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Antagonistic effect of lactic acid bacteria isolated from camel milk of south Algeria against methicillin-resistant *staphylococcus aureus* (MRSA)

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Abstract

Nosocomial infections due to methicillin-resistant *Staphylococcus aureus* (MRSA) are a major public health problem worldwide. The main aim of this study was to evaluate the inhibitory potential of lactic acid bacteria (LAB) against MRSA. Six strains of LAB isolated from camel milk, were identified by phenotypic method, which revealed their belonging to the species "*Lc.lactis subsp.lactis*"; "*Lc.lactis subsp.lactis var diacetylactis*"; "*Lc. lactis subsp. cremoris*"; "*Enterococcus durans*"; "*Ln.mesenteroides subsp. mesenteroides*"; "*Lb. Plantarium*". The antimicrobial activity of these selected strains is examined against five (05) strains of *methicillin-resistant Staphylococcus aureus* (MRSA) isolated from patients with infections in the intensive care unit of Slimane Amirate hospital, in the wilaya of Touggourt (Algeria) and also against two (02) control strain: *methicillin-sensitive Staphylococcus aureus* MSSA 25923 ATCC and *Methicillin-resistant Staphylococcus aureus* MRSA 43300 ATCC. Then the treatment of the culture supernatants of the isolated strains, selected antagonists "*Ln. mesenteroides subsp mesenteroides*" and "*Lb. Plantarum*" with proteolytic enzymes inactivated their inhibitory effect, indicating that the agents responsible for the inhibitions are bacteriocins. The physico-chemical characterization of bacteriocins revealed their thermo-resistance and their stability at acidic and basic pH. The results presented in this study provide a clearer idea of the antibacterial potential of bacteriocins produced by *Leuconostoc mesenteroides* and *Lactobacillus plantarum*, which represent a way forward to compensate for antibiotic treatments.

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Introduction

Staphylococcus aureus causes a wide range of syndromes, ranging from minor skin and soft tissue infection to life-threatening pneumonia and toxemia such as toxic shock syndrome. *S. aureus* can become Methicillin-resistant Staphylococcus *aureus* MRSA by the acquisition of the mecA gene, which encodes a penicillin-binding protein (PBP2a) with a low affinity for b-lactams (Hackbarth *et al.,* 1994; Niemeyeretal, 1996; Deresinski, 2005; Martins and Cunha, 2007). The PBP2a-producing MRSA strain is resistant not only to methicillin, oxacillin and nafcillin but also to all other b-lactam antibiotics including cephalosporins. The emergence of strains of *Staphylococcus aureus* multi-resistant to antibiotics represents a major problem in hospital settings. We must highlight the importance of finding solutions, and peptide molecules called "bacteriocins" can be the solution. The great interest in bacteriocins lies in their potential for use as bio-preservatives and their beneficial effect on health in inhibiting pathogenic or undesirable species. They are produced by certain lactic acid bacteria, which have been defined as a group of ubiquitous and heterogeneous bacteria that can ferment sugars mainly into lactic acid. They are GRAS (Generally Recognized as Safe) and are recommended for food and medical applications. This study aims to investigate the potential of bacteriocins to be an alternative to compensate for antibiotic treatments.

The objectives set for the realization of this work are as follows:

Isolation and identification of lactic isolates from camel milk.

Isolation of multi-resistant pathogenic bacteria (MRSA) in hospitals.

Antimicrobial effect of lactic isolates and Screening for the production of bacteriocins.

Physico-chemical characterization of bacteriocins.

Material and methods

Isolation and culture of lactic strains

Two tubes are filled with camel milk collected during the winter from herds of dromedaries (*Camelus dromedarius*) of the Saharawi population in mid-lactation living in extensive breeding in natural rangelands of the south-eastern Algerian region: Ouargla. The first is incubated at 30°C and the second at 45°C, until a coagulum is obtained under the effect of acidification due to the native, mesophilic or thermophilic lactic flora (Karam and Karam, 2006). Upon coagulation, the coagulum is homogenized. Decimal dilutions (10^{-1} to 10^{-3}) of the stock suspension are then made using sterile peptone water, then 1 ml of each dilution is inoculated into a specific agar medium (Table 1).

