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Full Length Research Paper

Technological characterization of lactic acid bacteria isolated from intestinal microbiota of marine fish in the Oran Algeria coast

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In spite of overwhelming information about lactic acid bacteria (LAB), only a few studies are available for the LAB of marine environment. The purpose of the present study was the isolation and characterization of new strains of LAB from gastrointestinal tract of coastal fish: sardine (*Sardina pilchardus)* and bug (*Boops boops)*. A total of 67 strains of LAB were isolated of which 16 strains displayed antibacterial activities against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae*, *Bacillus cereus*, *Salmonella* sp. and *Enterococcus faecalis.* Also an antifungal activity was detected against *Fusarium oxysporum* and *Aspergillus* sp. The strains selected for their antimicrobial activity were identified on the basis of phenotypic characters including API system as: *Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *diacetylactis, Leuconostoc* sp. and *Lactobacillus plantarum.* Three strains were screened for the study of their antibacterial compounds: *L. lactis* subsp*. lactis* MT, *L. lactis* subsp*. diacetylactis* BT4 and *L. lactis* subsp*. diacetylactis* BL10. It was observed that inhibitory activities of all three LAB strains were due to bacteriocin-like substances. This antimicrobial activity was inactivated by the addition of proteinase K, α-chymotrypsin, but not by lipase. All strains were able to hydrolyze casein, majority showed amylolytic activity and produces biogenic amines. However, they were non-hemolytic, showed no particular antibiotic resistance profile and none of them was found to possess lipolytic activity.

Key words: Marine fish, *Lactococcus, Enterococcus,* biogenic amines, antimicrobial activity.

INTRODUCTION

Lactic acid bacteria (LAB) constitute a diverse group of Gram-positive bacteria, possessing some common morphological, metabolic and physiological characteristics. The general description of the bacteria included in the group is Gram-positive, non sporulating, catalase and oxidize negative, rods and cocci ,that produce lactic acid as the major metabolite of the carbohydrate fermentation. LAB are anaero-aerotolerant and generally have complex nutritional requirements especially for amino acids and vitamins (González et al., 2000).

Lactic acid bacteria (LAB) are used for the production of a wide variety of fermented food products, in which they contribute to the improvement of flavor, texture and shelf-life. (Indira, 2011). They also help in keeping microbial quality by producing antimicrobial substances such as organic acids, diacetyl compounds, hydrogen peroxide and bacteriocins in dairy products (Ennahar et al., 2000; Lasagno et al., 2002). LAB are widespread in nature and commonly found in many food products (dairy, meat, fruit, vegetables, etc.), as well as in genital, intestinal and oral cavity of animal and human beings

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(Leroi, 2010). They are not considered as belonging to aquatic environments, but certain species (*Carnobacterium*, *Vagococcus*, *Lactobacillus*, *Enterococcus*, *Lactococcus*) have been found in freshwater fish and their surrounding environment (González et al., 2000). Indeed several studies have demonstrated that *Streptococcus*, *Leuconostoc*, *Lactobacillus*, and *Carnobacterium* belong to the normal microbiota of the gastrointestinal tract in healthy fish (Ishikawa et al., 2003; Franzmann et al., 1991). They are not dominant in the normal intestinal microbiota of fish, but some strains can colonize the gut (Denev, 2009) and inhibit adhesion of several fish pathogens bacteria (Balcázar et al., 2008). The aim of this study was to isolate, characterize and identify new lactic strains from gastrointestinal tract of coastal fish as well as some technological properties such as enzymatic activities, antimicrobial properties, ability to produce biogenic amines and antibiotic resistance.

