

## POTENTIAL TECHNOLOGICAL INTEREST OF INDIGENOUS LACTIC ACID BACTERIA FROM ALGERIAN CAMEL MILK

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### ABSTRACT

Nine isolates of lactic acid bacteria (LAB) obtained from the predominant microbiota of different camel milk samples collected in South-West Algeria, were selected in accordance with their growth ability in (cow) milk. The isolates were phenotypically and genotypically assigned to the following species: 4 *Leuconostoc mesenteroides* subsp. *dextranicum*; 2 *Lactobacillus brevis*; 2 *Lb. plantarum*; and 1 *Lactococcus lactis* subsp. *lactis*. One isolate from each of the *Leuconostoc* and *Lactobacillus* species were selected on the basis of their highest proteolytic and aminopeptidase activities. The selected isolates were used in combination with a commercial mesophilic O-type culture to make fermented milks. Sulfury flavor was detected as predominant in the sensory analysis of the milks made with only the *Ln. mesenteroides* and *Lb. brevis* adjuncts, which were characterized by the highest abundances of sulfur volatile compounds. Butter flavor was perceived in the milks made with the *Lb. plantarum*<sup>(cat)</sup> adjunct, and was related to the presence of acetoin. Finally, cheese flavor prevailed in the milks made with both the *Lb. brevis* and the *Lb. plantarum* adjuncts, characterized by their high contents of short-chain free fatty acids. The results suggest the potential interest of these microorganisms in the manufacture of dairy products, particularly the combination of the *Lb. brevis* and *Lb. plantarum* isolates for cheese making.

*Keywords:* camel milk, lactic acid bacteria, *Lactobacillus brevis*, *Lactobacillus plantarum*, sensory analysis, volatile compounds

## 1. INTRODUCTION

One of the main challenges of modern dairy industry is the development of a range of varied products to meet the needs and tastes of the various sectors of an increasingly numerous and demanding population. The request for novel cultured products development having different and improved sensorial attributes requires the use of microbial strains with interesting properties for application in dairy fermentations (WOUTERS *et al.*, 2002). The strict hygiene measures and the high standardization of the production systems make the milk and dairy cultured products manufactured in the developed countries a poor source of novel and distinctive LAB strains. So, novel strains should be searched in little standardized raw materials and products, and in ecological niches with particular and unusual environmental conditions.

Camel (*Camelus dromedarius*) milk, besides being produced in traditional low-tech systems, has compositional characteristics that make it very special milk with clearly different environmental conditions (FARAH, 1993; KONUSPAYEVA *et al.*, 2009) that may influence the metabolic abilities of the microbiota that settles it. The majority of the studies carried out on camel milk in the world, and particularly in Algeria focuses on the problem of its low clotting ability, which has been widely investigated (FARAH and BACHMAN, 1987; BOUDJENAH-HAROUN *et al.*, 2012). However, few works have been conducted regarding the microbiota of *Camelus dromedarius* milk or the biochemical and technological properties of LAB isolated from this source (ASHMAIG *et al.*, 2009; DRICI *et al.*, 2010; BENDIMERAD *et al.*, 2012; AKHMETSADYKOVA *et al.*, 2015).

In this context, the aims of the present study were: (i) to identify LAB isolates with potential technological interest belonging to the predominant microbiota of Algerian camel milk, and (ii) to characterize and to assay the isolates with a view to their use as adjunct cultures in the manufacture of fermented milks and cheeses with differentiated sensory characteristics.

## 2. MATERIALS AND METHODS

### 2.1. Bacterial cultures, media, and growth conditions

Nine LAB isolates obtained on pH 5.5 MRS medium (Oxoid Ltd., Basingstoke, UK) from six different camel milk samples collected in Bechar and Tindouf cities (southwest Algeria) were selected for this study. The selected isolates were able to clot cow milk after 12-24 h incubation at 30°C. The bacterial cultures were maintained at -30°C in MRS broth containing 20% glycerol (v/v). Working cultures were prepared by two consecutive transfers in MRS broth at 30 °C. Lyophilized commercial mesophilic O-type culture FD-DVS R-704 (Chr. Hansen, Denmark), containing *Lactococcus lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* (phage-resistant) strains was used alone or in combination with selected indigenous LAB cultures for the preparation of fermented milks. This culture was stored at -20°C and was directly inoculated in milk following the manufacturer's instructions.

### 2.2. Phenotypic identification of LAB isolates

Isolates were phenotypically assigned to the genus level as described by GARABAL *et al.* (2008). Utilization of citrate was also determined on citrate calcium agar (KCA) (NICKELS and LEESMENT, 1964), after 72 h incubation at 30°C. Criteria followed for phenotypic identification were those compiled by WOOD and HOLZAPFEL (1995). Assignment to species level was made by means of the API 50 CHL carbohydrate fermentation strips

(bioMérieux, Marcy l'Etoile, France), following the manufacturer's instructions. The results at 48 h were analyzed using the API CH Lab software package (bioMérieux).

### 2.3. Genotypic identification of LAB isolates

For molecular identification of (presumptive) leuconostocs and lactobacilli isolates, chromosomal DNA from bacterial isolates was extracted from single-colony by using the SPEEDTOOLS DNA Extraction kit (Biotools B&M Labs, S.A., Madrid, Spain) following the manufacturer's instructions. From the obtained DNA, 20 µL aliquots were made and stored at -20°C until needed.