Strain purification

Whitish or milky-colored colonies are picked and preliminary tests including GRAM staining and catalase test are performed. Only the GRAM-positive, catalase-negative and non-sporulated strains are retained and subcultured into the MRS and M17, MSE broth. The operation is repeated (4 successive subcultures) until a pure culture is obtained, the purity of which is estimated by microscopic observations. The lactic strains are preserved in skimmed milk with 0.2% yeast extract and 30% glycerol preserved in dense suspension and in Eppendorf tubes at -20°C.

Identification of isolated lactic strains

The strains are identified by applying conventional microbiology techniques based on the search for a certain number of morphological, physiological and biochemical characteristics (Kandler and Weiss, 1986; Stiles and Holzapfel, 1997; Guiraud, 2012; Merzouk, 2015).

Identification with galleries

Fermentation profiles of the strains were established using the API50 CHL and API20 STRP biochemical galleries according to manufacturer instructions. The identification of strains was performed using ApiwebTM software of Biomerieux.

Pathogenic strains

Quality control strain: methicillin-sensitive *Staphylococcus aureus MSSA* 25923 ATCC

and *Methicillin-resistant Staphylococcus aureus* MRSA 43300 ATCC (purchased from American Type Culture Collection) were used as negative and positive controls, respectively.

MRSA strains from clinical human isolates were identified at the Slimane Amirate hospital in the wilaya of Touggourt (Algeria) (Table 2).

Phenotypic detection of MRSA

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a staphylococcus on which certain antibiotics have lost their effectiveness. According to the recommendations of the antibiogram committee of the French Society of Microbiology CASFM-EUCAST, 2020, the detection of resistance requires the use of the diffusion method on Mueller-Hinton agar consisting of the deposit of discs.

The surface of the Mueller-Hinton agar culture medium is inoculated with 0.5 Mac Farland using a swab. Antibiotic discs are deposited on the surface of the agar plates using an automatic dispenser. The dishes are then incubated at a temperature of 37°C for (18-24 hours). The diameters of the zones of inhibition obtained around the discs of antibiotics are measured using *a vernier* caliper, and the interpretation of the results obtained (bacteria *sensitive* (S), Intermediate (I), Resistant (R)) is carried out according to the criteria defined by CASFM-EUCAST, 2020.

Detection of antimicrobial activity

Supernatant preparation

This step consists in inoculating a colony of lactic acid bacteria previously purified (inoculum) in 1 ml of appropriate broth (M17, MRS and MSE) and then incubating at the appropriate temperature: we then obtain the "intermediate culture1," then we proceed to a second subculturing which consists in inoculating 1 ml of the intermediate culture 1 in a tube containing 9 ml of medium (M17 MRS and MSE): we obtain the "intermediate culture 2" (Doumandji, 2008). After incubation at 37°C/18 to 24 hours, the tube containing intermediate culture 2 is used to inoculate 90 ml of medium.

The cultures obtained are centrifuged at 4000 rpm. for 10 mins. The supernatant is used for demonstrating the inhibitions.

Antagonistic assay LAB versus pathogenic strains

Antagonism tests against pathogenic strains are carried out using the well method (Schillinger and Lücke, 1989). Twenty milliliters of agar medium are covered with 5 ml of semi-solid medium (0.7% agar) previously inoculated with 0.05 ml of the suspension of the pathogenic strain (10^{-1} dilution). On the Petri dishes inoculated beforehand with the pathogenic strain, wells of 6 mm in diameter are made.

These are then filled with supernatant of LAB. The Petri dishes prepared are pre-incubated for 2 to 4 hours at +4°C, in order to allow radial diffusion of the inhibiting agent, then followed by incubation at 37°C for 18 to 24 hours (Doumandji, 2008; Allouche *et al.,* 2010; Khay *et al.,* 2011).

