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Isolation and Characterization of Bacteriocinogenic Enterococcal and Lactococcal Strains from South of Morocco Dairy Product

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AE, FA and AABA designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors MZ, RA and FH managed the analyses of the study. Author GAB managed the literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: To investigate the occurrence of bacteriocinogenic lactic acid bacteria (BAL) in different animal's milk of the south of Morocco.
Place and Duration of Study: Laboratory of Microbial biotechnologies and plant Protection, Faculty of Sciences, and Bioprocess and Environment laboratory (LASIME), EST-Agadir, Ibnou Zohr University, Agadir, Morocco, between January 2014 and January 2016.
Methodology: A total of 2000 different colonies, isolated from 42 samples of dromedary, ewe's, goat and cow spontaneously fermented milk collected from some southern regions of Morocco, were tested for antimicrobial activity. Three indicator strains were used; *Listeria innocua, Bacillus subtilis* and *Enterococcus hirae*. The selected strains are phenotypically and biochemically

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identified, especially by API 20 Strep galleries. In addition, the sanitary and technological aspects of these strains are studied.
Results: Among the active strains 150 strains were selected, and 91% among them were identified as lactic acid bacteria. Out of these, 11 strains isolated from dromedary and ewe's milk are shown to be active by the agar well diffusion assay (AWDA). Seven (7) strains were identified as *Enterococcus faecium*, three (3) as *Enterococcus faecalis* and only one (1) strain was identified as *Lactococcus lactis*. The twelve strains are active against a wide range of pathogenic and spoilage bacteria, including *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli*. In addition, all of these strains shown to lack haemolytic, decarboxylatic, proteolytic and lipolytic activities and to be susceptible to most tested antibiotics.
Conclusion: These results suggest a potential application of isolated strains of lactic acid bacteria in bio-preservation of fermented foods especially dairy products.

Keywords: Dairy animals; lactic acid bacteria; bacteriocin-like substance; antagonism; bio-preservation.

1. INTRODUCTION

Cows are the predominant dairying species in the world and their milks are the most produced types of milk [1]. According to Faye and Konuspayeva [2] Cow milk represented 74.8% of the global milk production in 2009. Sheep, goat and camel milk are predominant in arid regions, and their dairy products are rapidly gaining popularity because of nutritional benefits and medicinal properties [3].

Milk has traditionally been preserved through many means; including boiling and conversion into more stable products [4]. Morocco has a wide range of traditional dairy products that have a good nutritional quality. Among them we can mention Lben, Smen and Dhen [5]. Lben is fermented milk, made by spontaneous fermentation involving lactic acid bacteria (LAB) [3], followed by churning to separate Lben from raw butter, then this raw butter can be made into Smen for preservation, by washing, salting and conditioning under anaerobic conditions [5].

Lactic acid bacteria (LAB) have been associated with dairy products, especially fermented milks [6], and are generally considered beneficial microorganisms [7]. LAB are used in dairy product to improve their safety and keep their quality, and to enhance their added value properties. And such proprieties are due to their capacity to produce various specific substances, as citrate, acetaldehyde, acetoin, diacetyl [8], Volatile sulphur compounds [9], organic acids, hydrogen peroxide, antibiotics [10], casein-derived bioactive peptides as for example casecidins and isracidin [11] and bacteriocins [12]. *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Enterococcus*, and *Streptococcus* are the main bacterial genera isolated from dairy products in Morocco [13-17]. In this study we aim to isolate, identify and characterize bacteriocinogenic LAB with potential applications in the bio-preservation of foods, present in various dairy product samples collected from some rural locations in the south of Morocco.

2. MATERIALS AND METHODS

2.1 Collection of Milk Samples

Forty samples of different animal's milk were collected from different southern Moroccan locations by manual milking as normally practiced by the farmers. The samples (Table 1) were collected in clean plastic bottles (500 ml) and transported immediately. They were analyzed upon arrival to the laboratory.

Table 1. Samples of milk investigated in this study (number and origin of simples)

Samples	Regions	Count
Camel's milk	Laayoune	11
	Guelmim	7
	Tan-Tan	4
Gaot's milk	Tiznit	9
Cow's milk	Témsia	2
	Howara	3
	Tiznit	4
Ewe's milk	Tiznit	2
Total count		42

2.2 Bacterial Cultures and Detection of Inhibitors

Prior to isolation, milk samples were allowed to ferment for 48h, to promote the growth of lactic acid bacteria (LAB), then LAB were isolated by

homogenizing 1 ml samples of milk in 9 ml saline solution and then plating suitable serial dilutions onto two buffered media: MRS and GM-17 (M-17 medium added with 5% of glucose) (Biokar Diagnostics, Barcelona, Spain). The plates were incubated aerobically at 30℃ for 48 h. Several colonies were then picked at random and incubated in a candle jar overnight for bacteriocin screening and replicated onto 4 sets of agar plates, three of which were overlaid with 5 ml of overnight cultures of the indicator strains: *Listeria innocua* CECT 4030, *Bacillus subtilis* DSMZ 6633 or *Enterococcus hirae* F419. After incubation at 37℃, the plates were examined for zones of inhibition surrounding individual colonies. All cultures were routinely stored at 4℃ and maintained as frozen stocks at -20℃ in 35% glycerol. Before use they were propagated at 30℃ in their respective broth media.

