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Microbiological testing quality of raw and fermented milk of Moroccan cows and camels and isolation of antagonistic lactic acid Bacteria

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Abstract

Samples of raw and traditional fermented milk ("lben") of both camels and cows collected from different zones of southern Morocco were analyzed to evaluate their microbiological quality and to isolate lactic acid bacteria (LAB) producing bacteriocin-like inhibitory substances (BLIS). The following groups of microorganisms were investigated: aerobic mesophilic flora, coliform, fecal coliform, casein-degrading germs, staphylococci, lactic acid bacteria, yeasts and fungi. The analyzed samples were generally highly contaminated and the microbial counts were clearly varying among samples. A total of 1000 strains randomly isolated from different samples were screened for the production of BLIS. 55 LAB isolates were shown to be active against at least one of the indicator strains: *Bacillus subtilis*, *Staphylococcus aureus* and *E. coli*. Among the active isolates, two strains called Q37 and Q310 were selected for their inhibitory activity, heat stability and pH acid resistance. They were identified as *Streptococcus thermophilus*, throughout API 20Strep and API 50CH tests. Both active strains lack haemolytic activity and were sensitive to most of the antibiotics tested. These characteristics insure their safety aspect and their potential application in fermented food as starter and/or protective cultures.

Keywords: Microbiological quality, cow's milk and "lben", camel's milk and "lben", lactic acid bacteria, BLIS, *Streptococcus thermophilus*.

Introduction

The milk of different ruminant species, either directly or as derivative dairy products, constitutes a particularly important food for humans throughout their lives. This food can be considered as a source of macro- and micronutrients, further containing a number of active compounds that play important nutritional and protective roles (Tamime, 2006). Either for direct consumption or as by-products, cow's milk is the most used throughout the world, especially because of its greater availability, while the production of camel milk is very limited (FAO, 2013). Therefore, studies undertaken on camel milk, which constitute a key food in arid and sub-arid regions, are limited (Benkerroum *et al*., 2003). In rural areas, milk is produced in poor sanitary conditions and consumed in its raw state, and then, this milk can be a vehicle of many pathogens, as *Staphylococcus aureus* (Rahimi *et al*., 2013), *Salmonella*, *Escherichia coli* including *E. coli O157:H7, Listeria monocytogenes* (Singh & Prakash, 2008; Osman *et al*., 2014), *Brucella* (Zowghi *et al*., 20008) and some enterotoxins-producing spores (El-ziney & Al-turki, 2007). Lactic acid bacteria (LAB) have traditionally been associated with dairy products, especially fermented milks (Carr *et al*., 2002; Benkerroum & Tamime, 2004; Lore *et al*., 2005), and are generally considered beneficial microorganisms and some strains even as probiotics (Lahtinen *et al*., 2012). Thus, LAB becomes a focus of increasing international interest with a

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view to apply in food systems (Achemchem *et al*., 2006; Ananou *et al*., 2010). However, some strains are considered as potential pathogens (Franz *et al*., 2011). A thorough understanding of the taxonomy and characteristics of LAB is thus necessary to fully utilize the technological, nutritional and health-promoting aspects of LAB while avoiding health risks (Lahtinen *et al*., 2012).

Numerous researchers demonstrated that LAB are able to inhibit not only Gram positive bacteria (Achemchem *et al*., 2005, 2012; Khay *et al*., 2011), but also Gram negative bacteria (Maqueda *et al*., 2004; Yateem *et al*., 2008; Jrad *et al*., 2013) and some fungi as *Penicillium expansum*, *Mucor plumbeus*, *Kluyveromyces lactis* and *Pichia anomala* (Delavenne *et al*., 2012). The source of lactic acid bacteria may have an impact on their characteristics. In camel milk, thermophilic species are highly represented in comparison with cow's milk (Khedid *et al*., 2009; Merzouk *et al*., 2013). Additionally, 6.5% salt resistant *Lactococcus lactis* strain was isolated from camel milk (Zadi-Karam & Karam, 2006). This can be interpreted as an adaptation to the natural environment of these bacteria.