MATERIALS AND METHODS

Bacterial strains and media

Twenty five of sardine *(Sardina pilchardus)* and thirty of bug (*Boops boops)* were collected from the coast of Oran – Algeria. Each specimen was dissected aseptically after capture. The contents squeezed from the intestinal tracts were serially diluted with sterile saline solution up to 10^{-5} dilutions. Subsequently, 0.1 ml of each dilution was inoculated into MRS broth (De Man, Rogosa and Sharp, Merck, Germany) and incubated at 30°C for 18 h. The enrichments were then plated onto the following solid media: MRS adjusted to pH 6.2 and 5.4 .Colonies were selected randomly and purified by re-streaking (Leisner et al., 1997). Purified isolates of LAB were inoculated into MRS broth (pH 6.5) and incubated at 30°C for 24 h. All strains were investigated to determine their colony morphology, cell morphology, motility, Gram stain, spore formation, and catalase production as described by Harrigan and McCance (1976). The short term conservation of the pure isolates was done on solid media (Badis et al., 2003). After growth at optimal temperature, the culture was maintained at 4°C and cultures were renewed every month. The long-term conservation of the purified isolates was carried out in MRS broth containing glycerol (Merck, Darmstadt, Germany) (v/v 70/30) and were maintained as frozen stocks at -80°C (Papamanoli, 2002).

Technological properties

Screening for antagonistic activity

1. Antibacterial activity: Isolates of LAB were screened for antagonistic activity by the agar spot method of Schillinger and Lücke (1989). The indicator strains used for antagonisms included: *S. aureus* (ATCC 25923), *E. coli (ATCC25922), Pseudomonas aeruginosa (*ATCC27853*), K. pneumoniae*, *B. cereus*, *Salmonella* and *E. faecalis.*

Strains exhibiting antagonistic activities against undesirable bacteria were investigated for their antibacterial compounds as described by Ammor et al. (2005). In order to eliminate the inhibitory effect of lactic acid and/or H_2O_2, the supernatants were adjusted to pH 6.5 with 1 M NaOH and treated with catalase at final concentration of 65 (UI/ml), following by filtration through a 0.22 µm pore size filter (Type Minisart NML; Sartorius GmbH, Göttingen, Germany). 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 (Thompson et al., 1996).

2. Antifungal activity: The antifungal activity of LAB was investigated with an overlay assay (Lind et al., 2005; Magnusson and Shnürer, 2001). Microorganisms used for the antifungal activity are *F. oxysporum and Aspergillus* sp. The degree of inhibition was calculated as the area of inhibited growth in relation to the total area of the Petri dish and the scale was the following: - = no visible inhibition, (+) = weak inhibition in the soft agar above the bacterial growth, + = no fungal growth on 0.1–3% of plate area/bacterial streak, ++ = no fungal growth on 3–8% of plate area/bacterial streak, +++ = no fungal growth on >8% of plate area/bacterial streak.

3. Sensitivity of the bacteriocin-like substance to enzymes: Cell-free supernatant at pH 6.5 was treated with the following enzymes: proteinase K, α-chymotrypsin and lipase at a final concentration of 1 mg/ml in phosphate buffer (pH 6.5). The supernatants were incubated with these enzymes at 37°C for 2 h and the antagonist activity was detected using the well diffusion agar method described above (Corsetti et al., 2004).

Identification of antimicrobial effect strain

Strains with antimicrobial activity were characterized according to the methods and criteria of Buchanan et al. (1974) and Klein (2001) as follow: They were checked for gas production from glucose in MRS broth containing Durham tubes (Greco et al., 2005). Growth at different temperatures was observed in MRS broth after incubation for 5 days at 15, 37, 45°C then 12 days at 5 and 10°C (Badis et al., 2003). The ability to grow at pH3.9 and 9.6 was tested on MRS broth (Dykes et al. 1994). The tolerance to NaCl was studied by growth in MRS broth containing NaCl at concentrations (2, 3, 4 and 6.5%) as described by Stiles and Holzapfel (1997) . Arginine dihydrolase was determined in MRS broth supplemented with 0.3% arginine, which was incubated for 3 days, followed by NH3 detection by addition of Nessler's reagent (Gelman et al., 2000). Hydrolysis of esculin and the effect of bile salts were observed on bile esculin agar. Heat resistance was determinated in MRS broth at 60.5°C for 30 min (Samelis et al., 1994). Citrate utilization was studied on the media of Kempler and Mc Kay (1980).Production of dextran was recorded on MSE agar (Mayeux et al., 1962) and the production of acetoine from glucose was determined by using the Voges–Proskauer test (Clark and Lubs, 1915). Carbohydrate fermentation patterns of LAB were determined by means of miniaturized API 50 CH biochemical tests (BioMérieux, Marcy L_Etoile, France).