All DNA samples were tested using universal primers amplifying a 1000-bp region of the 16S rRNA gene 616V (forward): 5'-AGAGTTTGATYMTGGCTC AG-3' and 699R (reverse): 5'-RGGGTTGCGCTCGTT-3' (ARAHAL *et al.*, 2008). The primers were synthesized by Invitrogen Co. (Invitrogen, Carlsbad, CA, USA), diluted at a final concentration of 1 µg/µL with sterilized deionized water upon reception and stored at -20°C. PCRs were performed in a reaction volume of 50 µL, containing 20 pM each of forward and reverse primers and 1 mL DNA template and prepared by using the DreamTaq DNA polymerase kit (Thermo Scientific, Waltham, MA, USA).

PCR amplifications were monitored in a Gene Amp thermal PCR System 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: an initial cycle of 95°C for 15 min; then 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min; and a final elongation step of 72°C for 10 min. The PCR products were purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). DNA sequencing was carried out with the v3.1 BigDye Terminator Cycle Sequencing kit (Applied Biosystems) on the ABI 3130xl capillary automate sequencer (Applied Biosystems) following the manufacturer's instructions. The obtained sequences were aligned to 16S rRNA gene sequences in the Gen Bank NCBI data base (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov>) using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST>) database.

### 2.4. Technological characterization of the selected LAB isolates

Leuconostocs and lactobacilli isolates were characterized for the following abilities: (a) carbohydrate fermentation, using the miniaturized API 50CHL system (bioMérieux), after 48 h incubation at 30°C; (b) acidifying activity in sterile (110°C, 15 min) reconstituted (10%, w/v) skim milk (pH 6.70) (Oxoid) after 6 h incubation at 30°C (IDF, 1995); (c) proteolytic activity in sterile reconstituted skim milk (Oxoid) after 24 h incubation at 30°C, as evaluated by the OPA method (Church *et al.* 1983); (d) aminopeptidase (AP) activity in sterile reconstituted skim milk (Oxoid) using leucine- and lysine-*p*-nitroanilide (both reagents from Sigma-Aldrich Corp., St. Louis, MO, USA) as substrates (EL SODA and DESMAZEAUD, 1982); (e) diacetyl-acetoin production in sterile reconstituted skim milk (Oxoid) after 48 h incubation at 30°C (IDF 1997); (f) extracellular proteolytic activity on calcium caseinate agar (Merck GmbH, Darmstadt, Germany) (RODRÍGUEZ-ALONSO *et al.*, 2008); (g) extracellular lipolytic activity on 1% (w/v) tributyrin agar (Merck), containing 1% arabic gum (Panreac) (RODRÍGUEZ-ALONSO *et al.*, 2008); and (h) antimicrobial activity of the nine isolates against each other and against the commercial culture R-704 (Chr. Hansen, Hørsholm, Denmark), determined by the agar well diffusion assay as described by CENTENO *et al.* (2002).

All assays for testing technological abilities were performed in triplicate, and numerical results were expressed as the mean values obtained for each isolate.

## 2.5. Preparation of fermented milks

Eight fermented or acidified milks were prepared per assay. One of the milks was acidified with the mesophilic commercial culture R-704 alone, and the other seven milks were fermented with the commercial culture plus one among three selected isolates (*Leuconostoc mesenteroides* subsp. *mesenteroides* C8M, *Lactobacillus brevis* C21B, *Lactobacillus plantarum* C22P), or their combinations (C8M+C21B, C8M+C22P, C21B+C22P, and C8M+C21B+C22P). Fermented milks were prepared in 1 L of retail pasteurized (80°C, 20 s) homogenized whole (3.6 g fat 100 mL<sup>-1</sup>) milk (Leyma, A Coruña, Spain) contained in polyethylene bottles. The milks used for each assay corresponded to the same industrial batch and had been pasteurized the day before purchasing. The bottles were inoculated with 0.1 units of the DVS R-704 commercial culture. The DVS culture was previously rehydrated and strongly stirred in sterile (reconstituted) skim milk (Oxoid) at a ratio of 10 units per liter of milk, and then inoculated at 1% (v/v) in the pasteurized milk. The LAB isolates were cultured in sterile skim milk (Oxoid) at 30°C for 16 h, and then inoculated at 1% (v/v) in the pasteurized milk. The inoculated milks were incubated at 30°C for 24 h. Assays were made in triplicate.

## 2.6. Sensory analysis and volatile compounds produced in the fermented milks

For sensory analysis, the (clotted) fermented milks were intensely agitated and then distributed in 50 mL amounts in sterile polyethylene containers. Flavors of the fermented milks were perceived by smelling and tasting, as evaluated by a panel of ten regular consumers of fermented milks who had been trained as previously described (RODRÍGUEZ-ALONSO *et al.*, 2008). The judges were asked to agitate the milks before smelling and tasting. Odor and taste preference as well as overall acceptance were scored on a scale from 1 to 7. The acceptability indexes (AI) of the fermented milks were calculated by the formula: overall acceptance  $\times$  100/7 (DUTCOSKY, 1996).