Gram staining, morphology and catalase production with hydrogen peroxide as substrate were determined. Colonies of catalase negative and Gram-positive were presumed to be LAB. Also, the agar well diffusion assay (AWDA) [18] was used for the detection of antagonistic activity. MHA (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. LAB strains were grown in MRS broth at 30℃ for 24 h. Cultures were centrifuged at 10000 g for 20 min at 4℃, the cell-free supernatants (CFS) were collected, and 100 µl of CFS of the potential producer strains was placed in each well. Plates were refrigerated (4℃) for 1-2 h to allow the radial diffusion of the compounds contained in the supernatants, and then incubated at 37℃ for 10-16 h. The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the wells.

2.3 Inhibitory Activity Spectrum

The inhibitory activity spectrum was obtained using the agar spot test [19] against the strains listed in Table 2. To this end, 5 µl aliquots from an overnight culture of each producing strain grown in appropriate broth being spotted onto buffered appropriate agar plates and incubated for approx. 24 h. Subsequently the plates were then overlaid with 6 ml of soft agar medium (0.75% agar) seeded with actively growing cells of the test organisms and then incubated. The sensitivity of the strain in question was evaluated by checking for clear zones around spots.

2.4 Effect of Heat Treatments, pH and Enzymes on Bacteriocin-like Substances Activity

The effect of chymosin, α-chymotrypsin on bacteriocin activity was determined as described by Achemchem et al. [12]. To evaluate the effect of heat on bacteriocin activity, semi-purified bacteriocin was heated at temperatures of 60, 80, 100℃ for 30, 10 and 5 min, respectively, and at 121℃ for 15 min as described by Achemchem et al. [14]. The sensitivity of the active substances to different pH values was estimated by adjusting the pH of semi-purified bacteriocin sample between 2 and 10 using 5 M NaOH or 5 M HCl. The residual activity after each treatment was determined with the agar-well diffusion assay with *E. hirae* F419 as the indicator strain.

2.5 Taxonomic Identification

The isolated strains were selected as bacteriocin producer because of its broad antimicrobial activity, and subjected to phenotypic identification. Cell morphology and Gram-staining reaction were examined by light microscopy. A test for catalase activity was carried out. Phenotypic identification was based upon biochemical characteristics, including the ability to grow at 10 and 45℃ in the presence of 6.5% (w/v) NaCl on bile-aesculin agar (BEA). API- 20 Strep fermentation was carried out according to the manufacturer's instructions (bioMérieux SA, France) and used for species identification.

2.6 Haemolytic Activity

Investigation of haemolytic activity was performed as described by Achemchem et al. [12]. A fresh culture of strain was streaked on Columbia agar plates, containing 5% (w/v) sheep blood, and incubated for 48 h at 37 C. Blood agar plates were examined for signs of β-haemolysis (clear zones around colonies), α-haemolysis (green-hued zones around colonies) or γ-haemolysis (no zones around colonies).

2.7 Antibiotic Resistance

Susceptibility to some antibiotics from different classes, including chloramphenicol, tetracycline, penicillin, amoxicillin, ampicillin and streptomycin, was tested using a disc diffusion method on Mueller-Hinton Agar (MHA). The disc diffusion zone diameter was interpreted based on the methods studied by "Standardisation de

l'Antibiogramme en Médecine Vétérinaire" (2008) with modifications as mentioned by Ahmed et al. [20].

2.8 Biogenic Amine Production

The decarboxylase test for production of biogenic amines was done by spoting LAB isolates in plates containing Maijala agar medium [21] with the addition of 20 g/l final concentration of the following amino acids as precursors: lysine, ornithine, histidine tyrosine and arginine. Plates were observed for a purple colour in the producing and surrounding colonies to indicate production of biogenic amines from precursor amino acids.

2.9 Proteolytic Activity

For the screening of hydrolysis of milk casein, aliquot from each LAB strain was spoted on PCA agar supplemented with UHT skim milk (1.5%, v/v). Plates were incubated at 30℃ for 48 h to detect proteinases giving rise to clear haloes surrounding colonies, which were taken as a positive indicator of proteolysis [22].

2.10 Gelatinase Activity

Production of gelatinase was tested on PCA agar plates containing 30 g/l gelatine. After overnight incubation at 30℃, the plates were placed at 4℃ for 5 h before examination for zone of turbidity around the colonies indicating hydrolysis of gelatine [23].

2.11 Lipolytic Activity

Lipolytic activity was tested on PCA agar plates containing 10 g/l Tween 80. Plates were incubated for at 30 C and examined daily for halo formation around the colonies.

