in excess of the standard one (10 cfu/ml). But, given its habitat (teats) and its frequent question in the mastitis, the presence of *staphylococci* in milk appears almost inevitable (Soussy, 2005). In addition, their ability to colonize the skin and the environment is certainly due to their ability to form biofilm (Planchon et al., 2006). On the other hand, the presence of thermophilic or mesophilic *Clostridium* has not been detected.

The evolution of the microflora of the milk is fairly uniform and grows in the same direction and virtually equivalent thresholds in the reviewed collection centres. Nevertheless, the presence of the thermophilic and the psychrophilc bacteria represents a sufficiently serious threat to Tunisian quality raw milk collection (Michel, 2001). Gram positive bacteria (lactic acid bacteria, staphylococci and *Bacillus*) dominate in many samples. In most samples, rates of *Enterobacteria*, total and fecal coliforms are significantly increasing above the threshold of 10^6 cfu/ ml. *Pseudomonas* counts were highly variable. These results are in agreement with those obtained in previous studies (Bloquel et al., 1980; Esmasures et al., 1997). These results can be explained by the alternation of soda/acid cleaning cycles of milking machines, refrigeration machines, problems of storing silage, etc. (Michel, 2001). Indeed, neglecting hygiene, especially in teats, can bring high contamination of milk (between 10^4 and 10^5 cfu /ml) for mesophilic aerobic bacteria, psychrophilic, *Clostridium tyrobutyricum* and *Bacillus stearothermophilus* and generally, limited coliform microflora (Luquet, 1985).

Table 3. Species identification of collected milk.

	Mesophilic microflora	Thermophilic	Psychrophilic
GRAM +ve			
Lactic acid bacteria			
Lactococcus lactis lactis	30		
Lactobacillus lactis	10		
Lactobacillus paracasei	15		
Bacillus cereus	10	5	
Bacillus stearothermophilus	3	5	2
Bacillus licheniformis	10	5	
Staphylococci			
Staphylococcus xylosus	10		
GRAM -ve			
coliforms	40		
Enterobacter sakazaki	30		
Serratia liquefaciens	5		
Enterobacter cloacae	5		
Pseudomonas			
Chryseomonas luteola	5		
Pseudomonas fluoresens	25		
Psudomonas putida	15		
Yeasts			
Candida zylanoides	20		
Candida albicans	20		
Saccharomyces cerevisiae	10		
Fungi			
Geotrichum capilatum	10		
Alternaria	5		

Identification of different species

Biochemical identification of collection milk microflora was performed by API strips which helped to characterize different species of milk in collection centres (Table 3). This method confirmed that, 80% of these isolates belonged to the genus and species identified by the physiological and biochemical tests. Results are presented in percentage (%) compared to the bacteria isolated for each species.

In this study, our results indicate the predominance of the lactic acid bacteria compared to the total microflora. The results were in agreement with those of other workers, undertaken on the enumeration and isolation of the lactic acid bacteria from fermented milks. According to Beukes et al. (2001) and Savadogo et al. (2004), the number of lactic bacteria largely exceeds that of the other microflora of traditional fermented milk in South Africa and in Burkina Faso, respectively. The high rate of the lactic acid bacteria can be explained by the selectivity of media used, MRS, M17 and Rogosa, for this type of bacteria (Reuter, 1985). Thus, the results revealed the presence of diversity in the lactic microflora isolated from goat's milk. This can be related to several factors. First of all, these species are frequently isolated from the animals, such as bovines, sheep and caprines. The environment and the climate can play a very great role as indicated by Picque et al. (1992) and Remeuf (1992).

Among the identified lactic microflora in Eighty Samples of milk collection, *Lactococcus lactis* spp *lactis*, appears

Table 4. Values of microbiological references of Tunisian collected milk.

GERMS	Minimale values (m)	Maximale values (M)	Standards (g/ml)
MAB	10^5	10^{11}	510^5
Lactic acid bacteria	10^4	10^{10}	10^6
Enterobacteria	10^3	10^9	10^4
Total/ fecal coliforms	10^3	10^8	10^3
Staphylococci	10	10^5	10
Pseudomonas	10^3	10^7	10^2
Mesophilic *Bacillus*	10	10^4	Absence
Thermophilic *Bacillus*	10	10^3	Absence
Yeast/ Funghi	10	10^6	10^3

dominant in collect milk (40%). These results were in concordance with the results described by Moreno and Busani (1990) who found that, *L. lactis subsp. lactis*, was more frequently isolated from raw milk samples.

The yeasts and fungi are also present in all samples. *Candida albicans* and *Geotrichum capilatum* were found in 50 samples. Samples contain more than 10^6 coliforms/ml of fecal origin, especially *Enterobacter*, in 40 samples. *Micrococacceae* (*Staphylococcus xylosus*) also represents a microbial predominant group and exceeding the threshold of 10^5 cfu after total flora, lactic acid bacteria and *Enterobacteria*. In one third of the samples, maximum threshold of *Pseudomonas* (100 cfu/ml) of milk products contain up to 10^5 cfu/ ml and in dominant species, psychrophilic are *Pseudomonas Putida* and *Chryseomonas luteola.* These results were in concordance with the results described by Esmasures (1997) as part of the study of microbiological composition of milk. In addition, mesophilic and thermophilic *Bacillus* were present in all samples especially *Bacillus stearothermophilus and B. cereus*.