Volatile compounds were determined in 20 mL samples taken after agitation of the clotted milks, and stored at -80 °C until analysis. Volatile compounds were extracted by using the solid phase microextraction (SPME) technique and detected by gas chromatography coupled to mass spectrometry (GC-MS). For samples equilibration, 10 mL headspace vials containing 1 g of fermented milk were sealed with a PTFE-faced silicone septum (Supelco, Bellefonte, PA, USA) and maintained at 35°C in a thermo block (Memmert model 100-800, Schwabach, Germany) during 15 min. Then, a 75  $\mu$ m film thickness carboxen/polydimethylsiloxane (CAR/PDMS) fibre (Supelco) was exposed to the headspace while maintaining the sample at 35°C during 30 min. The SPME adsorbed compounds were injected to the chromatograph with a splitless mode injection at 260°C for 8 min. The separation of volatiles was performed on a Hewlett-Packard 6890N (Agilent Technologies, Santa Clara, CA, USA) gas chromatograph equipped with a DB-624 capillary column (30m  $\times$  0.25 mm id, 1.4  $\mu$ m film thickness; J&W Scientific, Folsom, CA, USA), following the method described by LORENZO and FONSECA (2014). Compounds were identified by comparing their mass spectra with those contained in the NIST05 (National Institute of Standards and Technology, Gaithersburg, MD, USA) library, and by comparing their mass spectra and retention time with authentic standards (Supelco). Abundances of volatile compounds are provided as peak area units/10<sup>6</sup> values.

## 2.7 Statistical analysis

Numerical data corresponding to technological characteristics, sensory analysis and volatile compounds were subjected to analysis of variance (ANOVA) and where statistical

differences were noted, differences among the distinct groups (isolates or fermented milks) were determined by the Duncan's test at a significance level of  $P < 0.05$ . All statistical procedures were performed with the SPSS software, version 20.0 (SPSS Inc., Chicago, IL, USA).

### 3. RESULTS AND DISCUSSIONS

#### 3.1. Isolation and identification of LAB isolates

Low quantities (mean value of  $2.50 \log \text{cfu mL}^{-1}$ ) of LAB were found in the six samples of camel milk used for LAB isolation (data not shown). This fact may be attributed to the high content of lysozyme and ascorbic acid in camel milk (FARAH, 1993). Among the 45 LAB isolates initially obtained, 27 (60%) were phenotypically assigned to *Leuconostoc/Weissella* genus (data not shown). Similarly to the results of this study leuconostocs have often been found as the major LAB in camel milk (ASHMAIG *et al.*, 2009; AKHMETSADYKOVA *et al.*, 2015), this may be attributed to the higher resistance to lysozyme of this microbial group as compared to other LAB (LIMONET *et al.*, 2004). As regards the remaining 18 isolates, eight were identified as mesophilic lactobacilli, one was assigned to *Lactococcus* genus, and nine LAB isolates (unable to clot cow milk) could not be assigned to genus level (data not shown).

The selected nine isolates able to clot (cow) milk after 12-24 h incubation at 30°C were identified by means of the API 50 CHL system as follows: four *Leuconostoc mesenteroides* subsp. *dextranicum* (similarity level of 95.2-99.8%); two *Lactobacillus brevis* (99.7% similarity); two *Lactobacillus plantarum* (77.8-79.3% similarity); and one *Lactococcus lactis* subsp. *lactis* (72.3% similarity) (data not shown). Both the *Lb. plantarum* and the *Lc. lactis* subsp. *lactis* isolates were able to metabolize citrate on KCA medium (data not shown). The low proportion of leuconostoc isolates showing the ability to coagulate milk may be explained by the fact that these microorganisms are adapted to growth on vegetables and roots and therefore lack sufficient proteolytic ability to grow in milk (VEDAMUTHU, 1994). The lactococcal isolate was not further considered in this study because of the known susceptibility of lactococcal cells to bacteriophage infection, as lytic phage infection is at present a major cause of fermentation failure (GARNEAU and MOINEAU, 2011).

Phenotypic identification of the selected (presumptive) leuconostocs and lactobacilli isolates was confirmed by sequencing of the fragments of the 16S rDNA gen amplified by PCR (data not shown). Sequences obtained from the four presumptive *Ln. mesenteroides* isolates produced significant (97-99%) alignments with the complete genome of *Ln. mesenteroides* subsp. *dextranicum* DSM 20484. Sequences from the two presumptive *Lb. brevis* isolates aligned (97%) with that of *Lb. brevis* ATCC 367. Lower percentages of similarities (91-94%) were found between the presumptive *Lb. plantarum* isolates and the *Lb. plantarum* JDM1 strain (genomes of all the type strains included in the NCBI data base). Similarly to the results of this study, the species *Lb. plantarum* and *Lb. brevis* have been isolated from Sudanese fermented camel milk (ASHMAIG *et al.*, 2009), and *Lc. lactis* subsp. *lactis*<sup>ca</sup> has been found in Algerian fermented camel milk (DRICI *et al.*, 2010; BENDIMERAD *et al.*, 2012).

#### 3.2. Technological properties of the selected leuconostocs and lactobacilli isolates

The most relevant carbohydrate fermentation abilities exhibited by the selected LAB strains are shown in Table 1. Despite of their ability to hydrolyze lactose in the API 50 CHL test tube, all the selected isolates (*Ln. mesenteroides* subsp. *dextranicum* C2M, C5M,

C8M and C14M; *Lb. brevis* C21B and C27B; and *Lb. plantarum* C14P and C22P) showed low acidifying activities (mean pH values of 6.42-6.65, and mean titratable acidities of 0.06-0.21 g lactic acid 100 mL<sup>-1</sup>) after 6 h incubation at 30°C (Table 1).

