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Characterization of Technological Properties of Lactic Acid Bacteria Isolated from Intestinal Microbiota of Marine Fish Caught in the Coast of Oran Algeria

F. Sahnouni, A. Matallah-Boutiba, D. Chemlal and Z. Boutiba
Environmental Monitoring Network, Faculty of Science, University of Oran, Algeria

Abstract: 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 characterization of new strains of LAB from gastrointestinal tract of coastal fish; sardine (*Sardina pilchardus*) and bug (*Boops boops*) with potential application as biopreservatives. A total of 67 isolates were isolated of which 16 isolates displayed antibacterial activities against *S. aureus*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *B. cereus*, *Salmonella* sp. and *E. 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: *L. lactis* ssp. *lactis*, *L. lactis* ssp. *diacetylactis*, *Leuconostoc* sp. and *Lactobacillus plantarum*. Three strains were screened for the study of their antibacterial compounds: *L. lactis* ssp. *lactis* MT, *L. lactis diacetylactis* BT4 and *L. lactis* ssp. *diacetylactis* BL10. As a result 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 the strains were no hemolytic showed no particular antibiotic resistance profile able to hydrolyze casein whereas none of them is found to possess lipolytic activity, however the majority produces biogenic amines.

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

INTRODUCTION

Food safety remains a major challenge to food producers and to legislators endeavoring to provide an adequate protection to consumers. Indeed a greater attention is being drawn towards application of the natural and safe antimicrobial proteins or peptides from LAB as biopreservatives (Cleveland *et al*., 2001). Their preservative effect is mainly due to various substances including acids, alcohols, carbon dioxide, diacetyl, hydrogen peroxide, bacteriocins, reuterin and other metabolites (Ennahar *et al*., 1996; Lasagno *et al*., 2002). Thus, the metabolic activity of LAB that may contribute in a number of ways to the control of bacterial pathogens and improvement of shelf life and sensory qualities might also have applications for preventing mold growth.

Lactic Acid Bacteria (LAB) constitute a diverse group of gram-positive bacteria, characterized by some common morphological, metabolic and physiological. The general description of the bacteria included in the group is gram-positive, catalase and oxidize negative, non-sporing, non-respiring cocci or rods which produce lactic acid as the major end product during the fermentation of carbohydrates (Dellaglio *et al*., 1994; Gonzalez *et al*., 2000). They 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. In deed, these microorganisms are found on several fresh fish species, fish products or in the intestinal contents of fish (Lyhs *et al*., 1999; Maugin and Novel, 1994; Ringo and Gatesoupe, 1998). Several investigations 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).

The aim of this study was to characterize and identify new lactic strains isolated 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. For this purpose, researchers chose as biological hardware two coastal fish; the sardine *Sardina pilchardus* and the bug *Boops boops* collected from coast of Oran Algeria.

Aim of this study: In Algeria, marine microbiology is very poorly exploited. There is no information on the microbiology of marine fish particularly lactic acid

Corresponding Author: F. Sahnouni, Environmental Monitoring Network, Faculty of Science, University of Oran, Algeria

bacteria. This It is the first study carried out in Algeria. The purpose of the present study was the characterization of new strains of LAB from gastrointestinal tract of coastal fish: sardine *Sardina pilchardus* and bug *Boops boops*) with potential application as biopreservatives.

MATERIALS AND METHODS

Bacterial strains and media: About 25 of sardine *Sardina pilchardus* and 30 of bug *Boops boops* were collected from the coast of Oran Algeria. Each specimen was aseptically dissected and the content from intestinal tract was homogenized in 1 mL of sterile saline solution. An aliquot of the homogenates was inoculated into De Man *et al.* (1960) broth (Biokar) 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 strains 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 and the production of cytochrome oxidase and catalase as described by Harrigan and McCance (1976). Then, they were kept in MRS broth containing 20% glycerol and were maintained as frozen stocks at -80°C.

Technological properties

Screening for antagonistic activity

Antibacterial activity: Isolates of LAB were screened for antagonistic activity by the agar spot method of Schillinger and Lucke (1989). The indicator strains used for antagonisms included: *Staphylococcus aureus* (ATCC25923), *E. coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC27853), *Klebsiella pneumoniae*, *Bacillus cereus*, Salmonella and *Enterococcus 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 NaOH 1N and treated with catalase at final concentration of 65 (UI mL^{-1}) following by filtration through a 0.22 μm pore size filter (Type Minisart NML; Sartorius GmbH, Gottingen, 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.

Antifungal activity: The antifungal activity of LAB was investigated with an overlay assay (Lind *et al.*, 2005; Maganusson and Shnurer, 2001). Micro-organisms used for the antifungal activity are *Fusarium 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

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, pronase E 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 before (Corsetti *et al.* 2004).

