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Molecular Appreciation of the Native Bacterial Flora of Raw Milk in the North-Western Region of Algeria

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ABSTRACT

Background: The appreciation of microbial milk communities, in order to promote useful flora and inhibit pathogenic one, has become one of the key factors in controlling the quality of cheeses.

Methods: In this context, an approach to assess the bacterial flora of raw cow's milk, was tested in 05 wilayas in north-western Algeria, on the one hand by a preliminary count of total flora and a phenotypic characterization based on morphological criteria and on the other hand by a genotypic identification of selected isolates, by the PCR method and sequencing of the gene encoding for the 16S ARNr.

Result: The results of molecular analysis obtained from 72 isolates allowed the identification of 12 bacterial species with a dominance of the lactic microflora, 37% *Lactococcus* and 29% *Enterococcus*. This milk-native lactic microflora has become the main vector that preserves the distinctiveness of dairy products, including local cheese.

Key words: Appreciation, Genotypic identification, Lactic microflora, Microbial milk communities.

INTRODUCTION

The production of cow's milk is often confronted with the problem of quality management which penalises both producers and processors (Jadhav *et al.*, 2008). The hygiene conditions at the farm level, the maintenance of the cold chain throughout the production process until the arrival of milk at the dairy, contain as many sources of contamination to be mastered in order to preserve the hygienic quality of milk (Aggad *et al.*, 2009; Faye and Loiseau, 2002; Ghazi and Niar, 2011; Labioui, 2009; Mennane *et al.*, 2007 and Srairi *et al.*, 2005).

Processors, in their quality approach, are convinced of the need to combine their producer and collector partners with the application of good production practices in order to improve the quality of the raw milk received. The importance of the concept of native quality of the raw material, in this case natural milk, is considerable in the development of typical products from each region (Montel *et al.*, 2014 and Vignola and Amiot, 2002).

However, these aspects of improving the quality of raw cow's milk, through the preservation of the original bacterial flora, have not been previously studied by prospective studies. The specificities of the context of cattle breeding with these practices in Algeria have imposed the management of this study with a molecular appreciation of the natural bacterial flora to raw milk of the north-western region of Algeria and the characterization of the autochthonous lactic microflora.

MATERIALS AND METHODS

The choice of regions

The regions studied are home to large dairy plants supplying the cheese processing units (Fig 1).

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Taking milk samples

Random sampling was carried out during the high lactation period (March 1, 2019 to May 30, 2019). In the wilayas of Mascara, Mostaganem, Oran, Relizane and Sidi Belabbes**.** Eighty-four samples of raw milk (at a rate of 7 samples per week per wilaya) were collected aseptically in sterile containers and stored at low temperature until the start of experimental analyses in the laboratory.

Microbiological analyses

Bacterial counting and isolation

The count was made after a series of decimal dilutions by transferring a volume of 1 ml of milk directly to 9 ml of sterile physiological water for dilution 10^{-1}; the latter is used to achieve a series of dilutions up to 10^{-7} (Gusils *et al.*, 2010). 100 µl of each dilution was spread on Petri dishes containing the solid isolation medium for incubation at 37°C for 72 hours.

Culture and isolation media used

The medium PCA milk was used to determine the total native flora of milk: Lactose: 5g, skimmed milk: 5g, Meat Peptone: 3g, Meat extract: 3g, yeast extract: 3g, Agar: 1g, Precipitated calcium carbonate: 15g, Neutral red aqueous solution: 5ml and 1000 ml distilled water, pH -6.8, autoclaved at 100°C for 20 min. This medium characterizes their presence; allows better recognition of the round white and lenticular colonies of native milk bacteria.

Petri's dishes are inoculated in the mass and incubated in an oven at 37°C for 48 hours (Labioui *et al.*, 2009). Enumeration of the various bacterial groups is carried out using a microbial colony counter (New Brunswick scientific CO Model C-100 6327). The results are expressed in cfu numbers per ml.

Isolation and purification

After counting, the colonies apparently characteristic of bacterial groups are taken for morphological study. Bacteria characterized in the form of isolated shells, diplococcis or shells associated with chains, gram-positive, negative catalase; without releasing oxygen when dissociated in a drop of oxygenated water, are retained as lactic strains (Gusils *et al.*, 2010).

Genotypic characterization of bacteria of technological interest

The identification of bacteria of technological interest by conventional methods does not reliably identify purified isolates (Reats *et al.*, 2011). We used, at the laboratory level; a tool based on molecular biology by amplification of 16 S ribosomal DNA with standard universal primers of prokaryotes and specific to lactic bacteria, a critical analysis of the sequence obtained and a positive control of the reference strains identified namely lactic enterococci and lactococci (*Enterococcus faecalis* ATCC 14506, *Enterococcus durans* ATCC 6056, *Lactococcus graviea* ATCC 49156 and *Lactococcus lactis* ATCC 49032).