The pH values of the milk cultures of the lactobacilli isolates were significantly ( $P < 0.05$ ) lower than those of the leuconostocs isolates. The lowest mean pH values corresponded to the two isolates of *Lb. brevis* (Table 1). On the basis of their low acidifying activities, *Lb. brevis*, *Lb. plantarum* and *Ln. mesenteroides* have been generally classified as adjunct LAB to be used together with a lactococcal starter in order to enhance the acidification of milk (SETTANNI and MOSCHETTI, 2010).

The proteolytic activities evaluated by means of the OPA test ranged between 0.124 lysine mM for one *Lb. brevis* isolate and 0.230 lysine mM for one *Ln. mesenteroides* isolate (Table 1). These values are much lower than those reported by HERREROS *et al.* (2003) for 17 isolates of leuconostoc and mesophilic lactobacilli obtained from raw goat's milk cheeses (mean value equivalent to 0.296 lysine mM) and by GARABAL *et al.* (2008) for 42 isolates of mesophilic lactobacilli from raw cow's milk cheeses (mean value equivalent to 0.298 lysine mM). Nevertheless, this low proteolytic activity could be a suitable trait if these bacteria are used as adjunct cultures, in order to prevent development of bitterness in the final product (NANDAN *et al.*, 2010).

All the leuconostocs and lactobacilli isolates exhibited both Leu- and Lys-aminopeptidase activities, with higher Lys-AP than Leu-AP values (Table 1). Differences among species were pronounced, with the two *Lb. brevis* isolates showing significant ( $P < 0.05$ ) higher values for both activities (highest mean values of 200 U for Leu-AP and 530 U for Lys-AP) than the *Lb. plantarum* and *Ln. mesenteroides* isolates. Values obtained for the *Lb. plantarum* isolates were also significantly ( $P < 0.05$ ) higher than those of the *Ln. mesenteroides* isolates (Table 1). High Leu- and Lys-aminopeptidase activities have been detected for cell-free extracts of some strains of *Lb. brevis*, *Lb. plantarum* and *Ln. mesenteroides* isolated from raw ewe's and goat's milk cheeses (MACEDO *et al.*, 2000; HERREROS *et al.*, 2003). Similarly to the results of the present study, NIETO-ARRIBAS *et al.* (2009) found low Leu- and Lys-AP activities for intact cells of 19 *Ln. mesenteroides* subsp. *dextranicum* isolates obtained from artisanal Manchego ewe's cheese, with Lys-AP activity being higher than Leu-AP activity for all the isolates. The same authors (NIETO-ARRIBAS *et al.*, 2010) reported higher values of both Leu-AP and Lys-AP activities for most of 10 *Lb. plantarum* isolates than those of *Ln. mesenteroides* isolates from the same cheese variety. Aminopeptidases play a key role in the degradation of bitter peptides and flavor formation during cheese ripening (URBACH *et al.*, 1995; NANDAN *et al.*, 2010).

Only the two *Lb. plantarum* isolates able to metabolize citrate on KCA produced diacetyl-acetoin in skim milk (Table 1). The production of diacetyl-acetoin by *Lb. plantarum* C22P (120 mg diacetyl L<sup>-1</sup>) was significantly ( $P < 0.05$ ) higher than those observed for *Lb. plantarum* C14P (45 mg L<sup>-1</sup>) and for the initially selected *Lc. lactis* subsp. *lactis*<sup>(G1+)</sup> isolate (35 mg L<sup>-1</sup>, data not shown). GARABAL *et al.* (2008) reported a mean production equivalent to 84 mg diacetyl L<sup>-1</sup> for 33 isolates of mesophilic facultatively heterofermentative lactobacilli obtained from Galician (northwest Spain) raw cow's milk cheeses. Production of diacetyl by LAB could be considered an interesting technological property since this compound is related to positive (butter) flavor and antimicrobial effect in dairy products (DRICI *et al.*, 2010).

None of the isolates exhibited extracellular proteolytic or lipolytic activities in spite of their growth on calcium caseinate agar and on tributyrin agar media (data not shown).

**Table 1:** Technological characterization of the selected LAB isolates obtained from camel milk (quantitative results are means of three replicates).

