academicJournals

Vol. 7(12), pp. 1002-1008, 19 March, 2013
DOI: 10.5897/AJMR12.488
ISSN 1996-0808 © 2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Antibacterial activity of Lactic acid bacteria isolated from Tunisian camel milk

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Accepted 15 February, 2013

The present study describes the composition of raw camel milk from south Tunisia in comparison with bovine milk and investigate the antimicrobial activity of isolated Lactic acid bacteria (LAB). The physical analysis of milk showed that camel milk was more acidic and viscous than cow milk while chemical composition showed that bovine milk was richer in fats (40 ± 3.4 g/l) and dry matter (121.54 ± 8.1 g/l). Mean microbial load in camel milk was recorded as follows: (7 ± 3)× 10^3 cfu/ml, (1.37 ± 12)× 10^2 cfu/mL and (1.8 ± 3) ×10^1 cfu/mL for the total mesophilic count, LAB and Coliforms respectively and they were lower when compared to samples of bovine milk. A total of 50 strains of LAB was isolated from camel and bovine milk, out of which 15 strains were selected as the strongest acidifying abilities. Screening of those strains led to the isolation of two strains from camel milk (BL_{C3} and BL_{C9}) demonstrated large and clear zones of inhibition of two indicators strains growth (*Staphylococcus epidermidis CIP 106510* and *Enterococcus faecalis ATCC 29212)*. Their antimicrobial compounds were detected in cell-free culture supernatant (CFS) after elimination of organic acids and hydrogen peroxide.

Key words: Camel milk, lactic acid bacteria, antimicrobial activity, microbiological quality, acidification ability.

INTRODUCTION

In hot and arid climate, camels (*Camelus dromedarius*) are the main source of human diet, providing mainly meat and milk. In these areas, camels can produce high volumes of good quality milk which may contain all essential nutrients found in milk from another species. In compareson with cow, camel milk contains less fats, inorganic salts but more proteins and lactose. Indeed camel milk has low milk fat made mainly from poly-unsaturated fatty acids, a high rate of vitamin-C. In addition, 1 kg of camel milk meets 100% of daily human requirements for calcium and phosphorus, 57.6% of potassium, 40% for iron, copper, zinc and magnesium and 24% of sodium (Nikkhah, 2011; Farah, 1993). Differences between camel and bovine milk lead to some differences in physiological and biological properties. Moreover, the Bedouin populations have long believed that raw camel milk is safe and even has therapeutic virtues. The health benefits of camel milk are due to their reported immuno-stimulating activity.

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Abbreviation: LAB, Lactic acid bacteria; **CFS,** cell free supernatant; **BL_C,** lactic acid bacteria isolated from camel milk; **BL_B,** lactic acid bacteria isolated from bovine milk; **cfu,** colony-forming unit; **NPN,** non protein nitrogen; **h,** hour; **MRS,** de Man. Rogosa and Sharpe; **PCA,** plate count agar; **VRBA,** violet red bile agar; **ATPC,** aerobic total plate count; **TSA,** tryptone soy agar.

Recent research suggests anti-diabetic, anti-oxidant, anti-microbial and Angiotensin Converting Enzyme (ACE) - inhibitory properties (Sboui et al., 2010; Salami et al., 2011). Camel milk is also considered helpful in the treatment of human viral related-decease like hepatitis C virus infectivity (El-Fakharany et al., 2008). This promotes the beneficial effect due to the combined action of a variety of protective factors present in these biological fluids such as in the whey proteins : immunoglobulin, α-lactalbumine, lactoferrin, Whey Acidic Proteins (WAP), lactophorin and peptidoglycan recognition protein (El Agamy et al., 1992; Kappeler et al., 2004 ; Salami et al., 2009), β-casein in camel caseins (Salami et al., 2011) and commensally and potentially benefit bacteria like lactic acid bacteria (LAB).