Screening of bacteriocinogenic LAB

Antibacterial activity of LAB may be due to the production of organic acids, the production of hydrogen peroxide, or the synthesis of bacteriocins. Bacteriocins are proteinaceous in nature (Klaenhammer, 1988; Le Lay, 2009), thermostable and sensitive to proteolytic enzymes (Ennahar *et al.,* 2000, Galvez *et al.,* 2007; Mechai, 2009).

These characteristics are used to verify the nature of the active substance contained in the supernatants. To ensure that the inhibitory substance produced by the selected lactic antagonist strains (Ln5 and Lb11) against MRSA is protein in nature (bacteriocin), proteolytic enzymes were used: trypsin and chymotrypsin. Each enzyme is dissolved in 0.1 mole/l phosphate buffer adjusted to pH 7.0 with HCl or 1N NaOH. The supernatant is added to these enzymes, and these supernatants are treated beforehand with catalase (1mg/ml) to eliminate the effect of hydrogen peroxide and neutralized at pH 6.5-7 with NaOH of 1N to eliminate the effect of organic acids, are

sterilized by filtration (0.45 µm in diameter) and incubated at 37°C for 2.5 hours. The plates were incubated at 30°C for 24 h and the diameters of the growth inhibition zones were then measured (Lachance, 2000; Hannachi, 2008; Doumandji *et al.,* 2010; Allouche *et al.,* 2010).

Characterization of bacteriocin

Effect of temperature

The supernatants (neutralized and treated with catalase) were heated at 70° C, 80° C, 90° C for 10 minutes, and at 100° C and 121° C for 30 and 15 min, respectively (autoclaving condition), followed by immediate cooling to 4°C (Lachance 2000; Labioui *et al.,* 2005; Allouche *et al.,* 2010; Khay *et al.,* 2011; Merzouk, 2015), The samples were tested by the well method described by Schillinger and Lücke, 1989.

Effect of pH

The pH of the supernatant of each strain was adjusted from 2 to 10 with 1 N HCl or 1 N NaOH. After incubation at 37°C for 5 h (Khay *et al.,* 2011), the pH was readjusted to 6.5 (to eliminate the effect of organic acids). All samples were tested for antimicrobial activity usingthe well diffusion assay.

Antibiogram of lactic strains

In order to determine the resistance or sensitivity to antibiotics (ATB) of the selected lactic antagonist strains in this study, Fifteen ATB belonging to different families were tested. 100 µl of an overnight culture of the Ln5 and Lb11 strains were spread on the surface of an MRS agar. After incubation at 37° C for 15 min, the ATB disks were deposited and incubated at 30° C for 24 to 48 hours.

Results and discussion

Identification of isolated lactic strains

Identification is based on macroscopic (Fig.1), microscopic (Fig. 2) observations, on physiological and biochemical tests.

The biochemical gallery API 50 CHL and API 20 STRP made it possible to retain 06 lactic strains belonging to four genera *Lactococcus, Lactobacillus, Leuconostoc* and *Enterococcus,* as shown (Table 3).

Table 1. Isolation media and culture conditions for lactic acid strains.

Culture media	bacteria	incubation	Condition of incubation
MRS + bromocresol green	Lactobacilli	37°C/24-72h	Anaerobic
M17	Lactocoques	37°C/48h	aerobic
MSE	Leuconostocs	21°C/4days	aerobic

The addition of bromocresol green to the MRS medium allows better differentiation of lactic acid bacteria which give acidifying colonies surrounded by a yellow halo while contaminants give weakly acidifying colonies with a blue halo.

Methicillin resistance test

The strain *Staphylococcus aureus* 25923 ATCC is sensitive to almost all the antibiotics tested during this study; however, all of the MRSA isolated (the human clinical isolates) and *Staphylococcus aureus resistant to methicillin* MRSA 43300 ATCC are resistant to methicillin (resistant to oxacillin and Céfoxitine) (Table 4).

Oxacillin and cefoxitin were tested instead of methicillin because methicillin is no longer commercially available and oxacillin maintains its activity during storage better than methicillin and is more likely to detect heteroresistant strains.