Identification of antimicrobial effect strain: Strains with antimicrobial activity were checked for gas production from glucose in MRS broth containing Durham tubes (Greco *et al.*, 2005). The growth of isolated LAB was also tested at different conditions (temperatures; 10/45°C, different pHs and MRS within 4 and 6.5% NaCl concentration) according to the procedures described by Hammes and Vogel (1995) and Stiles and Holzapfel (1997) as well as growth on bile esculin agar, NH_3 from arginine, heat resistance: 30 min at 63°C, search for citratase diacetyl and acetoin. Carbohydrate fermentation patterns of LAB were determined by means of miniaturized API 50 CH biochemical tests (BioMerieux, Marcy L_Etoile, France).

Acidification activity: The acidification activity was tested according to Ammor *et al.* (2005). It is followed 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 (Gordon *et al*., 1973) 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.

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

Safety characteristics: Biogenic amines production, Hemolytic activity and antibiotics resistance. These parameters were tested to ensure the safety of the strains for future use in biopreservation of food products (Rodgers, 2001).

The decarboxylase test for production of biogenic amines: It 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^{-1} final concentration as precursors: lysine, ornithine, histidine and tyrosine (Sigma). *Escherichia coli* (ATCC25922) was used as positive control.

Susceptibility to antibiotics: It was determined using agar diffusion discs of hloramphenicol, tetracycline, amikacin, erythromycin, vancomycin, penicillin, rifamycin and nalidixic acid as recommended by the supplier (Bio-Rad, Hercules, CA, USA). Analyses were performed in triplicate.

Haemolytic test: It 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 micro-organisms: *Staphylococcus aureus* (ATCC25923), *E. coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC27853), *Klebsiella pneumoniae*, *Bacillus cereus*, Salmonella and *Enterococcus faecalis*. A significant difference ($p<0.05$) was observed between strains. Inhibitory spectra of these isolates are showed in Table 1. These isolates seemed to belong to *L. lactis subsp lactis*, *L. lactis* ssp. *diacetylactis*, *Leuconostoc* sp. and *Lactobacillus plantarum*. According to the results, it is shown that two strains demonstrated antagonistic activity against seven target strains. *L. lactis* ssp. *lactis*

Table 1: Inhibitory spectra of LAB isolate exhibiting antimicrobial activity

	Inhibitory spectra of LAB isolates exhibiting antimicrobial activity									
	Isolates indicator strains								Indicator fungus	
Strain LAB	Code	*S. aureus*	*E. coli*	*K. pneumoniae*	*B. cereus*	*Salmonella* sp.	*P. aeruginosa*	*E. faecalis*	*F. oxy*	*A.* sp.
L. lactis diacetylactis	CT12	7±0.1	15±1.2	20±0.1	10±0.8	12±0.1	6±0.2	15±0.1	++	-
	BT5	0	15±0.7	13±0.8	11±0.4	15±0.7	0	0	++	+
	CT7	10 ±0.5	14±0.3	16±1.3	13±0.1	15±1.1	5±0.1	10±0.4	+	+
	MC	16 ±1.0	0	9±0.7	0	3±0.1	9±0.3	15±0.1	++	+
	CT11	1.1±0.3	13±0.1	23±1.1	16±0.3	11±0.1	0	0	++	++
	BT4	7 ±0.2	14±0.1	19±0.5	20±1.2	19±1.3	9±0.3	5±0.3	++	++
	ST1	8 ±0.1	13±1.0	15±0.4	17±0.1	15±1.1	0	0	++	++
	BL10	13 ±0.9	15±0.4	20±1.3	0	11±0.5	5±0.7	0	+	++
L lactis ssp. *lactis*	MT	10± 0.3	21±0.6	16±1.2	18±0.7	16±0.7	5±0.2	9±1.2	+	+
	ST2	19±0.1	13±1.2	20±0.7	16±1.3	11±1.2	7±1.2	0	++	+
E. faecium	BL2	11±1.5	14±1.3	20±0.3	0	10±0.4	5±0.1	0	++	+
	CT16	0	21±0.9	0	0	2±0.3	8±0.3	0	++	+
	MT1	15±0.7	0	15±0.5	0	10±0.3	0	0	++	+
Leuconostoc sp.	SC	13±1.1	20±0.8	0	16±0.5	0	0	0	++	+
Lb. plantarum	MT2	12±0.3	06±0.1	22±1.2	0	07±0.6	0	0	-	-
	CT1	15±0.8	20±0.7	23±0.3	16±1.2	11±0.2	0	0	-	-

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 fungal growth on >8% of plate area/bacterial streak. *F. oxy*: *Fusarium oxysporum* (*A.* sp.): *Aspegillus* sp.