LAB are Gram positive, non-spore forming, catalase-negative and acid tolerant microorganisms that produce lactic acid as the primary end-produced during carbohydrate fermentation (Carr et al., 2002). Several studies demonstrated that some live LAB- called probiotics- when consumed in an adequate amount as part of food confer health benefits on the host as is described by Reid and Burton (2002). Some of the LAB strains isolated from milk have the ability to inhibit the growth of a wide spectrum of pathogenic Gram-positive bacteria by producing antimicrobial compounds, such as organic acids, hydrogen peroxide or the bacteriocin (Olivares et al., 2006). Bacteriocins, peptides ribosomally synthesized by lactic acid bacteria (LAB), are generally recognized as safe microorganisms (Anastasiadou et al., 2008). The principal species of LAB producing bacteriocins belong to the genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus*, *Oenococcus*, *Enterococcus*, *Leuconostoc* and *Carnobacterium* (BACTIBASE:http://bactibase.pfba-lab-tun.org/main.php). The bacteriocin-producing bacteria are considered as promising natural food preservatives or preservatives that fulfill these requirements (Atanassova et al., 2001; Leroy and De Vuyst, 2003) mainly, regarding the great distrust of the consumer against food additives such as chemical preservatives used to increase the shelf life of certain food and the use of heat treatments which often deteriorate the organoleptic and nutritional properties of heat-sensitive foods. Preservation refers to the use of antagonistic microorganisms or their metabolic products to inhibit or destroy undesired microorganisms in foods to enhance food safety and extend shelf life (Schillinger et al., 1996).

Antimicrobial activity of bacteriocins in foods is affected by its levels, type and number of microorganisms, condition of application, interaction/inactivation by food components. However, at the authors' knowledge, no studies were undertaken on the camel milk to characterize its microflora especially LAB and characterize their antimicrobial substances. The aim of this study was to investigate the preliminary characterization of a bacteriocin-like substance produced by a LAB strain isolated from the raw one humped camel milk produced in Tunisia and the factors that affect its production and antimicrobial activity.

MATERIALS AND METHODS

Milk sample collection

Ten camel and bovine milk samples were collected aseptically in sterile bottles in the Arid Land Institute in Medenine (South of Tunisia). Samples were transported immediately to the laboratory for analysis.

Physical and chemical quality

pH and acidity

The pH of milk is determined using a Thermo Orion pH meter (Cumming Center Beverly, USA). The Dornic acidity is determined by titration of a known quantity of milk by the sodium hydroxide N / 9 in the presence of phenolphthalein (AFNOR, 1993).

Fat analysis

It is determined by the method of acid-butyrometric Gerber (AFNOR, 1993). This method consists of an attack of milk with sulfuric acid and separation of the fat released by centrifugation in the presence of iso-amyl alcohol and using butyrometers graduates.

Proteins analysis by the Kjeldahl method

The levels of crude protein (CP) of milk were determined by determining nitrogen by the Kjeldahl method (N× 6.38) (AFNOR, 1993) after distillation unit NITRO PRO-I and titration with 0.1 N hydrochloric acid. The non-protein nitrogen (NPN) is the fraction of nitrogenous matter soluble in trichloroacetic acid.

Viscosity

The viscosity was determined by applying a shear stress of 0.1 to 100 rpm at an oscillation frequency of 1 Hz for 1 min with a Brookfield type Viscometer (model DV-E, MA, USA). The viscosity was expressed in centipoises (cP).

Dry matter

Dry matter expressed in grams per liter milk is calculated after weighing the sample at 105°C for 24 h of its dry residue. The sample is 5 g, Ash content, expressed in g/l of milk was determined after drying at 505°C (AFNOR, 1993).

Bacteriological analysis of samples

The techniques used are conventional methods and reflect the recommendations of French law or official French method (Normes AFNOR, 1996) which gives details of the technique followed. All samples studied have undergone a preliminary treatment to obtain the dilutions according to standard NF V08-0IO (March, 96).