In addition, (Jrad *et al*., 2013) demonstrated that lactic acid bacteria isolated from camel milk have the strongest acidifying abilities in comparison with bovine bacterial strains which give their antimicrobial activity an important technological property. The present study aimed to assess the hygienic quality of some samples of Moroccan milk and fermented milk of both camels and cows collected from the South of the country and to isolate the bacteriocin-like inhibitory substances producing LAB.

Materials and methods

Collection of milk and fermented milk samples

Twenty samples of raw (n=10) and fermented (n=10) milk from cows and camels were collected from three different locations in the south of Morocco by manual milking as normally practiced by the farmers/nomads for raw milk. Cow's fermented milk samples were collected from traditional dairies and camel's ones were provided by nomads of Moroccan Sahara. Samples were collected in aseptic conditions and transported at 6°C immediately to the laboratory for pH and microbial count analysis.

Determination of microorganisms counts

Mesophilic aerobic bacteria (MAB), Coliform bacteria at 30°C (Total coliform bacteria -CBt-) and at 44.5°C (Fecal coliform bacteria -CBf-), Staphylococcus aureus (SA), yeasts and microscopic filamentous fungi (Y+MF) were enumerated using aerobic cultivation method as described by (Juhaniaková *et al*., 2014). Lactic acid bacteria (lactobacilli) (LAB1) and Lactic acid bacteria (cocci) (LAB2) were counted respectively on de Man, Rogosa and Sharpe Agar (MRS) and on M17 Agar supplemented with glucose (0.5%) (GM17). Plates were incubated under anaerobic cultivation conditions at 30°C for 48 h. Casein degrading bacteria as potential spoilage microorganisms (SM) were enumerated on sterile nutrient agar, supplemented with 10% sterile skimmed milk in aerobic cultivation conditions for 48-72 h at 37°C.

Cultivating media compositions correspond to the producer instructions (Scharlau, Barcelona, Spain). Viable cell counts were performed in three replications after serial dilutions in tryptone-salt solution (0.85% w/v, pH 7). For fermented milk, the cells were separated from substrate in shaking machine (10 minutes). Prepared basic solution was diluted to reduce the content of microorganisms below 300 CFU level.

Screening for antagonistic activity

The plates containing MRS or GM17 agar exploited in the CFU counts were used for the isolation of antagonistic lactic acid bacteria. For each sample, 100 colonies (50 on MRS agar and 50 in GM17 agar) were randomly picked and replicated onto four sets of agar plates, three of which were overlaid with 6 ml of an overnight culture of indicator strains: *Bacillus subtilis, Staphylococcus aureus* or *E. coli*. After incubation at 37°C, the plates were examined for zones of inhibition surrounding individual colonies. All cultures were routinely stored at 4°C and maintained as frozen stocks at -20°C in 35% glycerol. Before use, they were propagated twice at 30°C in their respective broth media.

Characterization and identification of isolates

Haemolytic activity was tested on fresh culture streaked on Columbia Blood Agar plates containing 5 % (v/v) sheep blood, and incubated for 48 h at 37°C. Subsequent clearing around the colonies indicated the production of β-haemolysin. Gram staining, morphology and catalase production were also determined. The Gram-positive, catalase-negative and β-haemolysin-negative isolates were selected for further studies. The selected strains were sub-cultured in appropriate culture broth overnight at 30°C. Cocci isolates were tested for growth at 10°C and 45°C, in 6.5% NaCl, at pH 9.6 and resistance to 60°C for 30 min, production of gaze in MRS medium and the growth in Bile Esculin Agar. The species identification was performed using API 20Strep and API 50CH galleries according to the manufacturer's instructions (bioMérieux, Marcy l'Etoile, France).