2.12 Kinetics of Growth and Bacteriocin Biosynthesis

Sterile MRS broth was inoculated with 1% (v/v) of an 18-h-old culture of each LAB strain and incubated at 30℃ with the pH of the culture not regulated. Samples were taken at appropriate intervals to determine the optical density (at 620 nm) of the culture, pH, viable cell count and the antimicrobial activity of the bacteriocin-like substances produced. Activity was expressed in arbitrary units (AU/ml) corresponding to the reciprocal of the highest dilution showing a definite zone of inhibition.

3. RESULTS AND DISCUSSION

3.1 Screening for Antimicrobial Activity

A total of 2000 strains, isolated from 42 samples of dairy products, were initially screened for antagonistic activity against *Bacillus subtilis, Enterococcus hirae* and *Listeria innocua* by the double layer agar method. Most of the strains produced an inhibition zone. 92.72% of the selected strains were characterized as LAB. Subsequently, the cell free supernatants of the 50 strains selected for their clear and large zone of inhibition were tested by the agar well diffusion assay. Only 11 showed a measurable clear zone of inhibition against tested indicatory strains, among which two (2) strains were isolated from camel's milk and nine (9) from ewe's milk.

3.2 Inhibitory Activity Spectra

All tested strains exhibited inhibitory activity against some indicator microorganisms in a plate assay. Inhibitory spectra of these isolates are presented in Table 2. These strains isolated from dromedary and sheep milk revealed a strong inhibitory activity towards a wide range of Gram-positive bacteria, including food-borne pathogens and spoilage bacteria as *Listeria*, *Staphylococcus*, and *Bacillus*, and some lactic acid bacteria, such as *Enterococcus faecium* F58 and *Leuconostoc mesenteroides* F332. Tested strains show also an antagonistic activity against one Gram negative bacteria (*Escherichia coli* K12). Although, none tested strains were able to inhibit *Proteus vulgaris CECT 484*.

The relevant result in our study was the occurrence of antagonistic activity towards *E. coli* strain. This result go hand in hand with the finding of Ivanova et al. [24], who reported that Bozacine 14 secreted by *Lactococcus lactis subsp. lactis* B14 was active against Gram positive bacteria and only one gram negative bacteria which was *E. coli*. In fact, it was largely reported that enterocins are either not active against Gram negatives bacteria [25-29] or has a weak activity [30,31], but some bacteriocins as nisin [32] and some enterocins as Enterocin AS-48 [33] exhibit an antagonistic activity against Gram-negative bacteria, especially, through their synergetic effects with other antimicrobials [34-36]. Geis et al. [37] reported that nisin, like other bacteriocins applicated lonely, cannot inhibit Gram negative bacteria. The activity of lactic acid bacteria can be due not only to bacteriocin, but nutrients, also to other reasons, such as

Table 2. Antibacterial spectrum of the selected producing strains by the agar spot test

Indicator strains (source)	Selected producing strains										
	M8.21	**G8.10**	**M13.3**	**M13.5**	**M13.12**	**M13.13**	**M13.16**	**G13.1**	**G13.4**	**G13.22**	**G13.24**
Staphylococcus aureus 976 (CECT)	+	++	+	+	+	+	+	-	-	-	-
Listeria innocua 4030 (CECT)	+	+	+	+	+	+	+	++	++	++	++
L. monocytogenes 4032 (CECT)	+	+	+	++	+	+	+	+	+	+	-
Bacillus subtilis 6633 (DSMZ)	+	+	+	+	+	+	-	-	-	+	-
Proteus vulgaris 484 (CECT)	-	-	-	-	-	-	-	-	-	-	-
Escherichia coli K12	+	+	+	+	+	+	+	+	++	++	+
Enterococcus spp. (our collection)											
E. hirae F419	+	+	+++	+	+++	+	+++	+++	+	+++	+
E. faecium F58	+	+	++	+	+	+	-	++	++	++	+
E. faecium A13	-	+	-	+	-	+	-	-	-	-	+
E. faecium A15	-	+	-	+	-	+	-	-	+	-	-
E. avium 117	+	+	+++	+	++	+	++	++	++	++	++
E. durans 2022A	+	+	++	+	++	+	++	++	+++	++	+++
Leuconostoc mesenteroides F332 *(our collection)*	+	+	+	+	+	+	+	-	+	-	+
Streptococcus spp. (our collection)											
S. acidomonimus 37	-	-	-	+	-	-	-	-	+	-	+
S. acidomonimus 310	-	-	-	+	+	+	-	-	+	-	+
Aerococcus viridans 2224C *(our collection)*	-	-	-	-	-	-	-	-	-	-	-
Gemella haemolysans 1823 *(our collection)*	+	+	++	++	++	++	++	++	+++	++	+++
Lactococcus lactis (our collection)											
L. lactis ssp *lactis* 1816B	+	+	++	+	++	+	+++	++	++	++	+++
L. lactis ssp *cremoris* A2	+++	-	+++	+++	+++	+++	+++	+++	+++	+++	+++

