



Research Article

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**Antibacterial activity of marine bacteriocinogenic *Lactobacillus casei* Lb 28 against clinical pathogens including multidrug-resistant organisms(MDROs).
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ABSTRACT

Lactobacillus (Lb.) casei Lb 28, isolated from gastrointestinal tract of marine fish (Sarda sarda), produce bacteriocin which has wide spectrum of inhibition against human pathogenic bacteria: S aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus mirabilis, Enterobacter cloacae, Shigella sp, Bacillus cereus, methicillin-resistant Staphylococcus aureus (MRSA), methicillin-resistant Staphylococcus epidermidis (MRSE) and penicillin-resistant Streptococcus pneumonia (PRSP). However, no activity was detected on vancomycin resistant Enterococcus faecalis (VRE), Acinetobacter sp. and Candida albicans. The inhibitory activity of bacteriocinogenic Lb. casei Lb28 was eliminated upon treatment with proteinase K, α -chymotrypsin and trypsin but was not affected by lipase and α -amylase. Likewise, the antibacterial activity of crude supernatant fluid was maintained after heating at 121 °C for 20 min, at acidic and neutral pHs (4 – 10). The results obtained in this study revealed that the ability of bacteriocins produced by LAB of marine fish in inhibiting a wide-range of human pathogenic is of potential interest for food safety and may have future applications as food preservative and/or possible antibiotic alternatives.

Key words: marine fish, *Lactobacillus casei*, bacteriocin, human pathogenic bacteria, antibiotic-resistant microorganisms.

INTRODUCTION

With the abusive use of antibiotics, in various areas, scientists all over the world are facing to development and spread of antibiotic-resistant bacteria. Drug resistance has become a large and growing problem which causes treatment failures and consequently more severe and longer lasting diseases, increased hospitalization rates, more deaths, and higher costs to society. Faced with this worrying situation, there is a continuous demand for novel antimicrobials for clinical, veterinary and food applications. Numerous antibacterial agents are now being considered, such as bacteriophage [1], probiotic bacteria [2-3] and antimicrobial peptides [4] and bacteriocins [5].

In the past decade, the interest research in bacteriocin, especially from lactic acid bacteria (LAB), has gained great momentum due to its potential as both a natural food preservative and as therapeutic antibiotics. Bacteriocins are antimicrobial, proteinaceous compounds with a bactericidal mode of action against bacteria closely related to the producer strain [6]. Some bacteriocins are active against foodborne pathogens such as *Listeria monocytogenes*, *Clostridium perfringens*, *Bacillus cereus*, *Staphylococcus aureus* and spoilage LAB [5].

In the food industry, have been proposed for a long time, as a solution to the problems of food spoilage and food-borne infections. Up to now, nisin remains the only commercially available and industrially utilized bacteriocin despite a vast array of bacteriocins being discovered in the past two decades [7]. Whereas for clinical applications, bacteriocins have been presented as a viable alternative to antibiotics due to the high specificity of some bacteriocins against Gram-positive human and animal pathogens, including multidrug-resistant nosocomial pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) [8].

Marine Environment contains a huge diversity of microbial populations, many of them are still relatively uncharacterized and therefore, represent a potentially enormous untapped resource [9]. Lactic acid bacteria,

especially those isolated from marine fish, have been shown to produce a number of bacteriocin-like substances [10]. However, few bacteriocins of marine origin have been fully characterized and identified to date [9].

Thus the aim of this study was undertaken to focus on the bacteriocinogenic potential of marine *Lactobacillus casei* strains, isolated from marine fish, against human pathogenic bacteria including multidrug-resistant organisms.

EXPERIMENTAL SECTION

2.1 Microorganisms and their maintenance

Previously, *Lb. casei* Lb 02, Lb 15, Lb 28 and Lb 42 strains were isolated from the gut of marine fish (*Sarda sarda*), collected from the coast of Oran – Algeria and were identified as a Gram-positive, nonspore-forming and catalase negative. Further, they were identified by physiological and biochemical tests as described by [11-12-13] Carbohydrate fermentation patterns were obtained using API 50 CH (bio Mérieux, France) according to the manufacturer's instructions.