Acidification activity

The acidification activity was tested according to Ammor et al.

(2005). It is determined by measuring the Dornic acidity that expresses the acidity developed in the medium by transformation of lactose into lactic acid. *Streptococcus thermophilus* of dairy origin was used as a control.

Proteolytic activity

Surface-dried plates of milk agar were streaked with 24 h old cultures, after incubation at -30°C for 4 days, and examined for any clearing of casein around and underneath the growth for assessment of proteolytic activity (Thapa et al., 2006).

Lipolytic activity

Lipolytic activity against tributyrin was detected by a clear zone surrounding the culture in the turbid tributyrin agar (Leuschner et al*.,* 1997).

Amylolytic activity

The assessment of amylolytic activity was tested on starch agar and incubation was for 4 days at 30°C. The plates were flooded with Gram`s iodine solution for 15 to 30 min . the positive result was detected by a clear zone around and underneath the culture. (Bridget, 2011).

Safety characteristics

1. The decarboxylase test for production of biogenic amines was made by inoculating cell suspensions of each isolate into microtiter plates containing 150 ml of the modified decarboxylation medium described by Maijala (1993) and modified by Bover-Cid and Holzapfel (1999). The following amino acids were added to 2 g/l final concentration as precursors: lysine, ornithine, histidine and tyrosine (Sigma). *E. coli* (ATCC25922) was used as positive control.
2. Susceptibility to antibiotics was determined using agar diffusion discs of chloramphenicol (Chl), tetracycline (Tet), amikacin (Ami), erythromycin (Ery), vancomycin (Van), penicillin G (Pen) , rifamycin (Rif) and nalidixic acid (Nal) as recommended by the supplier (Bio-Rad, Hercules, CA, USA). Analyses were performed in triplicate.
3. Haemolytic test was performed at 37°C in Columbia Agar with sheep defibrinated blood (Oxoid, Unipath, Basingstoke, UK).

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

Screening for antagonistic activity

A total of 67 isolates were isolated from various marine fish species, of which 16 isolates were shown to produce inhibition zones against some indicator microorganisms: *Staphylococcus aureus (*ATCC 25923), *E. coli (ATCC25922), Pseudomonas aeruginosa (*ATCC27853)*, K. pneumoniae*, *B. cereus*, *Salmonella* and *E. faecalis)*. A significant difference ($P < 0.05$). These isolates were identified on the basis of phenotypic characters including API system as *L. lactis* subsp *lactis*, *L. lactis* subsp *diacetylactis, Leuconostoc* sp and *L. plantarum*. According to our results, it was noticed, that two strains demonstrated antagonistic activity against seven target strains. *L. lactis* subsp*. lactis* MT and *L. lactis diacetylactis* BT4 (Table 1). The diameters of the inhibition halos were in all cases within the 15–21 mm range (Figure 1).

1. *L. plantarum* strains displayed inhibition zones against *S. aureus*, *E coli, K. pneumoniae*, *B. cereus* however, no inhibition zone was detected against *P. aeruginosa and E. faecalis*.
2. *E. faecium* exerted its inhibitory effect on *S. aureus*, *E. coli, P.aeruginosa,K. pneumoniae*, *Salmonella sp but not on B. cereus*, and *E. faecalis*.
3. *Leuconostoc* sp. has a weak inhibition against the indicators microorganisms. Indeed no antagonistic activity was observed against *P. aeruginosa, K. pneumoniae, Salmonella* sp and *E. faecalis.*

Varying degrees of inhibition were detected against the isolates of *F. oxysporum* and *Aspergillus* sp. (Table 1) except *Lb. plantarum.* Figure 2 illustrates the antifungal activity of the three lactic strains selected in our study: *L. lactis* subsp. *lactis* MT, *L. lactis diacetylactis* and *L. lactis* subsp*. diacetylactis* BL10.