Sensitivity was expressed as the size of inhibition zones: (-) : <1 mm; + : 1-10; ++ : 10-20 mm; +++ : >20 mm; ND : not determined. CECT: Spanish Type Culture Collection; DSMZ: German Collection of Microorganisms and Cell Cultures

competition for nutrients, lowering of pH, production of lactic acid, acetic acid and/or hydrogen peroxide. In the case of *Listeria monocytogenes*, inhibition can not only due to a decrease in pH, because this pathogenic strain is able to survive in acid conditions with pH as low as 4.8 in foods [38]. In general, *Listeria* ssp. are susceptible to nisin [39,40] and bacteriocins of class IIa [41], especially Pediocin-like bacteriocins [29]. In our study, *L. monocytogenes* is shown to be more sensitive towards one producing strains (M13.5) than *L. innocua,* this result concord with previous studies that reported that *L. monocytogenes* is more sensitive towards some antimicrobial compounds as compared to *L. innocua* [42]. *Lactococcus lactis* was sensitive to all isolated strains, this suggests that any one of them does not produce a pediocin like bacteriocin [26,29].

None of tested strains exhibited inhibitory activity against *Aerococcus viridans* 2, this can be explained by the fact that bacteriocins are known to inhibit the growth of similar or closely related bacterial strains.

The observed antibacterial activities exhibited by the studied strains suggest their potential application in bio-preservation of fermented foods and preventing listeriosis and other food-borne diseases.

3.3 Effect of Heat, pH and Enzymes on Bacteriocin-like Substances Activity

The results of thermal treatment showed that bacteriocins produced by all enterococcal LAB strains isolated from camel and ewe's milk are resistant to heat. In fact, heating at 60℃, 80℃ o r 100℃ for 30, 10 or 5 min did not affect the antimicrobial activity of compounds produced by these strains. In addition, even autoclaving at 121℃ for 15 min didn't affect their antimicrobial activity. Whereas, the lactococcal strain isolated from camel milk was completely disappeared under heating. In other hand, bacteriocins of all LAB strains were stable after exposure to lower and higher pH-value (Fig. 1). All bacteriocin-like substances produced were totally inactivated by proteolytic enzymes (chymosin and α-chymotrypsin).

3.4 Identification of LAB Strains

In this study, we investigated the occurrence of bacteriocinogenic lactic acid bacteria in the spontaneously fermented milk, the principal active stains were found in camel's and ewe's studied dairies. Isolated and selected strains were identified phenotypically on the basis of its morphological and biochemical characteristics and by API 20 Strep gallery. The results recapitulated in the Table 3 show that this strains can be grouped into three major groups:

Group 1: contains five (7) strains (M8.21, M13.5, M13.13, M13.16, G13.4, G13.22, G13.24) identified as *Enterococcus faecium*, this group is homofermentative Gram positive, catalase negative cocci, facultative anaerobic, grew at 10℃ and at 45℃, grew in the presence of 6.5% of NaCl and at pH 9.6, can reduce 0.1% Methylene Blue and do not ferments inulin [43] nor glycerol [44] and can survive under 60℃/30 min [45].

Group 2: contains five (3) strains (M13.3, M13.12, G13.1) and identified as *Enterococcus faecalis*, this group possess the same morphology as *E. faecium*, but can be distinguished by some biochemical characters, it ferments glycerol and starch, but not raffinose nor arabinose [15,43-46].

Group 3: contain one (1) strain (M8.12) identified as *Lactococcus lactis* subsp. *lactis*, this species is homofermentative Gram positive, catalase negative, grew at 10℃ but not at 45℃, grew in the presence of 4% NaCl but not at 6.5% NaCl, ferment lactose, mannitol and maltose, but not ribose. In addition, it can't forms carbon dioxide and diacetyl from citrate [43].

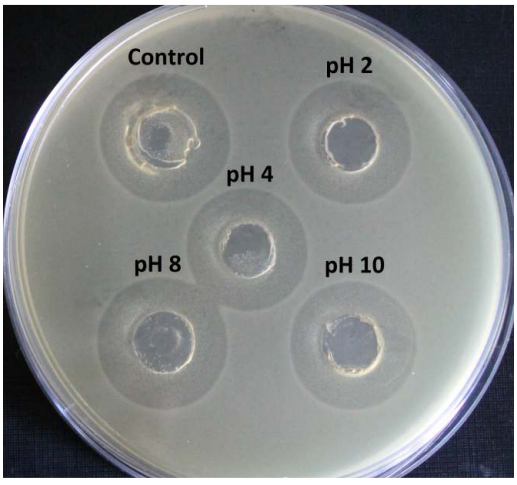


Fig. 1. Effect of pH on antimicrobial activity of the supernatant of M13.3 strain against *E. hirae* F419

Table 3. Morphological, physiological and biochemical characteristics of selected LAB strains