Values of microbiological references of Tunisian collected milk

Reference values have been established to determine the percentage of samples based on log cfu/ ml. These values were compared to the usual standards values required for raw count /ml (Tunisian standards and AFNOR standards) milk (Table 4). Microbiological characterisation of 80 milk samples showed that the most dominant species were the lactic acid bacteria (Kacem et al., 2002), the mesophilic aerobic microflora and the *Enterobacteria* at significantly higher rates reaching 10^7 in 10^{10} cfu/ml. Starting reference values could conclude that, original flora varies between 10 to 10^{11} cfu/ml, contaminating microflora, reaching the 10^9 cfu/ml and *Bacillus* spores were present at a very high order of 10^4 cfu/ml rates. It can be said that the application of the rules of hygiene is a necessary measure to ensure the safety of the product. In case of failure, major conformity foodstuffs may occur and this will cause an irreversible alteration of raw milk and milk products because we're far away from the quality of Europeans raw milk (Esmasures et al., 1997).

Molecular characterisation of LAB and *Bacillus*

Bacteriocin production, sugar fermentation, followed by a reduction in pH due to the production of lactic and other organic acids of lactic acid bacteria was an important factor for the inhibition growth of undesired micro-organisms. Thus, molecular identification was realised in the purpose of studied LAB/*Bacillus* interaction in the biofilm of pipelines of milk industry in the current study. These different isolates were identified by 16S rDNA sequences and were deposited in the NCBI nucleotide sequence databases as shown in (Table 5).

Among the identified lactic microflora, Lactoccocci appears dominant in the goat's milk with high rate of *L. lactis* subsp*. lactis*. In other works, it was found that *L. lactis* subsp*. lactis* is more frequently isolated from raw milk samples (Moreno and Busani, 1990), of cheeses manufactured, containing milk (Centeno et al., 1996). In addition, Crow et al. (1993) and Weerkamp et al. (1996) affirmed that the *Lactococci* isolated from natural sources were often identified as *Lc. lactis* subsp. *Lactis.* Concerning *Bacillus* characterisation, *Bacillus cereus* represents a significant part among our isolates. This specie was pathogenic and frequently isolated from soil, raw milk and dairy products (Table 5).

Conclusion

This study showed the high evolution of the microflora of the milk which is fairly uniform and grows in the same direction and equivalent thresholds for different collection centres. Mesophilic aerobic bacteria are the most dominant. It is followed by lactic acid bacteria. Often, the counts of coliforms exceeded the guidelines; almost 90% of samples exceeded 10 cfu/ml. The presence of coliforms confirms the lack of hygienic practices such as milk trafficking, milking equipment, tank collections, milk

Table 5. Species identification by sequencing of the 16s rDNA.

Isolate	Species	Similarities (%)
Lb1	*Lactobacillus lactis*	99
Lb2	*Lactobacillus lactis*	99
Lb3	*Lactococcus lactis* spp *lactis*	98
B1	*Bacillus cereus*	100
B2	*Bacillus licheniformis*	98
B3	*Bacillus stearothermophilus*	96
B5	*Bacillus cereus*	100
B20	*Bacillus weihenstephanensis*	98

spaces and the proper personnel and animal hygiene. Indeed, our visits to different collection centres, we found the misapplication of good hygiene, despite efforts to the smooth running of milk collection, cleaning and automatic disinfection protocol implemented. This observation can explain the high contamination of milk collection. Therefore, we may suggest that staff training is essential to improve the collection at farm-level and hygiene in collection centres. This suggests the implementation of quality control measures and the application of the hygiene rules so as to produce dairy product, such as raw milk, of good standards, and quality microbiological, from collection to packaging. These results, arising from microbial enumerations were important and helpful for understanding relationships between hygienic and organoleptic quality of milk production. So, management commitment, proper personal, animal, surfaces and process hygiene and equipments appear to be the primary issues to the addressed in order to curb undesirable contamination of the milk product. Therefore, in order to prevent the spread of infection in animal and human populations, the aforementioned should be instituted. Although, governmental regulation of milk pasteurization and sanitation in dairy processing plants has been established in Tunisia for many years, direct sale of unpasteurized milk and dairy products from producers to the consumer is not uncommon in many regions. In fact, the consumption of fresh, unpasteurized milk from goat is a traditional practice in some rural areas. The present results also suggest that, testing bulk tank milk as an easy and inexpensive method that could be used to assess the efficiency of control schemes aimed at controlling and/or preventing *Bacillus* infection in dairy herds. Further work is now required to characterize the epidemiology of the infection, more thoroughly (Rahimi, 2010).

The antagonistic activity exhibited by different *Lactobacillus* and *Lactococcus* strains was further evaluated by the well-diffusion method (Ebrahimi 2010). Based on these results, it is most likely that antagonistic activity is caused by production of organic acids and reduction of pH; although, inactivation of a bacteriocin during neutralization cannot be ruled out. It is also possible that antimicrobial substances are membrane associated. Similar observations have been reported, highlighting the importance of identification by molecular methods (Tannock et al., 1999).

There is still little information about the strain composition of the natural microbial population colonising the collect milk in Tunisia. This is a preliminary study and further investigation into strain characterisation of biofilm in the industrial lactoducs and the antagonist interaction within species, essentially *LAB/ Bacillus*.

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