However, cefoxitin is an even better inducer of the *mecA* gene, and tests using cefoxitin give more reproducible and accurate results than tests with oxacillinVancomycin currently represents one of the best antibiotics against MRSA infections; however routine use has led to the emergence of MRSA strains with sensitivities decreased to glycopeptides. Our results revealed that 2 strains were distinguished by their resistance to vancomycin.

Table 2. Clinical MRSA Isolates.

Clinical MRSA Isolate	Clinical Infection Site
Sa1	abdominal pus
Sa2	Vagina
Sa3	the nose
Sa4	thigh wound
Sa5	lungs

Table 3. Phenotypic characteristics of strains isolated from camel milk.

Form		Cocci	Cocci	Cocci	Cocci	cocci	Stick
Gram stain		+	+	+	+	+	+
Catalase test		-	-	-	-	-	-
CO_2 from glucose		-	-	-	-	+	-
Hemolysis		ɣ	ɣ	ɣ	ɣ	ND	ND
Arginine dihydrolase		+	+	-	+	-	-
Hydrolysis strach		-	-	-	-	ND	ND
Hydrolysis esculin		+	+	+	+	+	+
Growth at Température (°C)	10	+	+	+	+	ND	ND
	15	ND	ND	ND	ND	ND	+
	37	ND	ND	ND	ND	+	ND
	45	-	V	V	V	ND	V
Resistance to	63°C/30 min	+	+	V	+	ND	ND
	60°C/90 min	ND	ND	ND	ND	ND	+
	65°C/30 min	ND	ND	ND	ND	ND	+
	55°C/15 min	ND	ND	ND	ND	+	ND
pH	9.6	-	+	+	+	ND	ND
	3.9	ND	ND	ND	ND	ND	+
NaCl (%)	4	+	+	+	+	ND	+
	6.5	-	V	V	+	ND	+
Production of Acetoin		-	+	+	+	-	ND
CO_2 on citrate		-	+	+	-	ND	ND
Dextran		ND	ND	ND	ND	+	-
Use of citrate		-	+	+	-	ND	ND
Gelatinase		-	-	-	V	ND	ND
sherman's milk		+	+	+	+	ND	ND
Fermentation of sugars :							
Lactose		+	+	+	+	+	+
Maltose		+	+	+	+	+	+
Mannitol		-	-	-	-	-	+
Saccharose		+	+	+	+	+	+
Fructose		ND	ND	ND	+	+	+
Xylose		+	+	-	-	+	-
Arabinose		ND	ND	ND	ND	+	+
Raffinose		-	-	-	-	ND	+
Rhamnose		-	-	-	+	ND	-
Ribose		+	+	-	+	ND	+
Fermentation des sucres par galerie		Galerie api 50CHL	Galerie api 50CHL	Galerie api 50CHL	Galerie api 20 STRP	Galerie api 50CHL	Galerie api 50CHL
Species identified		Lc.lactis subsp. lactis	Lc.lactis subsp. Lactis var diacetylactis	Lc.lactis subsp cremoris	Enterococcus Durans	Ln.mesenteroides subsp mesenteroides	Lb.plantarum
Codes		Lc1	Lc5	Lc3	En22	Ln5	Lb11

+ : Positive reaction; - : Negative reaction ; ND : Not determined ; V : variable

These strains (Sa1, Sa4) are isolated from abdominal pus and thigh wounds, respectively.

Antagonism tests

In order to study the spectrum of activity of lactic acid bacteria against MRSA pathogenic bacteria, the well diffusion technique recommended by several authors (Schillinger and Luke, 1989; Ten Brink *et al.,* 1994; Jin *et al.,* 1996) used during this study showed the following (Table 5).

All of the Six lactic acid bacteria selected showed inhibitory activity against the control strain *Staphylococcus aureus* ATCC 25923 and two strains

Ln. Mesenteroide ssp mesenteroide (Ln 5) and *Lb.plantarum* (Lb11) which showed excellent inhibitory activity against the control strain *Staphylococcus aureus resistant to metheciline* ATCC 43300 and clinical human strains of MRSA. After this step, we selected the two strains Ln5 and Lb11 as antagonist strains against MRSA for subsequent tests.