	E. faecium **M8.21**	***L. lactis* ssp.** ***lactis* G8.10**	***E. faecalis*** **M13.3**	***E. faecium*** **M13.5**	***E. faecalis*** **M13.12**	***E. faecium*** **M13.13**	***E. faecium*** **M13.16**	***E. faecalis*** **G13.1**	***E. faecium*** **G13.4**	***E. faecium*** **G13.22**	***E. faecium*** **G13.24**
Gram	+	+	+	+	+	+	+	+	+	+	+
Form	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Catalase	-	-	-	-	-	-	-	-	-	-	-
10℃	+	+	-	-	+	+	+	+	+	+	+
45℃	+	-	+	+	+	+	+	+	+	+	+
4% NaCl	+	+	+	+	+	+	+	+	+	+	+
6.5 % NaCl	+	-	+	+	+	+	+	+	+	+	+
pH 6.5	+	+	+	+	+	+	+	+	+	+	+
pH 9.6	+	-	+	+	+	+	+	+	+	+	+
BEA	+	-	+	+	+	+	+	+	+	+	+
Gaz from glucose	-	-	-	-	-	-	-	-	-	-	-
Methylene blue 0.1%	+	+	+	+	+	+	+	+	+	+	+
Methylene blue 1%	-	-	-	+	+	-	+	+	+	-	-
Tetrazolium 0.01%	-	-	-	-	+	-	-	-	+	+	+
60℃/30min	-	-	+	-	+	-	-	-	+	+	-
Citrate	-	-	-	-	-	-	-	-	-	-	-
Glycerol	+	+	+	+	+	-	+	+	-	-	-
Xylose	-	-	-	-	-	-	-	-	-	-	-
Fructose	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+

Table 3. Morphological, physiological and biochemical characteristics of selected LAB strains (continued)

	E. faecium **M8.21**	***L. lactis* ssp.** **lactis G8.10**	***E. faecalis*** **M13.3**	***E. faecium*** **M13.5**	***E. faecalis*** **M13.12**	***E. faecium*** **M13.13**	***E. faecium*** **M13.16**	***E. faecalis*** **G13.1**	***E. faecium*** **G13.4**	***E. faecium*** **G13.22**	***E. faecium*** **G13.24**
Vp	+	+	+	+	+	+	+	+	+	+	+
Hip	-	-	-	-	-	-	-	-	-	-	-
Aesc	+	+	+	+	-	+	+	+	+	+	+
pyra	+	-	+	+	+	+	+	+	+	+	+
αgal	-	-	-	-	-	-	-	-	+	-	+
βgur	-	-	-	-	-	-	-	-	+	-	+
βgal	+	-	-	-	-	-	-	-	+	-	+
pal	-	-	-	-	-	-	-	-	-	-	-
Lap	+	-	+	+	-	+	+	+	+	+	+
Adh	+	+	+	+	+	+	+	+	+	+	+
Ribose	+	-	+	+	+	+	+	+	+	+	+
Arabinose	+	-	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	-	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	+	-	-	+	-
Starch	-	+	+	-	+	-	+	+	-	-	-
Glycogen	-	-	-	-	-	-	-	-	-	-	-

The results showed that *Enterococcus* dominated the other genera in camel fermented milk, followed by *Lactococcus*. Whereas the ewe's one is completely dominated by Enterococci. We noticed the absence of *Lactobacillus* species, whatever in camel's or ewe's fermented milk, which concords partially with the result reported by some other autors [13,16,47-49], this authors find that camel milk is dominated by *Enterococci* and *Lactococci*. Some other auteur who studied the diversity of microflora in ewe's milk and cheese found that this products are dominated by *Enterococcus* and *Lactococcus*, especially *E. faecium*, *E. faecalis* and *L. lactis* subsp*. Lactis* [23,50,51]. The occurrence of other genus is also reported, as *Streptococcus*, *Leuconostoc*, *Pediococcus* and *lactobacillus* [15,20,52,53], others genus are less reported, as *Weissella*, *Vagococcus* and *Aerococcus* [47,53,54]. The presence of *Lactococcus Lactis* ssp*. lactis* and *Enterococcus* spp*.* in camel fermented milk can be explained by the fact that this species have a high salt tolerance (4–6.5%) [15]. And camel milk is known by its high level of salt [55]. This tolerance is very requested in some dairy products manufacture [43]. *Enterococci* are recognized as a natural starter culture, contributing to the development of the organoleptic properties, the controlling of some pathogen bacteria and even promoting the health state of animals and humans [14]. In addition, Enterococci have a beneficial effect on some other LAB growth as lactococci. The farmer genus promotes Lactococci acid production, by their intense proteolytic activity, which have a beneficial effect on their growth [56], this fact can partially explain the co-occurrence of both Enterococcus and Lactococcus. The predomination of Enterococci can be explained either by a poor sanitary conditions during milking and handling or by the production of enterocins, which can inhibit the competitive strains. In the other hand, Geis, Singh [37] reported that among 280 lactococcal strains, only 5% were able to produce bacteriocins, however, bacteriocinogenic Lactococci have been used successfully in starter cultures to improve the safety and quality of some dairy product, especially in cheese [57]. Concerning ewe's milk, however both Enterococci and Lactococci are dominant, Rivas, Castro [23] suggested that *E. faecium* and *E. faecalis* can be considered as essentials representatives of microflora of ewe's dairy products, in addition, Feutry, Oneca [51] reported that the repining of ewe's cheese promote the predominance of Enterococci and disadvantage Lactococcus. In one other case, Gaya, Babín [58] demonstrated that the predominance of some *L. lactis* strains is related to the production of bacteriocins.