| Fermentation of:                            | <i>Leuconostoc mesenteroides</i> |                     |                    |                    | <i>Lactobacillus brevis</i> |                    | <i>Lb. plantarum</i> |                    | S.E.M. <sup>1</sup> | P value |
|---|----------------------------------|---------------------|--------------------|--------------------|-----------------------------|--------------------|----------------------|--------------------|---------------------|---------|
|   | C2M                              | C5M                 | C8M                | C14M               | C21B                        | C27B               | C22P                 | C14P               |                     |         |
| L- arabinose                                | –                                | –                   | –                  | –                  | +                           | +                  | +                    | +                  |                     |         |
| D-cellobiose                                | –                                | –                   | –                  | –                  | –                           | –                  | +                    | +                  |                     |         |
| D- fructose                                 | +                                | +                   | +                  | +                  | +                           | +                  | +                    | +                  |                     |         |
| D-galactose                                 | +                                | +                   | –                  | –                  | +                           | +                  | +                    | +                  |                     |         |
| D-glucose                                   | +                                | +                   | +                  | +                  | +                           | +                  | +                    | +                  |                     |         |
| D-lactose                                   | +                                | +                   | +                  | w <sup>2</sup>     | +                           | +                  | +                    | +                  |                     |         |
| D-maltose                                   | +                                | +                   | +                  | +                  | +                           | +                  | +                    | +                  |                     |         |
| D-mannose                                   | +                                | +                   | +                  | +                  | –                           | –                  | +                    | +                  |                     |         |
| D-melibiose                                 | –                                | –                   | –                  | –                  | +                           | +                  | +                    | +                  |                     |         |
| D-raffinose                                 | –                                | –                   | –                  | –                  | –                           | –                  | –                    | –                  |                     |         |
| L-rhamnose                                  | –                                | –                   | –                  | –                  | –                           | –                  | –                    | –                  |                     |         |
| D-ribose                                    | w                                | –                   | w                  | w                  | +                           | +                  | +                    | +                  |                     |         |
| D-saccharose                                | +                                | +                   | +                  | +                  | –                           | w                  | +                    | +                  |                     |         |
| L-sorbose                                   | –                                | –                   | –                  | –                  | –                           | –                  | –                    | –                  |                     |         |
| D-trehalose                                 | +                                | +                   | +                  | +                  | –                           | –                  | +                    | +                  |                     |         |
| D-xylose                                    | w                                | w                   | w                  | w                  | +                           | +                  | –                    | –                  |                     |         |
| Acidifying activity (skim milk; 30 °C, 6 h) |                                  |                     |                    |                    |                             |                    |                      |                    |                     |         |
| pH  | 6.60 <sup>b</sup>                | 6.65 <sup>a</sup>   | 6.63 <sup>a</sup>  | 6.65 <sup>a</sup>  | 6.42 <sup>d</sup>           | 6.45 <sup>cd</sup> | 6.48 <sup>c</sup>    | 6.50 <sup>c</sup>  | 0.003               | 0.000   |
| Titratable acidity <sup>3</sup>             | 0.10 <sup>bc</sup>               | 0.06 <sup>d</sup>   | 0.08 <sup>cd</sup> | 0.09 <sup>c</sup>  | 0.21 <sup>a</sup>           | 0.21 <sup>a</sup>  | 0.14 <sup>b</sup>    | 0.12 <sup>b</sup>  | 0.0002              | 0.000   |
| Proteolytic activity (OPA) <sup>4</sup>     | 0.210 <sup>ab</sup>              | 0.212 <sup>ab</sup> | 0.230 <sup>a</sup> | 0.189 <sup>b</sup> | 0.126 <sup>d</sup>          | 0.124 <sup>d</sup> | 0.180 <sup>bc</sup>  | 0.178 <sup>c</sup> | 0.009               | 0.012   |
| Aminopeptidase activity <sup>5</sup>        |                                  |                     |                    |                    |                             |                    |                      |                    |                     |         |
| Leu-AP                                      | 17.2 <sup>d</sup>                | 14.6 <sup>d</sup>   | 24.0 <sup>d</sup>  | 19.4 <sup>d</sup>  | 200 <sup>a</sup>            | 197 <sup>a</sup>   | 109 <sup>b</sup>     | 51 <sup>c</sup>    | 66                  | 0.000   |
| Lys-AP                                      | 73 <sup>c</sup>                  | 68 <sup>c</sup>     | 73 <sup>c</sup>    | 71 <sup>c</sup>    | 530 <sup>a</sup>            | 528 <sup>a</sup>   | 129 <sup>b</sup>     | 134 <sup>b</sup>   | 161                 | 0.000   |
| Production of diacetyl-acetoin <sup>6</sup> | 0.0 <sup>c</sup>                 | 0.0 <sup>c</sup>    | 0.0 <sup>c</sup>   | 0.0 <sup>c</sup>   | 0.0 <sup>c</sup>            | 0.0 <sup>c</sup>   | 120 <sup>a</sup>     | 45 <sup>b</sup>    | 6.9                 | 0.000   |

<sup>a-d</sup>Mean values within a row with different superscripts are significantly different ( $P < 0.05$ ; Duncan's test)

<sup>1</sup>S.E.M.: Standard error of the mean

<sup>2</sup>w: weak reaction

<sup>3</sup>Expressed as g lactic acid 100 mL<sup>-1</sup>

<sup>4</sup>Expressed as mM lysine

<sup>5</sup>Expressed as enzymatic units (1 enzymatic unit = amount of enzyme giving an absorbance of 0.001 units at 410 nm min<sup>-1</sup>)

<sup>6</sup>Expressed as mg of diacetyl per liter of milk

The absence of extracellular enzymatic activities in LAB other than enterococci has been generally reported (HERRERO *et al.*, 1996; BUFFA *et al.*, 2004; NIETO-ARRIBAS *et al.*, 2009, 2010). According to HERRERO *et al.* (1996), LAB used as starter cultures should ideally present low lipolytic activity, as the degradation of milk fat must be slight in order to induce aroma production without giving rancid flavors.

One isolate from each one of the identified species (*Ln. mesenteroides* subsp. *dextranicum* C8M; *Lb. brevis* C21B; and *Lb. plantarum* C22P) were selected in accordance with their expected better technological performance (highest proteolytic and AP activities, and production of diacetyl for *Lb. plantarum* isolates). It should be pointed out that the similarity in the results of biochemical and genotypic (data not shown), and technological assays for *Lb. brevis* C21B and C27B could indicate that these two isolates probably belong to the same strain, although they were obtained from different camel milk samples (data not shown). The selected isolates were used to make fermented milks in combination with the commercial culture R-704 (Chr. Hansen) after confirming the absence of antibacterial activity against each other and against the commercial culture (data not shown).