These results allow us to select three strains for the study of their antibacterial compounds: *L. lactis* subsp*. lactis* MT, *L. lactis* subsp. *diacetylactis* BT4 strains exhibiting antagonistic activity against all indicator microorganisms and *L. lactis* subsp*. diacetylactis* BL10 that inhibited *71.42%* against undesirable bacteria. This latter strain is the only one that does not produce biogenic amines. The three strains have exhibited inhibition zones for neutralized culture supernatants and catalase treated supernatants. Results from enzyme inactivation studies confirmed that antimicrobial activity was lost after treatment with the proteolytic enzymes like proteinase K, α chymotrypsin, whereas treatment with lipase, did not affect the activity of antimicrobial substance produced by the test isolates. The sensitivity of the found substance to proteolytic enzymes is a proof of its proteinaceous nature.

Acidification activity

Our result showed that the control strain ST produces a high amount of acidity during the first hours of growth

Table 1. Inhibitory spectra of LAB isolate exhibiting antimicrobial activity.

Strain LAB	code	Inhibitory spectra of LAB isolate exhibiting antimicrobial activity indicator micro-organisms								
		S. aureus	***E. coli***	***K. pneumoniae***	***B. cereus***	***Salmonella* sp.**	***P. aeruginosa***	***E. faecalis***	**F. *oxy***	***A.* sp.**
L. lactis subsp. *diacetylactis*	CT12	07 ±0.1	15±1.2	20±0.1	10±0.8	12±0.1	06±0.2	15±0.1	++	-
	BT5	00	15±0.7	13±0.8	11±0.4	15±0.7	00	00	++	+
	CT7	10 ±0.5	14±0.3	16±1.3	13±0.1	15±1.1	05±0.1	10±0.4	+	+
	MC	16 ±1.0	00	09±0.7	00	03±0.1	0 9±0.3	15±0.1	++	+
	CT11	1.1±0.3	13±0.1	23±1.1	16±0.3	11±0.1	00	00	++	++
	BT4	07 ±0.2	14±0.1	19±0.5	20±1.2	19±1.3	0 9±0.3	05±0.3	++	++
	ST1	08 ±0.1	13±1.0	15±0.4	17±0.1	15±1.1	00	00	++	++
	BL10	13 ±0.9	15±0.4	20±1.3	00	11±0.5	05±0.7	00	+	++
L. lactis subsp. *lactis*	MT	10± 0.3	21±0.6	16±1.2	18±0.7	16±0.7	05±0.2	09±1.2	+	+
	ST2	19±0.1	13±1.2	20±0.7	16±1.3	11±1.2	07±1.2	00	++	+
E. faecium	BL2	11±1.5	14±1.3	20±0.3	00	10±0.4	05±0.1	00	++	+
	CT16	00	21±0.9	00	00	02±0.3	08±0.3	00	++	+
	MT1	15±0.7	00	15±0.5	00	10±0.3	00	00	++	+
Leuconostoc sp.	SC	13±1.1	20±0.8	00	16±0.5	00	00	00	++	+
Lb . plantarum	MT2	12±0.3	06±0.1	22±1.2	00	07±0.6	00	00	-	-
	CT1	15±0.8	20±0.7	23±0.3	16±1.2	11±0.2	00	00	-	-

For indicator bacteria **:** Results are expressed as diameters of the inhibition zone and standard deviations in mm**.** For Indicator fungus: - = no visible inhibition; + = no fungal growth on 0.1–3% of plate area/bacterial streak; ++ = no fungal growth on 3–8% of plate area/bacterial streak and +++ = no ungal growth on > 8% of plate area/bacterial streak. (*F. oxy): Fusarium oxysporum, (A.sp) : Aspegillus* sp.

(47°C after 4 h of culture) and then stabilizes at this value during the fermentation (Figure 3). This stability is due to inhibition of the growth rate which is due to the high acidity of the medium. Bacterial strains display a low power acidifier compared to the ST but constant throughout the fermentation. *L. lactis* subsp. *lactis* MT *and L. lactis* subsp. *diacetylactis* BT4 strains produce lactic acid after fermentation similar to that produced by the control strain ST.