RESULTS AND DISCUSSION

Microbiological analyses

The results of microbiological analyses are as follows:

Bacterial count

Bacterial flora was enumerated in all raw milk samples studied (Table 1). Their number varies as follows: for the month of March from 1.8 10^6 to 2.13 10^7 cfu/ml for total flora and between 7.2 10^4 to 8 10^5 cfu/ml for lactic flora, for the month of April from 1.28 10^6 to 2.9 10^6 cfu/ml for total flora and between 3.25 10^4 to 8.42 10^4 cfu/ml for lactic flora, for the month of May from 0.7 10^6 to 2.1 10^6 cfu/ml for total flora and between 2 10^4 to 7.2 10^4 cfu/ml for lactic flora. This difference in counts in the samples analysed is the result of variability in the microbial dairy ecosystem within the dairy herd, farming practices and means of milk production. The number is determined by the quality of sampling in relation to the animal breed of the same farm as already described

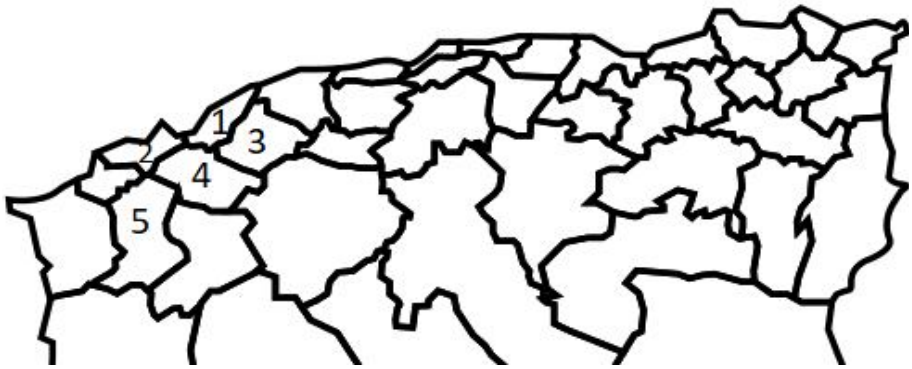

Fig 1: Sampling areas.

Table 1: Monthly average of bacterial count obtained by wilaya of the north-western region of Algeria: cfu/ml unit.

		05 wilayas studied in the north-western region of Algeria				
		Wilaya Mascara	Wilaya Mostaganem	Wilaya Oran	Wilaya Relizane	Wilaya Sidi-Belabbes
Month of March	Total flora	2.13 10^7	1.8 10^6	4.1 10^7	2.18 10^7	2.02 10^6
	Lactic flora	3 10^5	8 10^5	7.2 10^4	6.13 10^5	4.05 10^5
Month of April	Total flora	1.28 10^6	1.15 10^6	2.9 10^6	1.72 10^6	1.4 10^6
	Lactic flora	5.15 10^4	4.72 10^4	3.25 10^4	8.42 10^4	7.1 10^4
Month of May	Total flora	1.1 10^6	0.7 10^6	2.1 10^6	1.25 10^6	0.87 10^6
	Lactic flora	4 10^4	6 10^4	2 10^4	7.2 10^4	5.85 10^4

in the same context by Aggad *et al.*, 2009, Ghazi and Niar, 2011, Labioui *et al.*, 2009, Sekkar *et al.*, 2010 and Srairi *et al.*, 2005.

Isolation and purification

In this work, we focused only on lactic bacteria of applied interest. The dominance of lactic bacteria for raw milks was as follows: 72 isolates were selected for characterization by physiological and biochemical tests that showed that all isolates were found to be positive gram-positive and negative catalase which is characteristic of lactic bacteria (Labioui *et al.*, 2009).

Morphological characterization of lactic bacteria

The 72 isolates were purified on the PCA agar medium, the bacterial cells appeared small in size, round, with regular rims and whitish color. On broth, the strains have a homogeneous disorder that characterizes the group of lactic bacteria. Microscopic observation revealed a cell shape which is the shell shape. These bacterial cells have a micromorphology either in isolated diplococci or in chains (Table 2). This has been reported by numerous studies including Gusils *et al.*, 2008 and Montel *et al.*, 2014.