Lb. casei strains kept in MRS broth containing 20% (v/v) glycerol at -20 °C and were sub-cultured twice in MRS broth [14] for activation prior to experimental use. All pathogenic organisms (Table 1) were maintained as frozen stocks at -20°C in TSBYE (tryptic soy broth supplemented with 6 g l⁻¹ yeast extract) containing 20% (v/v) of glycerol. The cultures were propagated twice in TSBYE at 30°C for 18 h before use.

The isolates of *Klebsiella pneumonia*, *Enterobacter cloacae*, *Proteus mirabilis*, *Shigella* sp, methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *S. epidermidis*; (MRSE) , penicillin-resistant *Streptococcus pneumonia* (PRSP), vancomycin resistant *Enterococcus faecalis* (VRE) and *Acinetobacter* sp. were isolated from different clinical specimens at Mascara hospital – Algeria and were screened for antibiotic resistance according to criteria of National Committee for Clinical Laboratory Standards (NCCLS, 2002) and Manual of Antimicrobial Susceptibility Testing guidelines [15]. The strains (*Klebsiella pneumonia*, *Acinetobacter* sp, *Enterobacter cloacae*, *Shigella* sp and *Proteus mirabilis*) are resistant to several antibiotics.

2.2 Assay for antimicrobial activity

Antimicrobial activity was checked by using the agar-spot test described by [11]. An overnight culture of *Lb. casei* strains was spotted on the surface of an MRS plate and incubated for 18 h to 24h at 30°C. Spotted plates were overlaid with 7 ml of each test strain (10⁶ CFU/ml), imbedded in a thin layer of soft Mueller Hinton Agar (7% (w/v) agar). After incubation during 24h at 30°C, inhibition was recorded positive in presence of a detectable clearing zone around the colony of the producer strain.

2.3. Bacteriocin bioassay

Bacteriocin screening was investigated by Agar well diffusion method as described by [16]. The lactobacilli culture was grown in MRS broth (pH 5.5) at 37 °C for 18-20 h and then was centrifuged at 10,000 rpm for 5 min. The cell-free supernatant (CFS) of LAB was adjusted to pH 6.5 using 1M NaOH to exclude the antibacterial effect of organic acids. Inhibitory activity of hydrogen peroxide was eliminated by addition of catalase at a final concentration of 1mg/ml. Untreated and treated (neutralized and neutralized + catalase) cell free supernatants placed in the wells were allowed to diffuse into the agar for 1 h at room temperature. The plates were then incubated at 37°C in microaerophilic conditions for 24 h. The diameter of inhibition zone formed around the wells was calculated as the difference between the diameter of the total inhibition zone and the diameter of the well. The inhibition is noted positive if the diameter is superior to 2 mm.

2.4. Effect of enzymes, pH, and temperature on bacteriocin activity

Sterile cell-free supernatants (SCFS) at pH 6.5 were treated with the following enzymes (0.2 mg ml⁻¹): proteinase K (pH 7.0 Sigma USA); trypsin (pH 8.2 Merck, Germany); α chymotrypsin (pH 8.0, Sigma, USA); lipase (pH6 Sigma, USA), α -amylase (pH 7, Sigma, USA). All these solutions were filter-sterilized and then added to supernatants (v/v, 1/1). Untreated bacteriocin and enzyme solutions were used as controls. All samples and controls were incubated at 37°C for 2h.

To determine the activity of bacteriocins at different pH levels, SCFS was adjusted with sterile 2N NaOH or HCl to different pH values (2.0, 4.0, 6.0, 8.0, 10.0 and 12.0) and was incubated at 37°C for 2 h. The pH-treated sample was neutralized to pH 6 and then tested for antibacterial activity.

To evaluate thermostability, aliquots of sterile cell-free supernatant was heated to 60°C, 80°C, 100°C at 15min /30 min, and 121°C/20 min, immediately cooled in ice and tested for antibacterial activity.

In all the cases the residual activity of treated and untreated samples was determined against the indicator strain against methicillin-resistant *Staphylococcus aureus* (MRSA) by using agar well diffusion method described above [16]. All experiments were performed in triplicate.