### 3.3. Sensory analysis and volatile compounds produced in the fermented milks

The milks fermented with the commercial culture alone (control) and those cultured with the commercial starter in combination with both *Ln. mesenteroides* C8M and *Lb. plantarum* C22P, and in combination with the three selected isolates received the highest scores for sensory attributes (Table 2). The scores reached for the odor attribute were significantly ( $P < 0.05$ ) higher for these last two fermented milks than for the other milks, and the scores for the taste attribute were significantly ( $P < 0.05$ ) higher for the milk fermented with both the C8M and C22P adjuncts than for the other cultured milks. The highest acceptability index (86.1%) corresponded to the milk cultured with both the C8M and C22P isolates, followed by the milk fermented with the three selected isolates (76.7%) and by the control milk (74.5%) (Table 2).

**Table 2:** Mean sensory scores and main flavor descriptors of the fermented milks made with the commercial FD-DVS R-704 O-type starter alone (control) and in combination with cultures of the selected isolates obtained from camel milk.

|                            | Control            | +C8M                | +C21B             | +C22P             | +C8M<br>+C21B     | +C8M<br>+C22P     | +C21B<br>+C22P     | +C8M<br>+C21B<br>+C22P | S.E.M. <sup>1</sup> | P<br>value |
|----------------------------|--------------------|---------------------|-------------------|-------------------|-------------------|-------------------|--------------------|------------------------|---------------------|------------|
| Odor                       | 5.0 <sup>b</sup>   | 4.8 <sup>bc</sup>   | 4.7 <sup>bc</sup> | 4.9 <sup>bc</sup> | 4.4 <sup>c</sup>  | 5.8 <sup>a</sup>  | 4.8 <sup>bc</sup>  | 5.7 <sup>a</sup>       | 0.060               | 0.039      |
| Taste                      | 5.3 <sup>b</sup>   | 3.9 <sup>d</sup>    | 3.8 <sup>d</sup>  | 4.8 <sup>bc</sup> | 5.3 <sup>b</sup>  | 6.2 <sup>a</sup>  | 5.0 <sup>bc</sup>  | 5.0 <sup>bc</sup>      | 0.087               | 0.000      |
| Main flavor<br>descriptors | acid<br>yoghurt    | sulfury<br>metallic | sulfury<br>garlic | butter<br>vanilla | sulfury<br>sour   | butter<br>yoghurt | cheese<br>butter   | cheese<br>butter       |                     |            |
| AI (%) <sup>2</sup>        | 74.5 <sup>bc</sup> | 62.8 <sup>e</sup>   | 61.5 <sup>e</sup> | 70.1 <sup>d</sup> | 70.2 <sup>d</sup> | 86.1 <sup>a</sup> | 71.7 <sup>cd</sup> | 76.7 <sup>b</sup>      | 1.9                 | 0.000      |

<sup>1</sup>Mean values within a row with different superscripts are significantly different ( $P < 0.05$ ; Duncan's test)

<sup>2</sup>S.E.M.: Standard error of the mean

<sup>3</sup>AI (%): acceptability index of the fermented milk calculated by the formula: overall acceptance  $\times 100/7$  (in accordance with the used 7-point scale)

The flavor descriptors used by the majority of the judges include: acid and yoghurt for the control milk; butter and yoghurt for the milk fermented with both the C8M and C22P adjuncts; and butter and cheese for the milk cultured with the three selected isolates (Table



2). The butter and vanilla notes described for the milks made with the *Lb. plantarum* C22P adjunct may be related to the production of diacetyl and acetoin by this culture. These compounds are responsible for butter and nuts flavors in cheese (KONDYLI *et al.*, 2003; KAMINARIDES *et al.*, 2007). Sulfury and garlic nuances were detected by most of the judges in the milk fermented with the sole *Lb. brevis* C21B adjunct, which showed the lowest AI (61.5%) (Table 2). The sulfur flavors are related to the production of sulfur compounds principally from L-methionine (Met). It has been reported that sulfur compounds provide particular flavor notes in cheese such as garlic taste (KAMINARIDES *et al.*, 2007). The milks fermented with the C21B adjunct in combination with either the C8M or the C22P isolates received significant ( $P < 0.05$ ) higher scores for the taste attribute than the milk made with the C21B isolate as the only adjunct culture (Table 2). This suggests that the “sulfury defect” was moderated due to the presence of other flavor compounds.

Only ten volatile compounds were definitely identified in the fermented milks with the methodology used in the present study. These volatiles include: the alcohol 2-heptanol; the ketones 3-hydroxy 2-butanone (acetoin), 2-heptanone and 2-nonanone; the fatty acids acetic acid, butanoic acid, hexanoic acid and octanoic acid; and the sulfur compounds dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) (Table 3).

**Table 3:** Mean abundances (expressed as peak area units / 10) of each of the volatile compounds determined in the fermented milks made with the commercial FD-DVS R-704 O-type starter alone (control) and in combination with cultures of the selected isolates obtained from camel milk.