Dilution

Milk samples (1 ml) were diluted in buffered peptone saline (10^{-1} to 10^{-3}), mixed in stomacher bag. In order to quantify the various microbial groups, appropriate dilutions were surface plating.

Aerobic total plate count (ATPC) was carried out on plate count agar (PCA, Sharlau Chemie S.A), incubated at 32°C for 72h (EL-Ziney and AL-Turki, 2007).

Table 1. Indicator strains and their conditions of incubation.

Microorganism	Growth condition
Gram negative	
Escherichia coli ATCC 38218	TSB; 37°C; 24 h
Salmonella Typhimurium ATCC 14028	TSB; 37°C; 24 h
Gram positive	
Enterococcus faecalis ATCC 29212	TSB; 30°C; 24 h
Staphylococcus aureus ATCC 29923	TSB; 37°C; 24 h
Staphylococcus epidermidis CIP 106510	TSB; 37°C; 24 h

TSB, Tryptone Soy Agar; h, hour.

10^{-3}), mixed in stomacher bag. In order to quantify the various microbial groups, appropriate dilutions were surface plating.

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Yeast and moulds**:** was enumerated on Sabouraud Chloramphenicol (Pronadisa Micro and Molecular Biology) culture medium and incubated at 25°C for 3 to 5 days.

Total coliform**:** was grown in Violet Red Bile Agar (VRBA) (AppliChem. Biochemical. Chemical services) in double layer. After solidifying of the agar, the plates were incubated at 30 °C for 24 h (Federal Register, 1990).

The lactic acid bacteria **(LAB)**

MRS solid (de Man. Rogosa and Sharpe) (charlau Chemie S.A, De Man et al., 1960) is shown to cultivate LAB on the surface and then incubated 30°C for 48 h. After incubation, colonies are Gram stained and tested for catalase production. Only Gram positive and catalase negative strains are stored at – 20°C in MRS broth in the presence of glycerol (25%) until use. Before experimental use, cells were subcultured twice in appropriate media.

Acidifying ability of isolated LAB strains in MRS broth

Lactic acid bacteria were grown in MRS broth during 72 h at 30°C. The follow-up of pH culture were determined by using a pH meter (glass electrode, Thermo Orion) during incubation to evaluate the ability of strains to acidify the culture. Only the most acidifying strains are subjected for antimicrobial activity.

Elimination of effect organic acid and hydrogen peroxide as inhibitory agents

A youth culture of lactobacilli was carried out in MRS medium, incubated at 37°C. The supernatant containing the bacteriocin crude extract recovered by centrifugation 10 000 rev / min is adjusted to neutral pH of 6.5 to 7 with 1M NaOH. The neutralization of the extract bacteriocin eliminates the effect of organic acids. The antimicrobial activity is determined for each strain of Lactobacillus selected. To minimize the influence of the inhibitory effect of hydrogen peroxide, the Petri dishes are inoculated with the germ target in depth and double layer on the TSA (Tryptone Soy Agar) using the technique of diffusion wells advocated by Tagg and Mc Given then continued and modified by several authors Schillinger and Lucke (1989), Ten Brink et al. (1994) and Jin et al. (1996).

Antimicrobial activity

Strains and culture conditions

Indicator strains and their growth conditions are summarized in Table 1. The strains used in the present study are *Salmonella Typhimurium ATCC 14028*, *Enterococcus faecalis ATCC 29212, Escherichia coli ATCC 38218, Staphylococcus aureus ATCC 29923* and *Staphylococcus epidermidis CIP 106510*, a human clinical isolate, obtained from the American Type Collection Culture (ATCC) and Collection of Institute Pasteur (CIP). All strains are stored at – 20°C in Tryptone Soy Broth (TSB, Biokar) supplemented with 25% of glycerol. Before experimental use, strains were activated by two successive transfers in their appropriate broth and incubated overnight at 30°C.