Proteoltic, amylolytic and lipolytic activity

All strains of LAB screened for their antagonistic activity showed proteolytic activity (showing >2 mm hydrolysis zone in milk agar plate) and majority possess an amylolytic activity except *E. faecium* BL2 and *L. lactis diacetylactis* CT11. However none of them was able to hydrolyze tributyrin (Table 2).

Safety properties

According to our results, we note that no strain was hemolytic and sensitive to chloramphenicol,

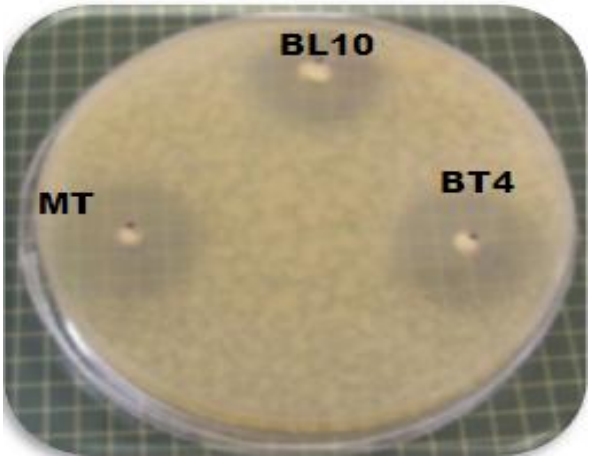

Figure 1. Inhibition of *E. coli (ATCC25922) by L.lactis* subsp*. lactis MT, L.lactis* subsp*. diacetylactis BT4* and *L. lactis* subsp*. diacetylactis BL10.*

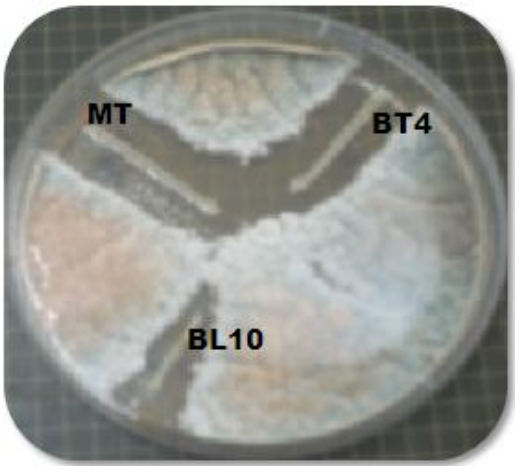

Figure 2. Inhibition of *Fusarium oxysporum by L.lactis* subsp *lactis MT, L.lactis* subsp *diacetylactis BT4and L.lactis* subsp *diacetylactis BL10.*

tetracycline, amicacin and rifamycin (Table 2). Isolates of LAB were screened also for their ability to produce biogenic amines. The results obtained showed that the majority of them produce biogenic amines in the applied method, except *L. lactis* subsp.*diacetylactis* BL10.

DISCUSSION

The main aim of this study was to isolate, identify and provide information on the technological properties of the new lactic strains from gastrointestinal tract of coastal fish: sardine (Sardina *pilchardus)* and bug (*Boops boops)*. Sixteen isolats out of a total of 67 LAB were screened for their antagonistic activities against indicators microorganisms such *S. aureus*, *E. coli, P. aeruginosa, K. pneumoniae*, *B. cereus*, *Salmonella* sp., *E. faecalis,* F. oxysporum and *Aspergillus* sp. LAB screened for their antimicrobial activity was identified as *L. lactis* subsp*. lactis, L.lactis diacetylactis Leuconostoc* sp*., Enterococcus faecium and L. plantarum.* This identification was based on phenotypic characters and