|                      | Control           | +C8M             | +C21B             | +C22P             | +C8M<br>+C21B    | +C8M<br>+C22P     | +C21B<br>+C22P     | +C8M<br>+C21B<br>+C22P | S.E.M. <sup>1</sup> | P<br>value |
|----------------------|-------------------|------------------|-------------------|-------------------|------------------|-------------------|--------------------|------------------------|---------------------|------------|
| ALCOHOLS             |                   |                  |                   |                   |                  |                   |                    |                        |                     |            |
| 2-Heptanol           | nd <sup>d2</sup>  | 4.1 <sup>c</sup> | 5.2 <sup>c</sup>  | 14.0 <sup>a</sup> | 8.6 <sup>b</sup> | 14.2 <sup>a</sup> | 10.8 <sup>ab</sup> | 9.8 <sup>ab</sup>      | 0.67                | 0.003      |
| KETONES              |                   |                  |                   |                   |                  |                   |                    |                        |                     |            |
| 3-Hydroxy 2-butanone | nd <sup>d</sup>   | nd <sup>d</sup>  | nd <sup>d</sup>   | 93 <sup>a</sup>   | nd <sup>d</sup>  | 20.1 <sup>b</sup> | nd <sup>d</sup>    | 5.4 <sup>c</sup>       | 21.8                | 0.031      |
| 2-Heptanone          | 1.1 <sup>b</sup>  | 2.8 <sup>b</sup> | 1.5 <sup>b</sup>  | 29.3 <sup>a</sup> | 0.8 <sup>b</sup> | 28.2 <sup>a</sup> | 4.8 <sup>b</sup>   | 4.7 <sup>b</sup>       | 3.06                | 0.001      |
| 2-Nonanone           | nd <sup>d</sup>   | 1.3 <sup>b</sup> | 0.4 <sup>c</sup>  | 3.1 <sup>a</sup>  | nd <sup>d</sup>  | 2.8 <sup>a</sup>  | nd <sup>d</sup>    | nd <sup>d</sup>        | 0.39                | 0.043      |
| FATTY ACIDS          |                   |                  |                   |                   |                  |                   |                    |                        |                     |            |
| Acetic acid          | 128 <sup>e</sup>  | 239 <sup>c</sup> | 149 <sup>de</sup> | 329 <sup>a</sup>  | 160 <sup>d</sup> | 265 <sup>bc</sup> | 131 <sup>e</sup>   | 281 <sup>b</sup>       | 18.9                | 0.005      |
| Butanoic acid        | 3.8 <sup>e</sup>  | 5.8 <sup>d</sup> | 9.7 <sup>bc</sup> | 9.2 <sup>bc</sup> | 8.1 <sup>c</sup> | 7.4 <sup>c</sup>  | 15.4 <sup>a</sup>  | 10.7 <sup>b</sup>      | 0.66                | 0.006      |
| Hexanoic acid        | nd <sup>f</sup>   | 2.7 <sup>e</sup> | 7.3 <sup>bc</sup> | 6.5 <sup>c</sup>  | 5.6 <sup>d</sup> | 6.7 <sup>c</sup>  | 12.7 <sup>a</sup>  | 7.8 <sup>b</sup>       | 0.56                | 0.004      |
| Octanoic acid        | nd <sup>c</sup>   | nd <sup>c</sup>  | 0.8 <sup>ab</sup> | 0.5 <sup>b</sup>  | 0.5 <sup>b</sup> | 0.5 <sup>b</sup>  | 1.2 <sup>a</sup>   | 1.1 <sup>a</sup>       | 0.073               | 0.019      |
| SULFUR COMPOUNDS     |                   |                  |                   |                   |                  |                   |                    |                        |                     |            |
| Dimethyl disulfide   | 0.7 <sup>cd</sup> | 2.0 <sup>a</sup> | 2.3 <sup>a</sup>  | 1.3 <sup>b</sup>  | 2.1 <sup>a</sup> | 1.0 <sup>bc</sup> | 0.5 <sup>d</sup>   | 0.6 <sup>d</sup>       | 0.21                | 0.011      |
| Dimethyl trisulfide  | nd <sup>b</sup>   | nd <sup>b</sup>  | 0.3 <sup>a</sup>  | nd <sup>b</sup>   | 0.2 <sup>a</sup> | nd <sup>b</sup>   | nd <sup>b</sup>    | nd <sup>b</sup>        | 0.01                | 0.001      |

<sup>a</sup>-Mean values within a row with different superscripts are significantly different ( $P < 0.05$ ; Duncan's test)

<sup>1</sup>S.E.M.: Standard error of the mean

nd: Compounds not detected (considered as 0.0 values for statistical analysis)

The poor volatile profiles of the fermented milks, with the absence of aldehydes and the practical absence of alcohols, may be partially explained on the basis of the SPME fiber performance. It has been reported that the porous CAR/PDMS fiber coating shows a higher affinity for low-molecular-weight compounds, including a high proportion of ketones (LORENZO, 2014). In addition, absorption for the most compounds may reach equilibrium with a longer time of exposure than that assayed in this work (MARCO *et al.*, 2004), although longer exposure times could result in inappropriate due to oxidation phenomena that can produce competitive effects between compounds (LORENZO, 2014). The secondary alcohol 2-heptanol, and its probable precursor 2-heptanone were present in all the fermented milks made with the adjunct cultures, but not in the control milk. The highest abundances of (2-heptanone and) 2-heptanol were found in the milk fermented with the *Lb. plantarum* C22P adjunct. The abundances of 2-heptanol in the milks made with the sole C22P adjunct and with the combination of the C8M and C22P adjuncts were significantly ( $P < 0.05$ ) higher than those of the milks made without the C22P adjunct culture. This compound, associated to herbaceous and fruity aromas has been considered as a key odorant in many cheese varieties (DELGADO *et al.*, 2011).