Table 4. Antibiogram of the pathogens strains.

Antibiotics (disk content)	Sa1	Sa2	Sa3	Sa4	Sa5	ATCC 43 300	ATCC 25 923
Penicillin (10 units)	R	R	R	R	R	R	S
Oxacillin (1 ug)	R	R	R	R	R	R	S
Céfoxitin (30 ug)	R	R	R	R	R	R	S
Amoxicillin/clavulanic acid (20/10 ug)	R	R	R	R	R	R	S
Gentamycin (10 ug)	R	R	R	R	R	R	S
Clindamycin(2ug)	R	R	R	R	R	R	S
Fusidique acid (10 ug)	R	R	R	R	R	R	S
Rifampicin (5ug)	R	R	R	R	R	R	S
Chloranphenicol (30ug)	R	R	R	R	R	R	S
Doxycyclin (30ug)	R	R	R	R	R	R	S
Tobramycin (30ug)	R	R	R	R	R	R	S
Ceftaroline (30 ug)	R	R	R	R	R	R	S
Vancomycin (30 ug)	R	S	S	R	S	S	S
Azithromycin (15 ug)	R	R	R	R	R	R	S
Erythromycin (15 ug)	R	R	R	R	R	R	S

S : sensitive ; R : resistant.

Table 5. Inhibitory effect of lactic acid bacteria supernatants on pathogenic bacteria.

Pathogenic bacteria	Inhibition zone diameter (mm)					
	Lb11	Ln5	En22	Lc1	Lc3	Lc5
S.aureus ATCC 25923	20	22	10	12	12	12
S.aureus ATCC 43300	20	21	0	0	0	0
Clinical humain strain :						
Sa1	19	23	0	0	0	0
Sa2	18	20	0	0	0	0
Sa3	20	21	0	0	0	0
Sa4	19	22	0	0	0	0
Sa5	20	21	0	0	0	0

0 : No zones of inhibition.

Production of bacteriocin by lactic strains

The treatment of the supernatants of the cultures of the selected lactic strains (Ln5 and Lb11) by two proteolytic enzymes: trypsin and α-chymotrypsin show a total absence of ZI for the supernatants treated with the enzymes and clearly distinguishable zones for the supernatants not subjected to the action of proteases (Fig.3) which shows that the inhibiting agent produced by the two strains is true of protein nature, it is therefore bacteriocins.

t: control e1: trypsin e2: α-chymotrypsin

Characterization of bacteriocins

Effect of heat treatments

The results show ZI comparable to those of the control (supernatant not treated with heat) for the two selected strains (Table 6). In this context, some authors have shown the thermoresistance of bacteriocins, such as (Bouricha, 2011) author has shown resistance to an antimicrobial substance

secreted by L*euconostoc* cameline to 100°C. (Labioui *et al.,* 2005) showed the heat resistance of bacteriocins produced by a strain of *streptococcus*.

Effect of pH

Our results show ZI comparable to those of the control (untreated supernatant) for the two strains (Ln5 and Lb11) (Table 7); this indicates the stability of this proteinaceous substance at acidic and basic pH. These results are in similar comparable agreement with those presented in Morocco by (Khay *et al.,* 2011), who report the stability of antibacterial substances of protein nature isolated from camel milk of the species *Enterococcus durans*, *Lactococcus lactis*, *Enterococcus faecium*, *Lactococcus cremoris* and *Enterococcus avium* over a wide pH range (2-10).

Table 6. The spectrum of activity of antibacterial substances (bacteriocin) after heat treatment, Zone of inhibition expressed in mm.

Heat treatment	**Ln5**	**Lb11**
Control (not heat treated)	15	13
60°C/10min	14	14
70°C/10min	13	14
80°C/10min	14	15
90°C/10min	14	14
100°C/30min	12	12
121°C/15min	12	12

Table 7. The spectrum of activity of bacteriocin for two strains screened at different pH values, Zone of inhibition expressed in mm.