The absence of *Pediococcus* and *Lactobacillus* genus in our case and many other studies, is probably due to the lack of essential growth factors, camel milk for example is particularly pour on some essential vitamins like folic acid [59,60], which is essentially required for *Pediococcus* growth [43]. Some autors reported that thiamin, retinol and riboflavin are less important in camel milk than other types [60], this can have a negative influence on *Lactobacillus* growth. Benkerroum, Boughdadi [13] suggested that camel milk can contain some natural inhibitors what can inhibit the mentioned genus. In addition, this fact can be explained by a non promoting competition for nutrients, since *Lactobacilli* metabolize lactose more slowly than *Lactococci* [15,61]. Gaya, Babín [58] who monitored the biodiversity of lactic acid bacteria in ewe's milk found that *Lactobacillus* clearly depend on the season, since it is more abundant in spring than winter or autumn.

However, In contrary to our results, the predominance of *Lactobacillus* in dairy products including camel's and ewe's ones is largely reported, especially *Lb. plantarum* [6,15,53,54,58,62,63]. It seem that manufacturing process influence the microflora diversity of dairy products, since the dominance of plant associated lactic acid bacteria as *L. plantarum*, *L. curvatus* and *L. mesenteroïdes* is widely reported in the fermented milk prepared in smoke-treated gourd [6,63].

3.5 Safety Characterization

Before using any strains in dairy product or any other foods, some important characteristics should be assayed, as haemolytic activity, resistance to antibiotics and decarboxylase activity.

3.5.1 Haemolytic activity

In fact, all investigated 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 [64].

3.5.2 Resistance to antibiotics

In addition, all tested strains were susceptible to penicillin, chloramphenicol, tetracyclin, ampicillin and amoxicillin, only one strains identified as *Lactococcus lactis* were sensible to Streptomycin, when the others strains identified as *Enterococcus faecium* and *Enterococcus faecalis* were all resistant to this antibiotic (Table 4). In fact, the evolution of resistance to antibiotics, especially to β-lactam, in streptococci is reported in literature [65]. This can be cause for concern, due to the problem of antibiotic resistance associated with some LAB strains.

Table 4. The antibiotic susceptibility of LAB strains

	P	C	TE	AX	S	AM
G8.10	S	S	S	S	S	S
M8.21	S	S	S	S	R	S
M13.3	I	S	S	I	R	S
M13.5	S	S	S	S	S	S
M13.12	I	I	S	R	R	S
M13.13	S	S	S	S	R	S
M13.16	I	S	S	S	R	I
G13.1	I	S	S	S	R	I
G13.4	S	S	S	S	R	S
G13.22	I	S	S	S	R	S
G13.24	S	S	S	S	R	S

R: Resistant, I: Intermediate Susceptible, S: Susceptible. P: Penicillin; C: Chloramphenicol, TE: Tetracyclin, AX: Amoxicillin, S: Streptomycin, AM: Ampicillin

3.5.3 Decarboxylase activity

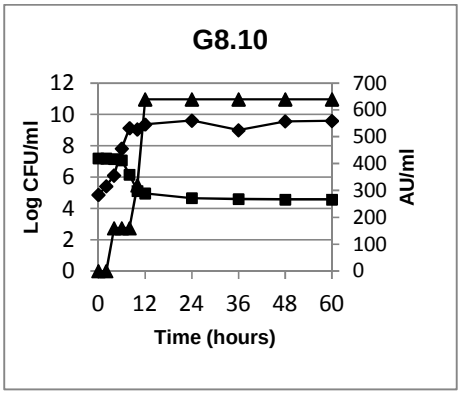
Biological amines, like histamine and tyramine, are nitrogenous compounds that occur naturally in wide variety of food. High level of amines can be produced by bacteria during amino acids decarboxylation, which can causes food-borne intoxication [66]. For this reason, is very important to investigate the LAB capacity to decarboxylate amino acids before any application in foods. In this study, all presented strains lack such undesirable activity. Even enterococcal strains are generally discarded for their potential carboxylatic activity and other undesirable characters, other autors isolated some important *Enterococci* strains without such activity [64], Achemchem et al. [12] found *Enterococcus hirae* F420 to be a tyrosine decarboxylating strain, although it was not able to decarboxylate any other tested amino acids (histidine, lysine and ornithine), in fact, none of our strains was identified as *Enterococcus hirae*. Bonetta et al. found *Enterococcus faecalis* to be the most intensive tyramine-former, but none of the strains identified in this study as *E. faecalis* produce tyramine or any other biogenic amines [67].