Agar well diffusion assay

Twenty milliliter (20 ml) of agar medium (TSA) are covered with 5 ml of semi-solid medium (0.7% agar) previously inoculated with 0.05 ml of the suspension of target strain 10^{-1} dilution. On inoculated Petri dishes, wells with 4.5 mm of diameter were performed and filled by 80 µl of neutralized crude bacteriocin extract. The Petri dishes thus prepared are pre-incubated for 2 to 4 h at 4°C to allow the radial diffusion of the inhibitor agents, then followed by incubation for 18 to 24 h at 37°C anaerobically to avoid the presence of air needed to produce hydrogen peroxide. At the end of incubation, the reading is done by measuring the diameter in mm of inhibition zones formed around the wells (Zi). Inhibition is considered positive if the diameter is greater than 2 mm (Thompson et al., 1996). The diameter of inhibition Zi is performed using the following formula:

Z (mm) = diameter of inhibition zone obtained (mm) - diameter wells (4.5 mm)

Sensitivity of antimicrobial compounds at different temperatures and pH values

In order to study the stability of antibacterial compounds to heat, the neutralized CFS of each strain is incubated in water bath at 60°C for 15 min, 100°C for 15, 20 and 30 min and autoclaved for 20 min at 121°C.

Sensitivity of antimicrobial substances to pH was investigated by adjusting the pH levels of supernatant of each strain to pH value 2, 6, 8 and 11 by using 1 N HCl or 1M NaOH.

Table 2. Principal physical and chemical composition of bovine and camel milk.

Parameter	pH	Dornic acidity (°D)	Fats (g/l)	Dry matter (g/l)	Proteins (g/l)	Viscosity (Cp)	NPN (g/l)
Camel milk	6.38 ± 0.1	18 ± 3.2	20± 4,9	115 ± 7.8	31.5 ± 5.9	192±4.4	0.8 ±0.05
Bovine milk	6.52 ±0.3	15±2.1	40±3.4	121.54 ±8.1	20.84 ±6.1	184±5.3	0.07± 0.08

Table 3. Microbiological examination of raw camel and bovine milk samples (cfu/ml).

Sample	ATPC	Yeast	mould	LAB	Coliform
Camel milk	$(7 \pm 3) \times 10^3$	$(1 \pm 13) \times 10^1$	$(1.8 \pm 2) \times 10^1$	$(1.37 \pm 12) \times 10^2$	$(1.8 \pm 3) \times 10^1$
Bovine milk	$(2 \pm 4) \times 10^4$	$(0.1 \pm 4) \times 10^1$	$(1 \pm 20) \times 10^1$	$(2.14 \pm 10) \times 10^2$	$(2.3 \pm 0.8) \times 10^1$

After heating and exposure at different pH values, CFS of each strain was tested for antimicrobial activity as is described above.

Statistical analysis

All measurements performed in triplicate were calculated for standard deviation, and results were presented as mean value ± S.D.

RESULTS AND DISCUSSION

Physical and chemical quality

Information on composition and physical-chemical characteristics of camel milk is essential for the success of development of camel dairy industries as well as marketing the products. There are distinct differences in physical-chemical characteristics between camel and bovine milks. Our results show that camel milk is more acidic and viscous than bovine milk (Table 2). The chemical composition showed that bovine milk is richer in dry matter (121.54 ± 8.1 g/l) and fat (40 ± 3.4 g/l). The protein and non protein nitrogen content in camel milk was significantly higher than that of cow milk. The fractions (NPN) have a biological importance due to their richness in acidic amino acids as well as in Vitamin B. These results are in perfect agreement with those repor-ted by other studies (El Hatmi et al., 2000). This increa-ses the nutritional value of camel milk. Therefore, chan-ges in camel milk composition occur by seasons, because towards the end of the lactation, the fat, protein, solids and mineral contents increase, while the lactose content decreases (Konuspayeva et al., 2009). Camel milk differs from cow milk as it has better digestibility and higher acidity, buffering capacity and certain therapeutic values in medicine and human nutrition. Camel milk has also higher specific composition, viscosity, and lower fats and dry matter than average cow milk (Table 2).