Molecular analyses

PCR Results

Molecular analysis involved 72 preserved isolates. After revitalization, colonies were analyzed by PCR according to the protocol defined by Reats *et al.,* 2011. The results of the chain amplification reaction, were verified on 3% agarose gel in the presence of a coloured reagent (Midori Green) to detect positive amplification results (Fig 2).

Sequencing results

The isolates that tested positive after PCR were sent to sequencing. Sequencing results show 45 identifiable strains. Analysis on BLAST allowed us to identify 41 strains. The identification results are shown in (Table 3).

Molecular analysis results from 72 isolates identified 12 bacterial species from 7 different genera varieties, with *lactococcus* dominance 37% followed by *Enterococcus* 29%, *Klebsiella* 15%, *Enterobacter* 12%, *Proterobacteria* 3% , *Citrobacter* 2% and *Chryseobacterium* 2% (Fig 3). It appears that 66% of the isolated strains are identified as lactic bacteria with 37% of lactococci representing the species *Lactococcus garvieae* and *Lactococcus lactis* as well as 29% of lactic enterococci with *Enterococcus faecalis* and *Enterococcus durans.* The remaining, 34% are contaminating bacteria, some of which have also been found by other researchers on raw milk samples, such as *Citrobacter, Klebsiella* (Faye *et al.*, 2002), *Enterobacter* (Aggad *et al.*, 2009 and Labioui *et al.,* 2009), *Stenotrophomonas rhizophila* (Nuwan *et al.,* 2014). On the other hand, the bibliography does not fall under work citing *Chryseobacterium* on raw milk.

Although the results confirm the microbiological profile of cow's milk established by (Montel *et al.,* 2012), the comparison between phenotypic and genotypic identification suggests differences in appreciation (Ouadghiri, 2009), since 33% of the initial strains presumed to be lactic are represented by contaminant bacteria, mainly coliforms. This clearly shows the limits of conventional identification methods (Bousbia et *al.*, 2018).

Table 2: Morphological criteria for identifying presumed genus of lactic strains.

Macro-morphologie	Micro-morphologie	Temperature °C	Groups
Round white or lenticular colonies	Diplococical and chained hulls	37	Enterococci Lactococci and Lactic Streptococci

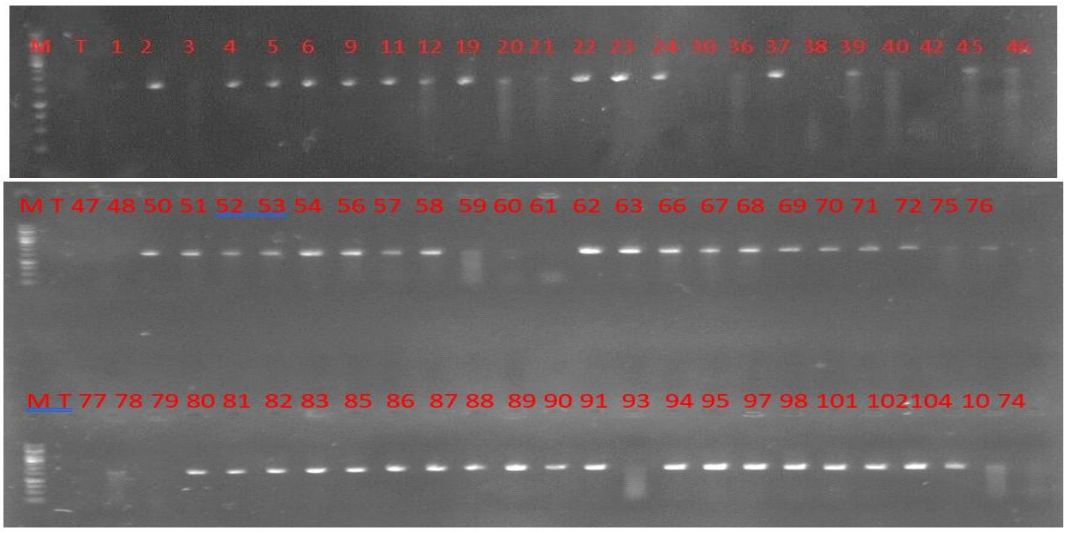

Fig 2: Results of amplification reaction on 3% agarose gel.

Table 3: BLAST Isolate Identification Results.