Regarding the ketones group, 3-hydroxy 2-butanone (acetoin) was only detected in the milks cultured with the *Lb. plantarum* C22P adjunct (Table 3), as it could be expected from the results of the technological assays. Acetoin is produced by the reduction of diacetyl originated from citrate metabolism, or by decarboxylation of  $\alpha$ -acetolactate (McSWEENEY *et al.*, 2000). Diacetyl, and to a lesser extent acetoin are generally appreciated for their buttery and nut-like notes (CURIONI *et al.*, 2002), and may be responsible to some extent for the butter flavors described in the sensory analysis. The abundances of the methyl ketones 2-heptanone and 2-nonanone were significantly ( $P < 0.05$ ) higher in the milks made with the sole *Lb. plantarum* C22P adjunct culture and with the combination of the C8M and C22P adjuncts than in the other fermented milks (Table 3). The methyl ketones 2-heptanone and 2-nonanone are associated with cheesy odors, particularly with blue cheese notes (CURIONI *et al.*, 2002). Methyl ketones containing odd numbers of C atoms are produced from  $\beta$ -oxidation of fatty acids (McSWEENEY *et al.*, 2000), which may have been released through the esterase and/or lipase activities of the lactobacilli.

The four fatty acids identified were detected in all the fermented milks made with the adjunct cultures, with the exception of octanoic acid in the milk fermented with the sole C8M leuconostoc adjunct. The abundances of acetic acid were significantly ( $P < 0.05$ ) higher in the milks made with the sole *Lb. plantarum* C22P adjunct culture than in the other fermented milks (Table 3). Citrate-fermenting microorganisms convert this compound to pyruvate, carbon dioxide, and acetic acid, together with various carbonyl compounds such as diacetyl and acetoin. Facultatively heterofermentative lactobacilli such as *Lb. plantarum* may also produce acetate from lactose or from amino acids (BUFFA *et al.*, 2004). Acetate is primarily formed from citrate and this may be the main reason why this compound is more abundant in the milks fermented with the *Lb. plantarum* C22P adjunct. Acetate also comes from pyruvate originated in the glycolysis from carbohydrates, and this would explain its presence in milks fermented with adjuncts unable to metabolize citrate. Acetic acid is responsible for sour flavor in dairy products (KAMINARIDES *et al.*, 2007). The abundances of butanoic and hexanoic acids were significantly ( $P < 0.05$ ) higher in the milks made with the combination of *Lb. brevis* C21B and *Lb. plantarum* C22P adjunct cultures than in the other fermented milks (Table 3). MENÉNDEZ *et al.* (2000) found higher volatile free fatty acids contents (3.05 vs. 1.33 meq/100 g) in 1-day samples of cow's cheeses made with a *Lb. plantarum* adjunct culture than in control samples. Butanoic acid may be derived from lipolysis of milk fat or produced through the fermentation of lactose and lactic acid (KAMINARIDES *et al.*, 2007). However, and despite the fact that the strains tested in the present study did not exhibit (exocellular) lipolytic activity when growing on

tributyryn agar, hexanoic and octanoic acids are probably released from triglycerides through the action of non-specific bacterial esterases and lipases as reported by GONZÁLEZ DE LLANO *et al.* (1996) and BUFFA *et al.* (2004). Short-chain fatty acids are related to rancid and pungent flavors (McSWEENEY *et al.*, 2000; DELGADO *et al.*, 2011), and butanoic acid has been found to be a potent odorant in cheese (KAMINARIDES *et al.*, 2007). The high contents of butanoic and hexanoic acids may relate to the cheese flavors detected in the milks made with both the C21B and C22P adjuncts.

As regards sulfur compounds, the abundances of DMDS were significantly ( $P < 0.05$ ) higher in the milks made with the *Ln. mesenteroides* C8M and the *Lb. brevis* C21B adjuncts, both alone or in combination, than in the other fermented milks (Table 3). DMTS was only detected in the milks made with the sole C21B adjunct and with the combination C8M+C21B. The thioesters DMDS and DMTS are expected to be formed mainly from the amino acid Met by the metabolism of LAB and secondary microbiota (URBACH, 1995; ENGELS *et al.*, 1997). It has been reported that a number of lactobacilli strains, including *Lb. brevis* are able to degrade Met (ENGELS *et al.*, 1997; SREEKUMAR *et al.*, 2009) which agrees with our findings regarding the *Lb. brevis* C21B adjunct assayed in the present study. The sulfury flavors detected in the sensory analysis of milks made with the *Ln. mesenteroides* C8M and the *Lb. brevis* C21B adjuncts, and the garlic nuance described in the milk made with the sole C21B adjunct culture may be attributed to the high contents of sulfur compounds.

#### 4. CONCLUSIONS

The selected leuconostocs and lactobacilli isolates from camel milk used in the present study as adjunct cultures to manufacture fermented milks in combination with an acidifying starter, conferred different flavor nuances and were responsible for different volatile profiles of the products. Sulfury flavor was detected as a main flavor in the milks made with only the *Ln. mesenteroides* and *Lb. brevis* adjuncts, characterized by the highest abundances of volatile sulfur compounds. Butter flavor, related to the presence of the volatile 3-hydroxy 2-butanone (acetoin) was perceived in the milks made with the *Lb. plantarum*<sup>(st+)</sup> adjunct, and cheese flavor, associated to high contents of volatile free fatty acids prevailed in the milks made with both the *Lb. brevis* and the *Lb. plantarum* adjuncts. The results suggest the potential interest of these LAB isolates in the manufacture of dairy products, in particular the combination of the *Lb. brevis* and *Lb. plantarum* isolates for cheese making.

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