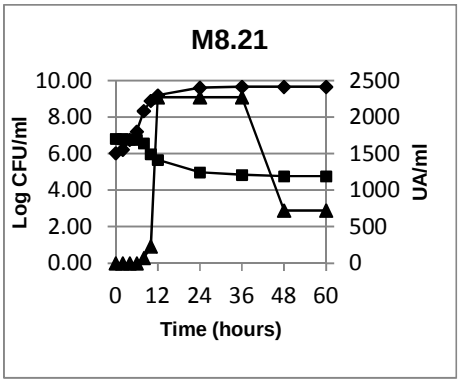
3.5.4 Proteolytic and gelatinase activity

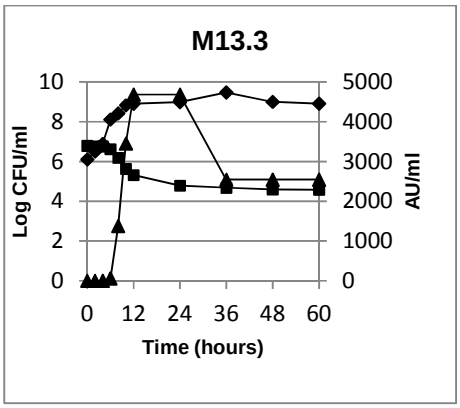
Proteolysis is very important in some dairy product, like most cheeses, because it affects texture and flavor [14]. Some LAB produces proteolytic enzymes involved in the repining [46]. But, other authors consider it as a putative virulence factor, especially in *Enterococci* [12]. In our study, all strains (*Lactococcus* and *Enterococci*) were negative for proteolysis and gelatinase and lipolytic activity, which can be considered as a safety character. According to Ahmadova (2012), thereabouts 10% of LAB isolated from dairy products are proteolytic, especially, *Enterococcus faecalis*, *Lactobacillus helveticus* and *Lactobacillus paracasei* subsp. *paracasei*. According to the same author, cocci LAB are more proteolytic than bacilli.

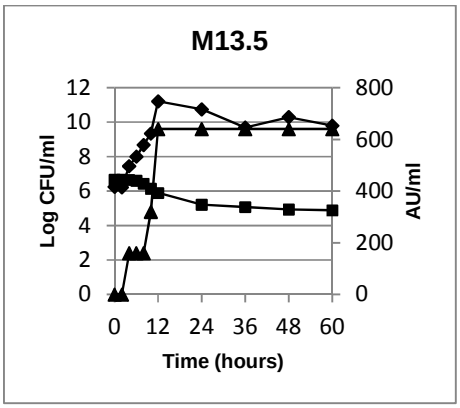
3.6 Kinetic of Growth and Production

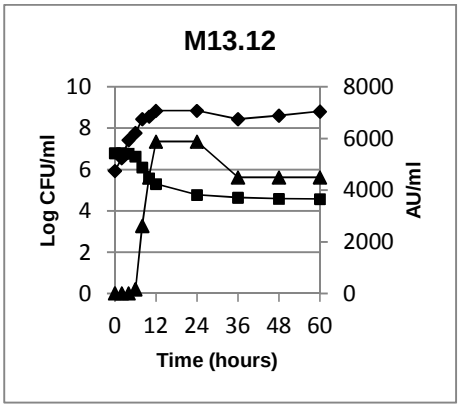
In the current study, strains investigated increased gradually in MRS broth, reached a proximately maximum population level of 9 log CFU/ml after 12 hours and remained stable up to 60 hours of incubation (Fig. 2). Bacteriocins were secreted in the early exponential phase of growth. The detectable levels of antibacterial activity against *E. hirae* F419 were detected between 2 and 6 h of growth. Then, the antibacterial activity increased gradually to reach the maximal level after 12 hours, and remains constant along the stationary phase. The maximum levels of antimicrobial activity were depending on the producer strain. Among the strains tested, *E. faecalis M13.12* exhibited the maximum inhibitory activity (5886 AU/ml) after 12 h of incubation at 30 C. For other strains maximum production differ between 650 and 4684 AU/ml depending on the producer strain. These results show that the antibacterial compounds investigated are primary metabolites, and their secretion was maximal at the end of the exponential phase, as many autors reported for bacteriocins [14, 23].