2.5. Mode of action

Mode of action was checked as described by [17]. 02 ml of SCFS were added to 08 ml of the indicator strain at the beginning of the exponential phase (4h). Growing cells of Methicillin-resistant *Staphylococcus aureus* (MRSA) in TSB broth without SCFS was used as control. Bacterial growth was monitored by measuring the optical density at 600 nm at different time intervals (every 02 hours).

2.6. Statistical analyses All experiments were carried out in triplicate. Statistical analyses were performed using the STATGRAPHICS. Version 1.4 software (Manugistics Inc., Cambridge, MA). Analysis of variance (ANOVA test) was used to determine differences between means.

RESULTS AND DISCUSSION

In the present study, four autochthonous strains of *Lb. casei* were isolated from gastrointestinal tract of coastal fish: Atlantic bonito (*Sarda sarda*). They were screened for their antagonistic activities against human pathogenic microorganisms as shown in Table 1. All strains showed antibacterial activity against at least one of the target strain (Figure 1).

Table -1: Inhibitory spectra of *Lb. casei* strains exhibiting antimicrobial activity (mm)

Indicator strain	Strain No	Lb02	Lb15	Lb28	Lb42
<i>S. aureus</i>	ATCC 25923	23±0,03	27±0,01	28±0,12	26±0,14
<i>E. coli</i>	ATCC 25922	25±0,12	12±0,01	24±0,03	18±0,02
<i>P. aeruginosa</i>	ATCC 27853	14±0,01	12±0,00	26±0,08	23±0,17
<i>K. pneumonia</i>	CHU12	-	21±0,05	17±0,02	-
<i>Acinetobacter sp</i>	CHU 03	23±0,00	-	-	18±0,04
<i>Shigella sp</i>	CHU 24	-	23±0,01	25±0,00	24±0,01
<i>E. cloacae</i>	CHU 19	22±0,02	17±0,00	21±0,02	26±0,00
<i>P. mirabilis</i>	CHU 23	25±0,23	22±0,16	27±0,05	27±0,14
<i>B. cereus</i>	CHU 08	16±0,06	26±0,04	19±0, 23	25±0,00
Methicillin-resistant <i>S.aureus</i> (MRSA)	MR 04	18±0,01	-	28 ±0,06	27±0,08
Methicillin-resistant <i>S.epidermidis</i> ; (MRSE)	MR 09	-	13±0,02	20±0,10	-
Vancomycin-résistant <i>E. faecalis</i> (VRE)	MR 25	19±0,03	21±0,03	-	28±0,01
Penicillin-resistant <i>S. pneumonia</i> (PRSP)	MR 10	14±0,01	13±0,00	22±0,08	17±0,03
<i>C.albicans</i>	LUV 01	-	-	-	-

The inhibitory effect, which was observed by the formation of clear and distinct zones around the colony of the producer strain, may be due to the production of several antibacterial compounds; organic acids, H₂O₂ or bacteriocins [18]. No strain has inhibited *Candida albican*.

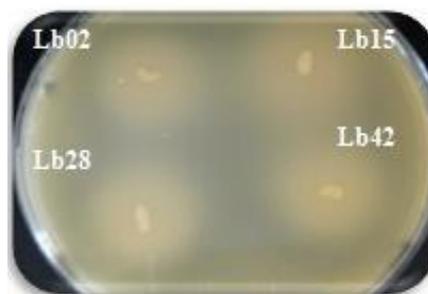


Figure 1. Inhibition of Penicillin-resistant *S. pneumonia* (PRSP) by *Lb. casei* strains

Lactobacillus casei Lb. 28 and Lb. 42 displayed broad antibacterial activity against several genera of Gram-positive and Gram-negative bacteria. Moreover a high level of inhibitory activity against *S. aureus* ATCC 25923, Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Proteus mirabilis* was observed.