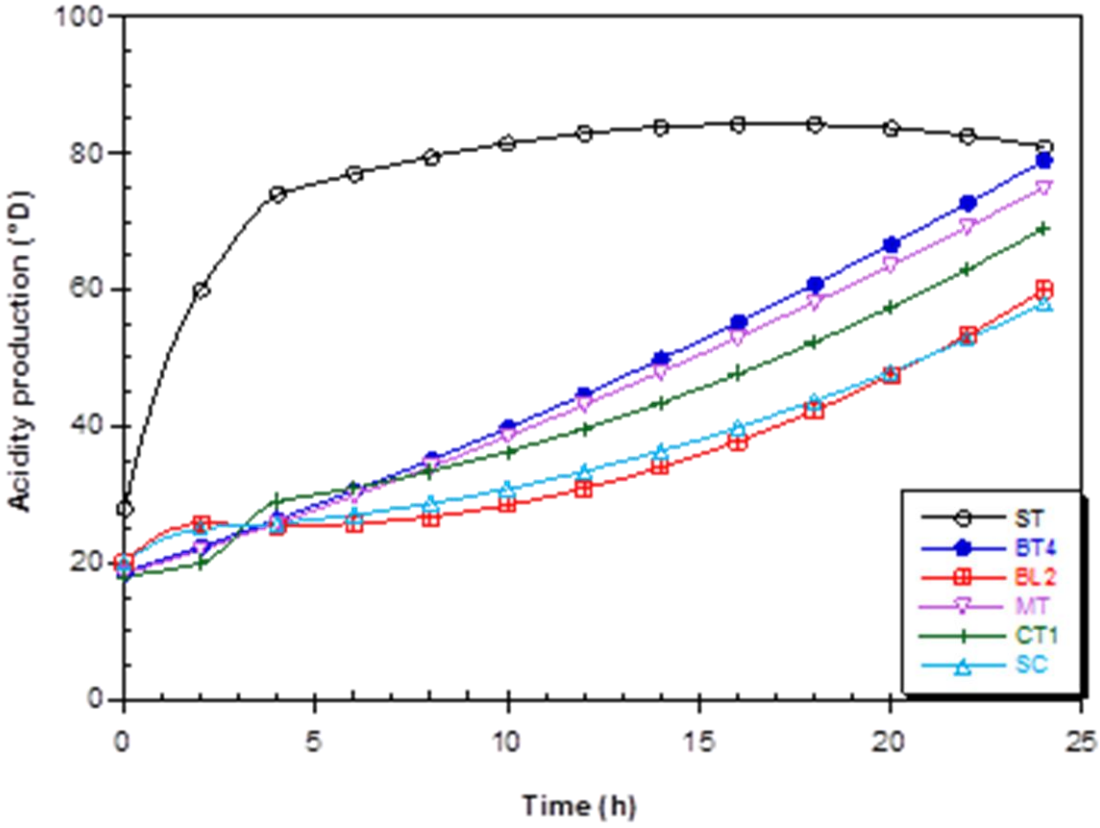

Figure 3. Acidification activity of LAB strains isolated from intestinal tracts of marine fish.

Table 2. Antibiotic activity and enzymatic activity of LAB strains isolated from gastrointestinal tract of coastal fish.

LAB	Pen	Chl	Tet	Ami	Ery	Van	Rif	Nal	Proteolytic activity	Lipolytic activity	Amylolytic activity
CT12	I	S	S	S	I	R	S	R	+	-	+
BT5	S	S	S	S	I	R	S	R	+	-	+
CT7	S	S	S	S	I	R	S	R	+	-	+
MC	S	S	S	S	S	R	S	R	+	-	+
CT11	I	S	S	S	S	R	S	R	+	-	-
BT4	I	S	S	S	S	R	S	R	+	-	+
ST1	S	S	S	S	S	R	S	R	+	-	+
BL10	S	S	S	S	S	R	S	R	+	-	+
MT	S	S	S	S	I	R	S	R	+	-	+
ST2	I	S	S	S	I	R	S	R	+	-	+
BL2	S	S	S	S	S	R	S	R	+	-	-
CT16	S	S	S	S	S	R	S	R	+	-	+
MT1	S	S	S	S	S	R	S	R	+	-	+
SC	S	S	S	S	S	R	S	R	+	-	+
MT2	I	S	S	S	S	R	S	R	+	-	+
CT1	S	S	S	S	I	R	S	R	+	-	+

S- sensible ;I- intermediate; R- resistant . chloramphenicol (Chl), tetracycline (Tet), amikacin (Ami) , erythromycin (Ery), vancomycin (Van), penicillin G (Pen) , rifamycin (Rif) nalidixic acid (Nal).

the API system. The identity of LAB species seems to agree with that of LAB typically reported from other fresh fish species, fish products, or in the intestinal contents of fish (Ringø and Gatesoupe, 1998; Campos et al., 2006; Balcázar et al., 2008; Itoi, 2008; Valenzuela, 2010). The presence of these bacteria in the intestinal tract of marine fish may result from the high adaptability to various environments.