Stability of antagonistic substances

The stability of BLIS in liquid medium was investigated by the well diffusion assay (Tagg & McGiven, 1971) with modifications: Mueller Hinton Agar (Biokar Diagnostic) plates were overlaid with 5 ml of molten TSB agar (0.75% agar) inoculated with 100 µl of an overnight culture of the indicator microorganism. Wells (10 mm in diameter) were cut in the plates. The selected LAB isolates were grown in appropriate broth at 30°C for 16-24 h, subsequently cultures were centrifuged at 10000 g for 10 min at 4°C and the cell-free supernatants (CFS) were collected. 100 µl of CFS were placed in each well. Plates were pre-incubated at 4-10°C for 2 h to allow the radial diffusion of the compounds contained in the supernatants, and then incubated at 37°C for 10-16 h. The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the wells. Cell-free supernatants from cultures isolates were tested for stability in the presence of several physicochemical agents, using *Staphylococcus aureus* as indicator strain. To evaluate their thermo-resistance, aliquots of supernatants were heated to 60, 80 and 120°C for 30, 20 and 15 min respectively, then immediately cooled in ice and tested for antimicrobial activity. Samples were also dissolved in sterile buffers (pH 4 and 8) prepared at double concentration and kept at room temperature for 2 h before being neutralized and tested for residual activity. Non Buffered supernatants were used as controls.

Results and Discussion

Microbial enumerations

Samples of Camel's and cow's milk and fermented milk ("lben") were analyzed on appropriate culture media for the enumeration and isolation of the microflora. The analyzed samples were generally highly contaminated and the microbial counts markedly variable among samples of milk (Figure 1) and "lben" (Figure 2). The MAB recorded exceed the international standard. In the case of camel's milk, the recorded values are

production and collection, and the potential hazard associated to its consumption. In our samples, counts of coliforms were variable and have fluctuated from less than 10 CFU/ml to more than 10^7 CFU/ml. Therefore, the significance of coliforms as a fecal indicator to assess the hygienic quality of camel's milk appears to be questionable. These results go hand in hand with those reported by Benkerroum *et al*. (2003) and El Agamy *et al*. (1992) who reported the absence of coliforms in camel's milk, explaining this with the inhibition system of pathogenic bacteria in camel milk, which is stronger than cow's milk. Counts of LAB ranged between 5.6 10^4 and 3.2 10^6 CFU/ml, which are comparable to reported results (Benkerroum *et al*., 2003). LAB were predominant in "lben" and ranged between 8.5 10^7 and 9.9 10^8 CFU/ml.

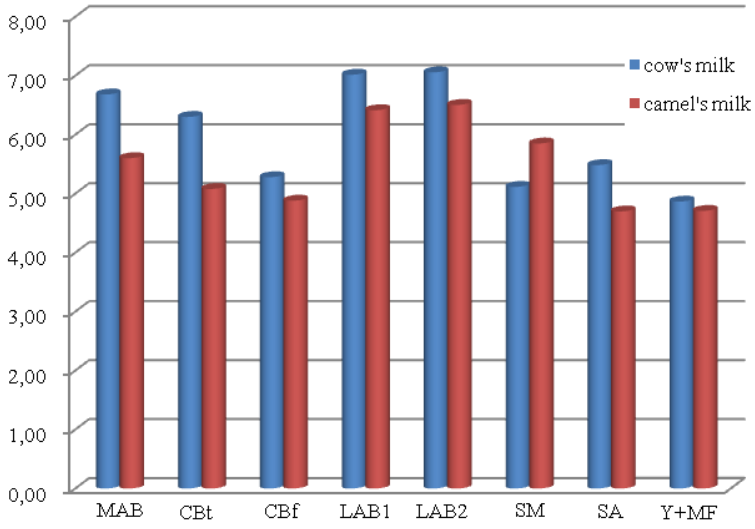

Figure 1. Microbiological quality of cow and camel milk samples (Log CFU). **MAB,** Mesophilic Aerobic Bacteria; **CBt,** Total Coliforms Bacteria; **CBf**, Fecal Coliforms Bacteria; **LAB1**, Lactic Acid Bacteria Isolated in MRS Medium; **LAB2**, Lactic Acid Bacteria Isolated in M17 Medium; **SM**, Spoilage Microorganisms; **SA**, *Staphylococcus*; **Y+MF**, Yeasts and Microscopic Filamentous Fungi.