Only *Lb. casei* Lb28 kept its antibacterial activity against Methicillin-resistant *Staphylococcus aureus* (MRSA) in neutralized and catalase-treated culture supernatants. Also, Addition of proteinase K; trypsin and α -chymotrypsin stopped their antibacterial activity. The other enzymes tested in our study (α -amylase and lipase) did not cause

inactivation. This confirmed that carbohydrate and lipid moieties if existing were not required for the inhibitory activity.

The destruction of the antibacterial activity by proteases suggested that this compound could be a peptide or bacteriocin-like inhibitory substances (BLIS). These results were comparable to those obtained by [19]. These authors have confirmed that *Lactobacillus casei* AP8 and *Lactobacillus plantarum* H5 isolated from intestinal bacterial flora of marine fish : beluga (*Huso huso*) and Persian sturgeon (*Acipenser persicus*) were able to produce

Table 2. Effect of enzymes, pH and temperature on antibacterial activity of *Lb. casei* Lb 28

	Treatment	Lb 28
Enzyme	α -Chymotrypsin	-
	proteinase K	-
	trypsin	-
	Lipase	+
	amylase	+
	Catalase	+
pH		4-10
Heat treatment	60,80,100°C /15min	+
	60,80,100°C /30min	+
	121°C/20min	+

bacteriocin which have a potential inhibitory against pathogenic and spoilage microorganisms such as *A. hydrophila*, *A. salmonicida*, *C. perfringens*, *B. cereus* and *L. monocytogenes*.

Indira K. et al. [20] have revealed that bacteriocin from *Lb. casei*, fish gut (*Mugil cephalus*) and prawn muscle (*Peneaus monodon*), seems to be ideal for industrial scale production and commercial utilization.

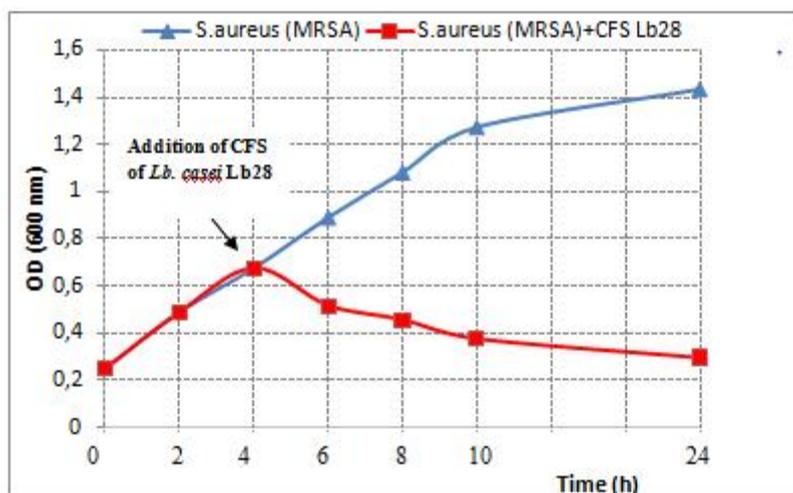


Figure 2. Effect of CFS of *Lb. casei* Lb 28 on the growth of Methicillin-resistant *S. aureus* (MRSA)

On the other hand, it was observed in other reports that some bacteriocins produced by other strains of LAB exhibited broad antimicrobial spectrum to both Gram-positive and Gram-negative bacteria including some antibiotic-resistant strains [21]. Likewise, the antibacterial activity of crude supernatant fluid was maintained after heating at 121 °C for 20 min, at acidic and neutral pHs (4– 10). These results were similar as some bacteriocins [19-22].

Exposure of Methicillin-resistant *Staphylococcus aureus* (MRSA) to active culture supernatant of *Lb. casei* Lb28 resulted in a strong decrease of the optical density (Figure 2). After four hours, the optical density (OD600) declined from 0.89 to 0.52 indicating the bacteriostatic mode of action. Bacteriocins that are produced by LAB can have bactericidal or bacteriostatic activity [23].

CONCLUSION

Bacteriocinogenic *Lb. casei* Lb28 showed a wide spectrum of antibacterial activity against human pathogenic bacteria including multidrug-resistant organisms. Accordingly, LAB strains derived from marine fish may be of great interest as a viable alternative for clinical, veterinary and food applications.

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