L. lactis subsp.*lactis MT and L.lactis diacetylactis* BT4 showed the broadest spectrum by inhibiting target both Gram positive and Gram negative strains. This antimicrobial activity due probably to the bacteriocins secretion. These results were comparable to those obtained by Campos (2006) and by Ayad et al. (2002) who find that 41% of wild lactococci produced bacteriocins. Balcazar et al., (2008) shows that *L. lactis* isolated from fish had also inhibitory activity Against *Aeromonas hydrophila, Aeromonas salmonicida, Yersinia ruckeri and Vibrio anguillarum.*

The results of antimicrobial activity due to *L. plantarum* partially confirm those of Nieto- Lozano et al. (2002) who find that strains of *L. plantarum* exhibit an inhibitory activity against *S. aureus* but no antagonism was observed against *P. aeuroginosa, E. coli and Salmonella* sp.

According to Valenzuela et al. (2010) *Enterococcus faecium* isolats from seafoods were able to inhibit *S. aureus* and other enterococci, but no antibacterial activity was detected towards *B. cereus* or *E. coli*. However, in our study, no antibacterial activity against *E. faecalis* was noticed.

The antibacterial activity of *L. plantarum Enterococcus faecium* and *Leuconostoc* sp. may be due to the production of many metabolites such as organic acids (lactic and acetic acid), hydrogen peroxide, diacetyl and bacteriocins (Ennahar et al., 2000; Lasagno et al., 2002;Valenzuela et al., 2010).

The detected antifungic activity against *F. oxysporum* and *Aspergillus sp* may be due to organic acids, produce hydrogen peroxide (H_2O_2), proteinaceous compounds or reuterin (Magnusson and Schnurer, 2001). Several authors (Roy et al., 1996) have reported that the antifungal activity of LAB is lost after treatment with proteolytic enzymes. Thus, Batish et al. (1989) suggest that the nature of antifungal substance is produced by LAB isolate was of proteinaceous since activity disappeared with proteinase treatment.

All the sixteen strains could hydrolyze casein and majority showed amylolytic activity however none of the strains was lipolytic .These results partially confirm those of Thapa et al. (2006) who find that *E. feacium* isolated from fish products was lipolytic.

Isolates of LAB were found to be sensitive to chloramphenicol, tetracycline, amicacin and rifamycin.

Some strains showed intermediate resistance to erythromycin and penicillin. All isolates were resistant to vancomycin, and nalidixic acid. These resistances are widely described among LAB and are usually considered as intrinsic and nontransferable (Mathur and Singh, 2005).

Nowadays, increasing attention is given to biogenic amine (BA) because the consumption of food containing high concentrations of BA has been associated with toxic effects and constitutes a potential health hazard (Suzzi and Gardini, 2003). The compounds implicated mainly in these toxic effects are histamine and tyramine. Histamine is the most significant biogenic amine in fish and fish products (Leisner et al., 1994; Emborg et al., 2002). Indeed in our study, only *L. lactis* subsp. *diacetylactis* BL10 did not produce biogenic amines.

Hence, these results enable, on the one hand, to exploit the bacteriocin produced by *L.lactis* subsp.*lactis MT and L. lactis diacetylactis* BT4.On the other hand, the inability of *L.lactis* subsp.*diacetylactis* BL10 to produce biogenic amines is a pre-requist criteria for a potential use as a starter culture.

In conclusion , the results obtained in this study revealed that LAB strains derived from marine fish possess potentially important properties. Selected strains in this study , might be valuable for practical application as starter, adjunct and protective cultures or as source of bacteriocin, providing future scope for the biopreservation of seafood products. In future, we prospect to characterize the nature of bacteriocin and the antifungal agent of selected strains. Also, These strains will be further evaluated for their in situ antimicrobial activity, as well as their safety and sensory acceptability for a food application.

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