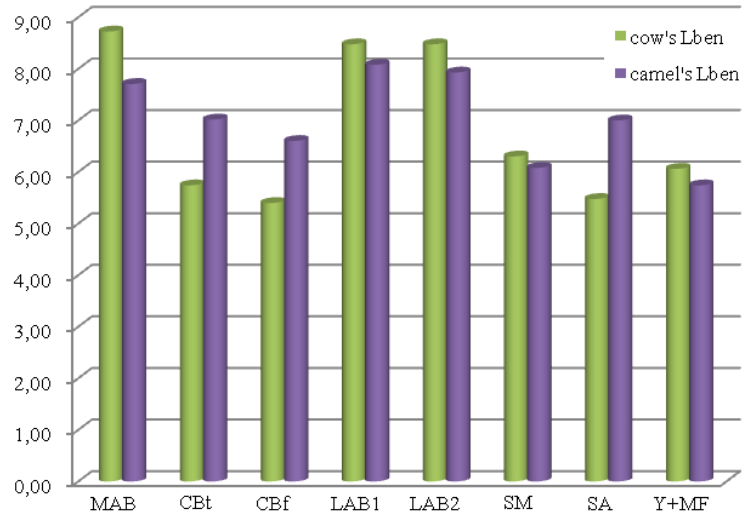

Figure 2. Microbiological quality of cow and camel "lben" samples (Log CFU). **MAB**, Mesophilic Aerobic Bacteria; **CBt**, Total Coliforms Bacteria; **CBf**, Fecal Coliforms Bacteria; **LAB1**, Lactic Acid Bacteria Isolated in MRS Medium; **LAB2**, Lactic Acid Bacteria Isolated in M17 Medium; **SM**, Spoilage Microorganisms, SA- *Staphylococcus*; **Y+MF**, Yeasts and Microscopic Filamentous Fungi.

Staphylococci were present in all analyzed samples. In the camel's "lben" it was 1000 times greater than the international standard, 100 times in the cow's milk and "lben" and 10 times in the camel's milk. The microflora of milk deterioration was very important in the milk as well as in "lben" which affects the sensory quality of these dairies products. All samples of cow and camel's "lben" analyzed are inedible, because they contain undesirable germs exceeding international standards. Microbiological fragility of "lben" is increased follows the poor manufacturing process and hygienic practices during production and storage, which leads to an unsatisfactory quality and can be responsible for severe food poisoning. One of the objectives of our study is to

investigate antagonist lactic acid bacteria to inhibit pathogens and spoilage microflora in these dairy products.

Screening for antimicrobial activity

A total of 1000 strains, isolated from 10 samples of milk, were initially screened for antagonistic activity against *Bacillus subtilis, Staphylococcus aureus and* E. coli by the double layer agar method (Photo 1). 478 produced an inhibition zone, and only 55 among them were characterized as LAB. Subsequently, the cell free supernatants of the 55 strains were tested by the agar well diffusion assay against *Bacillus subtilis, Staphylococcus aureus and E. coli*. Only 15 showed a measurable clear zone of inhibition against all tested indicatory strains, among of which 14 were isolated from camel dairy product.

The antimicrobial activity exhibited by five producing isolates against the indicator strain *Staphylococcus aureus* as seen by a clear zone of inhibition around the producer isolates growth (central spots).

Identification of lab strains and stability of inhibitory substances

In this screening for LAB bacteriocin producers, the strains of camel milk called Q37 and Q310 were selected for its inhibitory activity and stability under heat and pH acid. Both strains were identified phenotypically on the basis of its morphological and biochemical characteristics and the results was performed following the recommendations of Carr *et al*. (2002) and Harnett *et al*. (2011).

According to these results: cocci in pairs and chains, some of which were very long, Gram positive, catalase negative, capacity to grow at 45°C but not at 10°C, CO_2(-), ADH(-), production of acids from a limited number of sugars including, fructose, sucrose, and glucose; both strains were identified to belong to *Streptococcus thermophilus* (Table 1). This identification has to be confirmed by genetic characterization. The isolation of a thermophilic species as *S. thermophilus* concord with other reports on LAB of raw dromedary milk in Morocco (Khedid *et al*., 2009) and in Algeria (Merzouk *et al*., 2013). This can be explained by the fact that the dromedary grow in regions where the ambient temperatures can reach 40 °C during the lactating season (Merzouk *et al*., 2013). In other hand, *Streptococcus thermophilus* is frequently isolated from milk, to which is highly adapted. *Streptococcus thermophilus* strains have been used as probiotics for farm animals and had the ability to modulate immune responses. *S. thermophilus* has long been used in the manufacture of fermented milks and various cheeses and yogurt in combination with lactobacilli (Harnett *et al*., 2011).