Isolate code	Kind	Species
63	*Citrobacter*	*Citrobacter freundii*
50-37-38-71	*Enterobacter*	*Enterobacter xiangfangensis*
19		*Lelliottia amnigena*
5-24-30-97	*Klebsiella*	*Klebsiella oxytoca*
9		*Klebsiella pneumonia*
101		*Raoultella ornithinolytica*
69-70-81-82-85-86-87-88-90-91	*Enterococcus*	*Enterococcus faecalis*
72-104		*Enterococcus durans*
98	*Chryseobacterium*	*Chryseobacterium sediminis*
80-83-95	*Lactococcus*	*Lactococcus garvieae*
1-2-4-6-11-22-23-24-62-90-94-102		*Lactococcus lactis*
93	*Proteobacteria*	*Stenotrophomonas rhizophila*

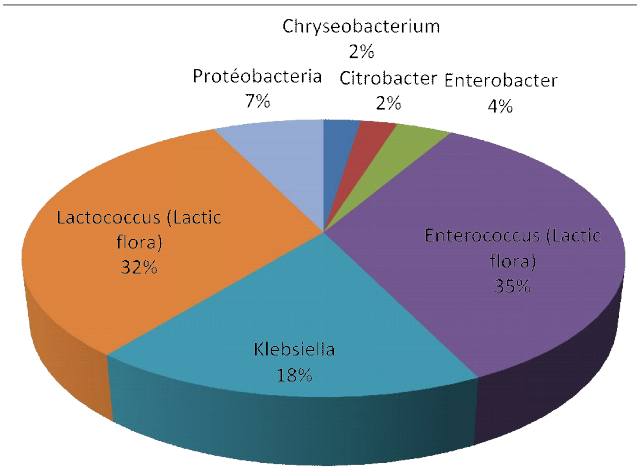

Fig 3: Percentage of genders identified in raw milk samples.

All the undesirable germs identified are indicators of faecal contamination and a lack of hygiene at the level of livestock farms and more specifically during milking of cows.

The variety of the native bacterial flora comes mainly from the composition of the herds in relation to the wilayas studied. It is the practices of dairy farmers by their methods, their habits, their empirical know-how that promote the presence of bacterial flora of interest to be identified before any milk transformation essential to improve the quality of the dairy products manufactured.

Indeed, according to studies initiated with the same perspective, the most commonly characterized enterococci in raw milks are *Enterococcus faecium*, *Enterococcus faecalis* and *Enterococcus durans* (Labioui *et al.*, 2009 and Montel *et al.*, 2012), as well as in cheeses made from raw milk, from goats, sheep or cows; *Enterococcus casseliflavus* is less often found (Burdychova and Komprda, 2007). Enterococci play an important role in the ripening of several varieties of cheese, probably due to their proteolytic, lipolytic activity, diacetyl production capacity and other volatile components contributing to the aroma, pleasant flavor and characteristic taste (Sakore *et al.*, 2007).

In addition, the main habitat of lactococci is formed by various niches of the environment of dairy and cheese production. They are found in large quantities in fermented milks or in cheeses made from raw milks and at temperatures favourable to their development (Ouadghiri, 2009). With their enzymatic equipment, they participate in the proteolysis of casein in amino acids precursors of many aromatic molecules. Some are studied for their biopreservative capacities, which are their ability to inhibit pathogenic contaminants in food. For example, like those identified in our milk samples, *Lactococcus lactis* and *Lactococcus garvieae* which according to the research of Montel *et al.*, 2014, can inhibit growth but also strongly affect the metabolism and virulence of *Staphylococcus aureus*.

Understanding the influence of farming and dairy production practices is crucial to preserving the integrity of bacterial biodiversity. On the other hand, mastering hygiene practices will reduce the level of undesirable microorganisms in raw milks to avoid any health risks for milk and by-products. Several authors (Ouadghiri, 2009, Montel *et al.*, 2014 and Sakore *et al.*, 2007) have confirmed that any decrease in the total flora of milks is accompanied by a reduction in microorganisms useful for the development of the organoleptic characteristics of derived products, including cheeses.

The results of this preliminary study are only one step between researchers, producers and processors in order to assess the path of the natural microflora of milk from the farm to the human microbiota by passing through the cows and finally the hands of the cheese processor.

CONCLUSION

The current state of knowledge shows that the preservation of the technological flora native to raw milks requires the establishment of a good practice protocol, breeding and dairy production. These actions depend upon a common purpose between dairy companies and producers to validate necessary incentives with regulatory obligations to improve the hygienic quality of raw milks and to preserve the rich heritage of this region in native bacterial flora giving a distinctiveness to dairy products and more specifically to

local cheeses. The perspective of our research team at the laboratory level is to build a microbiological control and identification plan at the dairy cow milking stage in order to maintain microbial reservoirs especially the flora of the teats and on the other hand to safeguard the ecosystem in favor of native bacterial flora of interest.

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