PH	Ln5	Lb11
2	14	13
3	14	13
4	15	14
5	14	13
6	15	14
7	13	14
8	14	13
9	13	13
10	13	12

Study of the antibiotic resistance of lactic strains

The study of antibiotic resistance in lactic bacteria constitutes an important criterion of the probiotic potential of lactic strains. A probiotic organism must not be resistant to antibiotics; if it is resistant, the resistance gene should be located on the chromosome (Chesson, 2002) but not on the plasmid or on other elements transferable to other microorganisms. We tested 14 ATB belonging to different families against the lactic acid bacteria used in this study (Table 8).

Table 8. Antibiogram of LAB.

Antibiotics (disk content)	Ln5	Lb11
Penicillin (10 units)	S	S
Oxacillin (1 ug)	S	S
Céfoxitin (30 ug)	S	S
Amoxicillin/clavulanic acid (20/10 ug)	S	S
Gentamycin (10 ug)	S	S
Clindamycin(2ug)	S	S
Fusidique acid (10 ug)	S	S
Rifampicin (5ug)	S	S
Chloranphenicol (30ug)	S	S
Doxycyclin (30ug)	S	S
Tobramycin (30ug)	S	S
Ceftaroline (30 ug)	S	S
Vancomycin (30 ug)	R	S
Azithromycin (15 ug)	S	S
Erythromycin (15 ug)	S	S

According to the results, the strains of *Ln.mesenteroides* and *Lb.plantarum* are sensitive to the majority of ATB (Aarestrup *et al.,* 2000). Resistance to ATB depends on the country of origin of the strains and the ATB used there. However, we note the resistance of *Leuconostoc mesenteroides ssp. Mesenteroides* (Ln5) to vancomycin, according to (Salminen *et al.,* 1998), the Resistance of *Leuconostoc spp* to vancomycin is natural and not acquired by gene transfer. Work by Hummel *et al.* (2007) reported the determination of phenotypic resistance profiles; they showed that *Leuconostoc* resistance is intrinsic and generally considered non-transferable.