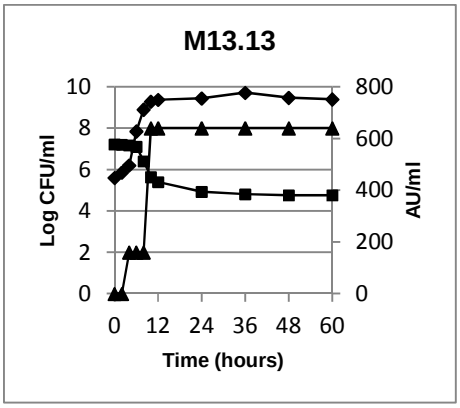

G8.10
Log CFU/ml
AU/ml
Time (hours)

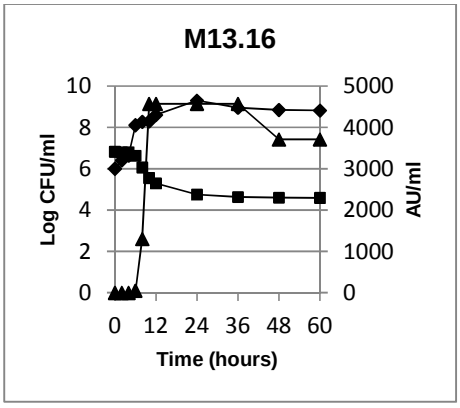

M8.21
Log CFU/ml
UA/ml
Time (hours)

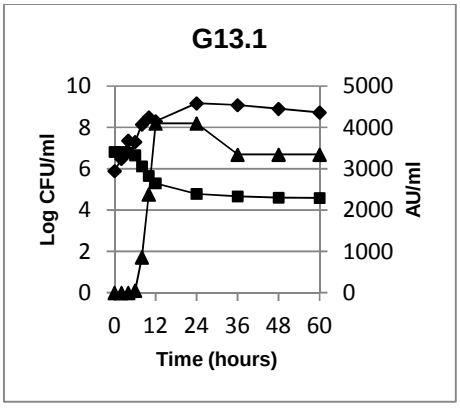

M13.3
Log CFU/ml
AU/ml
Time (hours)


M13.5
Log CFU/ml
AU/ml
Time (hours)


M13.12
Log CFU/ml
AU/ml
Time (hours)


M13.13
Log CFU/ml
AU/ml
Time (hours)


M13.16
Log CFU/ml
AU/ml
Time (hours)


G13.1
Log CFU/ml
AU/ml
Time (hours)

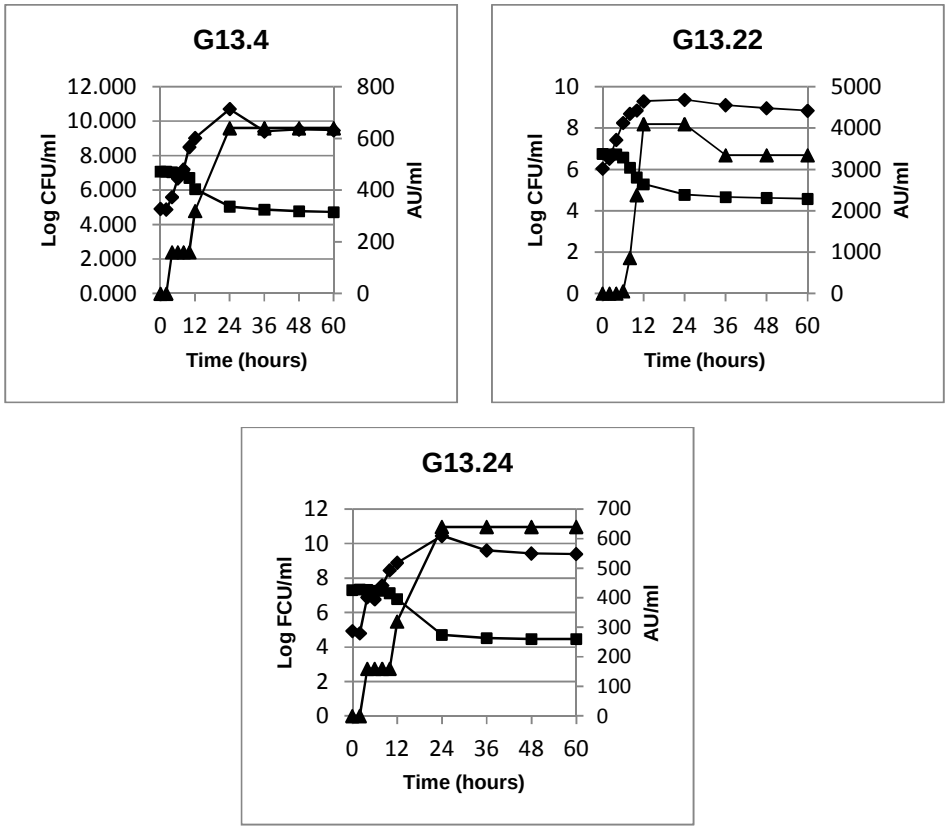


Fig. 2. Growth of selected LAB strains in MRS broth. Growth (♦), antibacterial activity against *E. hirae* F419 (▲) and pH (■)

4. CONCLUSION

This work aimed to search bacteriocinogenic lactic acid bacteria from some dairy product of south of Morocco. The results showed that selected LAB strains have a potential application in food products, as protective culture against eventual contamination of milk or curd with pathogenic or spoilage microorganisms. Our results showed also that strains isolated from camel and sheep dairy product were stronger and more important than other types.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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