Enumeration of microorganisms

Results shown in Table 3 demonstrate that aerobic total plate counts (ATPC) in camel and bovine milks were detected with an average value of 7×10^3 and 2×10^4 (ufc/ml), respectively. The samples of bovine milk contain a higher ATPC number than those of camel milk. Higher results for the ATPC observed in camel milk were reported by Benkerroum et al. (2003), Moustafa et al. (2000) and Zahran and Salah (1997).

The bacteriological results found were compared with those of raw cow milk according to EU standards because there is no standard for raw camel milk. All milk samples did not meet the cow milk standard ($< 5\times10^4$ ufc/ml). This result is due to good health of milking dromedaries (with no mastitis) and two precautions taken to avoid any contamination of milk.

Coliforms counts in our samples were variables. We have recorded the absence of Coliforms in some samples and they did not exceed 8×10^1 cfu/ml (for camel milk) and 2.3×10^1 cfu/ml (for bovine milk) in others. This count of camel milk presented a relatively lower profile than those reported by Benkerroum et al. (2003) (7×10^3 cfu/ml); Khedid et al. (2003) (3×10^3 cfu/ml), Aly and Abo Al Yazeed (2003) (9.51×10^4 cfu/ml), Semereab and Molla (2001) (7×10^3 cfu/ml) and Al Mohizea (1986) (2.09×10^2 cfu/ml). The presence of less than 100 Coliforms per ml of milk indicates a good hygienic measure during milk production, handling and distribution.

The determination of microorganisms involved in the milk technological process included yeasts, and molds bacteria with an average: $(1 \pm 13) \times10^1$ and $(1.8 \pm 2) \times10^1$ (cfu/ml), respectively for camel milk while $(0.1 \pm 4) \times10^1$ cfu/ml and $(1 \pm 20) \times10^1$ cfu/ml for bovine milk. Generally, molds and yeasts are considered as normal flora of camel wool (Nasser et al., 1998) but the presence of a large number of molds in camel milk may be responsible for undesirable changes and inferior quality of the milk. By growth and metabolic activity, lipolytic mould may cause spoilage through the production of lipase enzyme leading to off flavors (Mohamed, 1990; Hubbert et al., 1996 and Conesa et al., 2001).

Isolation of lactic acid bacteria

One hundred and thirty-seven (137) strains were isolated from camel milk and 214 colonies from bovine milk. Fifty

Table 4. Acidifiying activity of lactic acid bacteria strains isolated from raw camel's and bovine's milk incubated in broth MRS at 30°C during 72 h.

Strain	Initial pH	Final pH	∆ pH
BL_{C1}	7.07	3.97	3.1
BL_{B2}	7.07	4.004	3.066
BL_{C3}	7.07	4.007	3.063
BL_{C4}	7.07	4.015	3.055
BL_{B5}	7.07	4.002	3.068
BL_{B6}	7.07	4.003	3.067
BL_{C7}	7.07	4.01	3.06
BL_{C8}	7.07	4.006	3.064
BL_{C9}	7.07	4.012	3.058
BL_{C10}	7.07	4.084	2.986
BL_{C11}	7.07	3.743	3.327
BL_{B12}	7.07	3.773	3.297
BL_{C13}	7.07	3.744	3.326
BL_{C14}	7.07	3.741	3.329
BL_{B15}	7.07	3.753	3.317

$BL_{C,}$ Lactic acid bacteria isolated from camel milk; BL_B, lactic acid bacteria isolated from bovine milk.

Table 5. *In vitro* sensitivity testing of different lactic acid bacteria strains to bovine and camel's milk by the well diffusion assay on Tryptic Soy Agar (TSA). Inhibition zone is in mm.