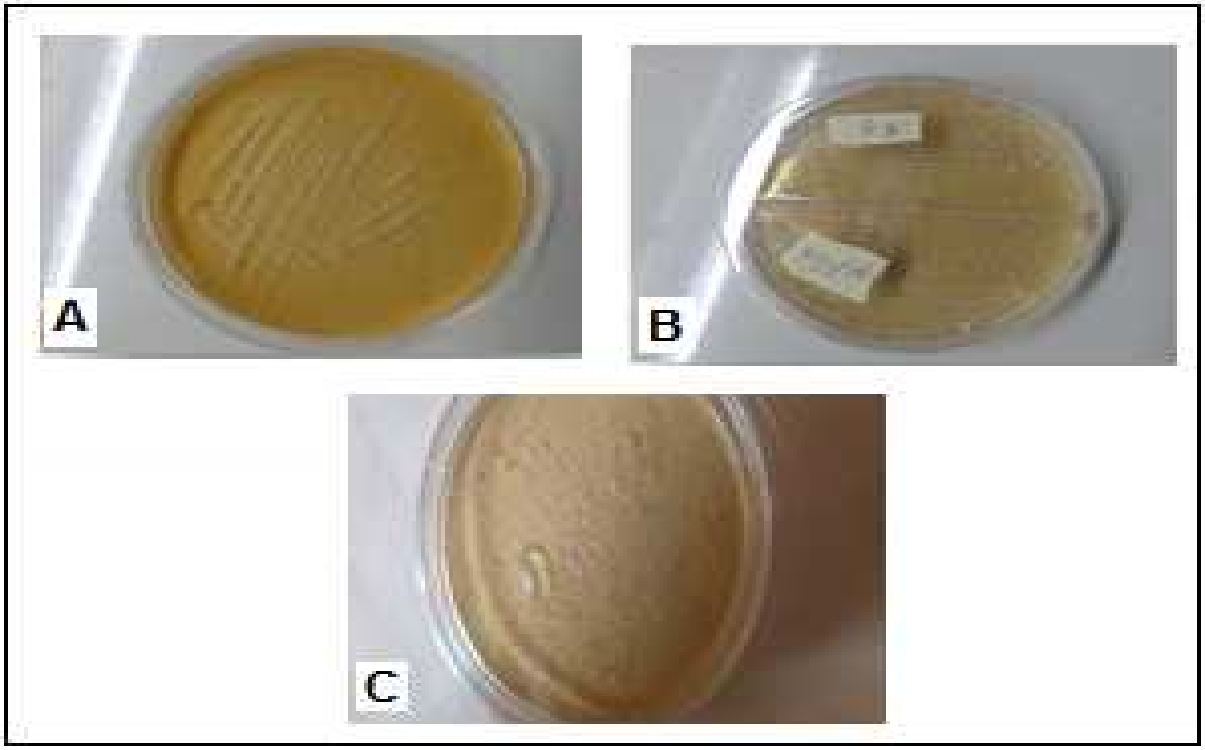

Fig. 1. Macroscopic appearance of colonies isolated from camel milk, A: on M17 medium B: on MRS medium C: on MSE medium.

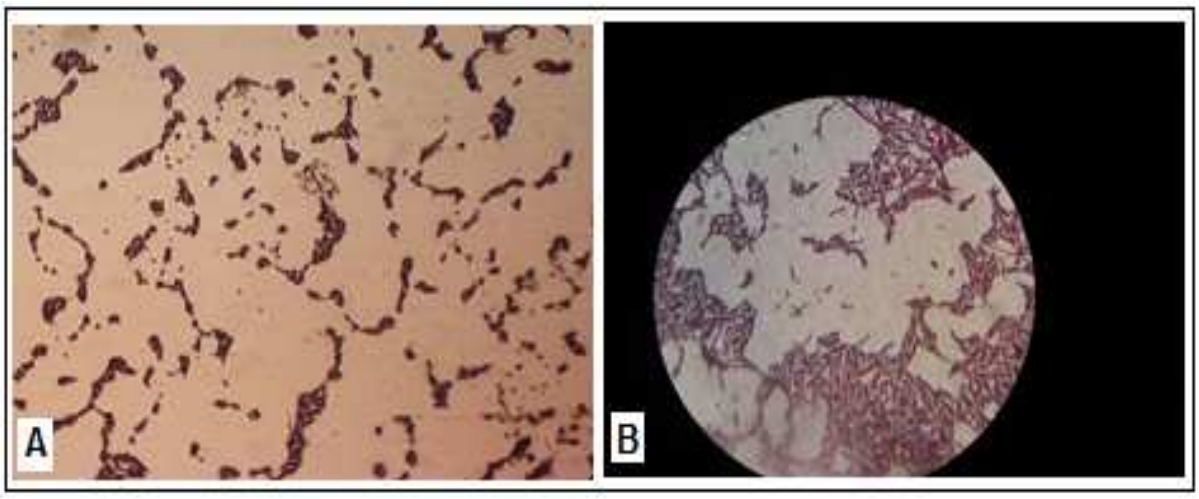

Fig. 2. Microscopic examination of A: *Leuconostoc,* B: *Lactobacillus.*

According to the European Food Safety Authority, the use of the Leuconostoc bacterium in the production of dairy and other products represents a low potential for the spread of genes encoding resistance.

Fig. 3. Zones of inhibition created by supernatants of strains of leuconostoc and lactobacilli after treatment with enzymes.

Conclusion

Among the antibacterial substances produced by lactic strains isolated from camel milk in this study, substances proteinaceous in nature and thermostable (bacteriocin), keeping their effect over a wide range of pH, these bacteriocins have anti- MRSA activities in vitro. The results presented in this study provide a clearer idea of the antibacterial potential of bacteriocins produced by *Leuconostoc mesenteroides* and *Lactobacillus plantarum*, which represent a way forward to compensate for antibiotic treatments.

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