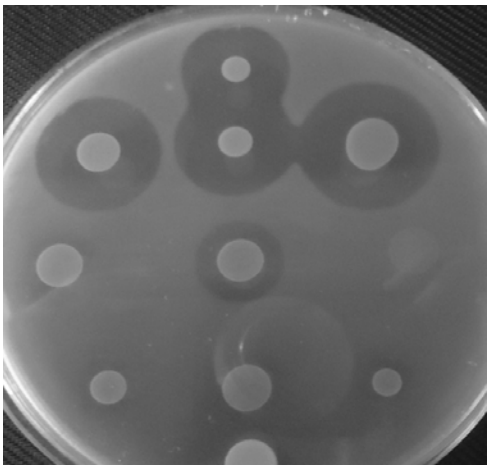

Photo 1. Agar-spot assay on GM17 medium.

The antimicrobial activity of Q310, a strain of camel milk appeared to be highly heat-stable as the inhibitory activity of this strain culture was not modified even after being heated to 120°C for 5 min. where the antimicrobial activity of Q37, also a strain of camel milk, appeared to be intermediate heat-stable. Inhibition of both strains was also preserved in supernatants with pH4. All of which are important characteristics for its potential application in acid food as starter and preservative.

Table 1. Identification of isolates using API 20strep.

TESTS	Q37	Q310
Acetoin production (Voges Proskauer)	+	+
hydrolysis (Hippuric acid)	+	+
ß-glucosidase hydrolysis (Esculin)	-	-
Pyrrolidonyl Arylamidase	-	-
α-Galactosidase	-	-
β-Giucuronidase	+	-
β-Galactosidase	-	-
Alkaline Phosphatase	-	-
Leucine AminoPeptidase	+	+
Arginine Dihydrolase	-	-
D-ribose	-	-
L-arabinose	-	-
D-mannitol	+	+
D-sorbitol	-	-
D-lactose	-	-
D-trehalose	+	+
Inulin	-	-
D-raffinose	-	-
Starch	-	-
Glycogen	-	-

Haemolytic activities and antibiotic resistance

Before using any strains in dairy product, some important characteristics should be assayed, as haemolytic activity and resistance to antibiotics. In fact, both isolated Streptococci strains shown to lack haemolytic activity when tested on Columbia Blood agar. The absence of such activity should be a criterion for selecting strains to be used as starter cultures in dairy products (Achemchem *et al*., 2006, 2012). Also, in our study, both of tested strains were susceptible to tetracycline, chloramphenicol, erythromycin, streptomycin and vancomycin; nevertheless they were resistant to penicillin and ampicillin (Results not shown).

This resistance to antibiotics is similar to what was mentioned for some enterococci strains as reported by (Ahmed *et al*., 2012) who found that *S. acidominimus* were susceptible to all antibiotics investigated in the present work, while *S. thermophilus* was resistant to chloramphenicol and erythromycin and susceptible to penicillin, ampicillin, tetracycline and erythromycin. Generally, *S. thermophilus* isolates were much more frequently observed with combined resistance to chloramphenicol, lincomycin, kanamycin, neomycin, and gentamycin, without tetracycline and streptomycin (Zhou *et al*., 2012).

Therefore, antibiotic susceptibility cannot serve as a criterion for LAB classification. In addition, the evolution of resistance to antibiotics, especially to β-lactam in streptococci is well documented in literature especially for clinical strains (Al-Swailem *et al*., 2004).

Conclusion

This work aimed to investigate the microbiological quality of milk and "lben" of camel and cow in south of Morocco and search bacteriocinogenic lactic acid bacteria. The results showed a pour microbiological quality. A total of 1000 strains of bacteria were isolated, among of which 478 showed antagonistic activity against some indicator microorganisms (*S. aureus*, *B. subtilis* and *E. coli*). Two strains called Q37 and Q310, identified as *Streptococcus thermophilus,* was selected for their inhibitory activity, heat stable and pH acid resistance. Both strains had shown to be generally safe and to have a potential application in fermented food as starter and bio-preservatives.

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