Indicator strain	*Tested strain*	Inhibition diameter zone (mm)[a]					
		BL_{C3}	BL_{C4}	BL_{C7}	BL_{C9}	BL_{B12}	BL_{B15}
Salmonella Typhimurium	*ATCC 14028*	0	0	27.5	0	0	0
Enterococcus faecalis	*ATCC 29212*	0	0	0	22.5	0	0
Escherichia coli	*ATCC 38218*	0	12.5	0	0	0	0
Staphylococcus epidermidis	*CIP 106510*	31.5	0	0	0	19.5	14.5
Staphylococcus aureus	*ATCC 29923*	0	0	0	0	0	0

[a]Mean of two determinations in triplicate

(50) isolates were Gram positive, catalase negative and oxidase negative confirming the selectivity of MRS. In general, the isolated bacteria presented a rod-like morphology. Those characteristics suggested the correspondence of the isolates with the group of LAB.

Acidification ability

Monitoring the pH of 50 strains grown in MRS broth three days at 30°C shows a progressive decline in pH for all strains. It seems that 15 strains of the 50 LAB isolated are the most efficient in terms of acidifiying activity (Table 4). Nevertheless, after 72 h of incubation the ∆ pH of LAB isolated from camel milk was higher than that of strains isolated from bovine milk. Indeed, the ∆ pH (72 h) ranged between 2.986 - 3.329 pH units and 3.066 – 3.317 pH units for camel's and bovine's milk, respectively. The strongest acidifying activity of strains isolated from camel milk confers not only the antimicrobial activity of isolates but also is an important technological property and might facilitate the fermentation of camel milk by using them as starter organisms.

Antimicrobial activity of lactic acid bacteria selected

Different strains of Lactobacillus selected have a very similar spectrum of activity against indicator germs tested. It was noted that two of the isolates showed antimicrobial activity against the Gram negative bacteria considered in this study: only strain (BL_{C7}) is active against *Salmonella typhimurium* and (BL_{C4}) against *Escherichia coli*. However, four isolates showed antimicrobial activity against Gram positive strains: three against *Staphylococcus epidermidis* and one (BL_{C9}) against *Enterococcus faecalis*. Areas inhibitions are clear with distinct borders, the diameter of inhibition varies between 12.5 and 31.5 mm depending on the strain tested (Table 5). Inhibition is denoted positive when it exceeded 1 mm (Schillinger and

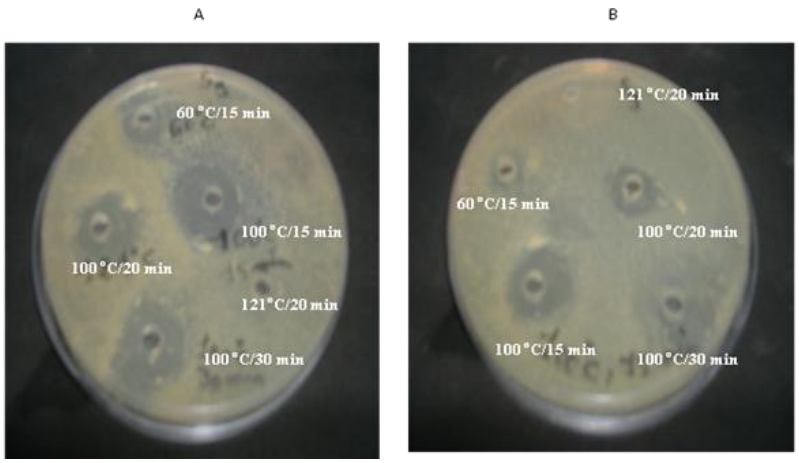

Figure 1. Effect of heat treatment on antimicrobial activity of BL_{C3} strain against *Enterococcus faecalis ATCC 29212* (A) and BL_{C9} isolate against *Staphylococcus epidermidis CIP 106510* (B).

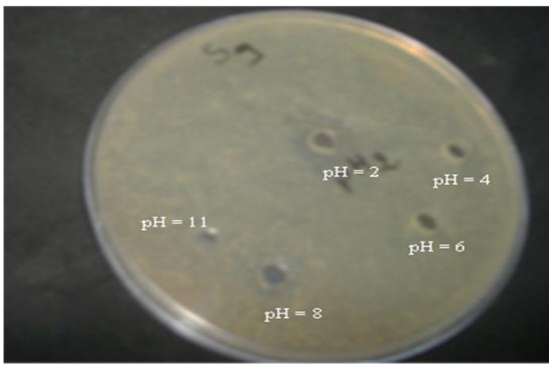

Figure 2. Effect of pH on bacteriocin activity of BL_{C9} strain against *S. epidermidis*.

Lucke, 1989). In fact, bacteriocins are most active on Gram-positive pathogens and act by forming pores in the cytoplasmic membrane disruptive function cell (Biswas et al., 1991). The bactericidal activity of the strains (BL_{C3} and BL_{C9}) is found exclusively in the culture medium. We have therefore the formation of extracellular substances. The latter differs from organic acids and peroxide oxygen. Indeed, the neutralization of the supernatant and the addition of catalase do not result in decreased inhibition diameter. Eliminating the effect of lactic acid and hydrogen peroxide rather promotes the activity of antibacterial substances. Therefore, the CFS (Cell Free Supernatant) from those strains was considered to contain bacteriocin-like substances and it will be chosen for further studies, including genotypic identification.

Effect of thermal and pH treatments on antimicrobial compounds of LAB

The results of thermal treatment showed that antimicrobial compounds produced by the two LAB strains isolated from camel milk (BL_{C3} and BL_{C9}) are resistant to heat. In fact, heating at 60°C for 15 min and at 100°C for 15, 20 and 30 min did not affect the antimicrobial activity of compounds produced by the two LAB isolates. Whereas, no antibacterial activity was observed after autoclaving at 121°C of CFS of BLC3 and BLC9 during 20 min, as reported by Lyon et al. (1995) and Labioui et al. (2005).

The exposure of BL_{C3} and BL_{C9} strains to different pH values showed that antimicrobial substances remained active at lower pH-value against *E. faecalis* as well as *S. epidermidis,* as shown in Figure 2*.* Although the two LAB strains (BL_{C3} and BL_{C9}) exhibited no antibacterial activity against *E. faecalis* and *S. epidermidis* within the pH range 4-11. The maintenance of the antimicrobial activity in the pH 2 exposed above suggests a promising applica-tion of this bacteriocin-like substance in acidic foods.

This finding is in contrast with the study of Bayoub et al. (2006); who indicated that the bacteriocin –the antimicrobial peptide produced by LAB, is active in a pH range between 2 and 11, with decreased activity by about 25% at pH 11. A bacteriocin should resist pH variations so as to be used as a potential antimicrobial agent.

Conclusion

Regarding the inhibitory compouds produced by lactic acid bacteria isolated from camel milk, it was found that BL_{C3} and BL_{C9} inhibits only *E. faecalis* and *S. epidermidis.* As such, it is characterized as having a narrow spectrum of inhibition. The results presented in this article provide a clearer idea on the potential antimicrobial Lactobacillus strains selected and which represent a way forward for the production of antimicrobial substances used in the fermentation and biopreservation food. The molecular identification of lactobacillus strains (BL_{C3} and BL_{C9}) and analysis of physicochemical properties of their inhibitory substance type bacteriocin is necessary and can be the

basis to guide future research on production of bacteriocins at different scales. Its potential applications in dairy product is needed. As an additive or as inoculums bacteria, it produces bacteriocins in the process manufacturing and as a natural preservative, it inhibits the growth of undesirable microorganisms in food.

ACKNOWLEDGMENTS

We thank Dr Khaled JEBAHI (The Higher Institute of Applied Studies in Humanities at Medenine, University of Gabes, Tunisia) for his kind help with English.

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