Table 2: Technological properties of LAB strains isolated from intestinal microbiota of fish from the coast of Oran Algeria

Strain LAB	Code	DEX	LDC	ODC	HIS	HEM
L. lactis ssp. *diacetylactis*	CT12	-	-	+	+	-
	BT5	-	+	-	+	-
	CT7	-	+	+	+	-
	MC	-	+	-	-	-
	CT11	-	+	-	+	-
	BT4	-	-	+	+	-
	ST1	-	+	+	+	-
	BL10	-	-	-	-	-
L. lactis ssp. *lacti*	MT	-	+	+	+	-
	ST2	-	+	+	+	-
E. faecium	BL2	-	+	+	+	-
	CT16	-	+	+	+	-
	MT1	-	+	+	+	-
Leuconostoc sp.	SC	+	+	+	+	-
Lb. plantarum	MT2	-	+	-	-	-
	CT1	-	+	-	-	-

HIS: Histidine decarboxylase; ODC: Ornithine Decarboxylase, tyrosine decarboxylase; HEM: Haemolytic test

MT and *L. lactis diacetylactis* BT4 (Table 1). The diameters of the inhibition halos were in all cases within the 15-21 mm range (Fig. 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*.

- *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*
- *Leuconostoc* sp. has a weak inhibition against the indicators micro-organisms. Indeed no antagonistic activity was observed against *P. aeruginosa*, *K. pneumoniae*, *Salmonella* sp. and *E. faecalis*

Therefore, the rate inhibition of target strains is as follows:

- *Salmonella* sp. was inhibited by 93.75% of LAB strains
- *S. aureus*, *K. pneumoniae* and *E. coli* were inhibited by 62.5% of LAB strains
- *B. cereus* by 87.5% of LAB strains of LAB strains
- *P. aeuroginosa* by 56.25% of LAB strains
- *E. faecalis* by 31.25% of LAB strains

Varying degrees of inhibition were detected against the isolates of *Fusarium oxysporum* and *Aspergillus* sp. (Table 2) except *Lb. plantarum*. The following Fig. 2 shows the antifungal activity of the three lactic strains selected in the study: *L. lactis* ssp. *lactis* MT, *L. lactis diacetylactis* BT4 and *L. lactis* ssp. *diacetylactis* BL10. These results allow us to select three strains for the study of their antibacterial compounds: *L. lactis* ssp. *lactis* MT,

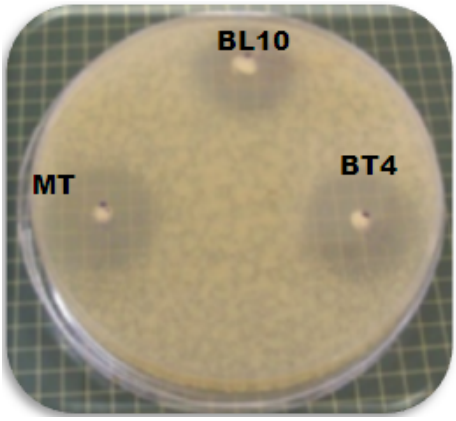

Fig. 1: Inhibition of *E. coli* (ATCC25922) by *L. lactis* ssp. *lactis* MT, *L. lactis* ssp. *diacetylactis* BT4 and *L. lactis* ssp. *diacetylactis* BL10

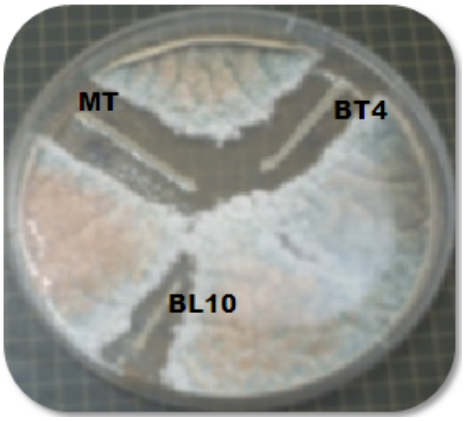

Fig. 2: Inhibition of *Fusarium oxysporum* by *L. lactis* ssp. *lactis* MT, *L. lactis* ssp. *diacetylactis* BT4 and *L. lactis* ssp. *diacetyl* BL10

L. lactis diacetylactis BT4 strains exhibiting antagonistic activity against all indicator micro-organisms and *L. lactis* ssp. *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 exhibit inhibition zones for neutralized culture supernatants and catalase treated supernatants. Such observations strongly suggest that the antimicrobial activities against indicator microorganisms used in this study would be based on the biosynthesis and secretion of bacteriocin-like substances.

Results from enzyme inactivation studies confirmed that antimicrobial activity was lost after treatment with the proteolytic enzymes like proteinase K, α chymotrypsin and pronase E 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.

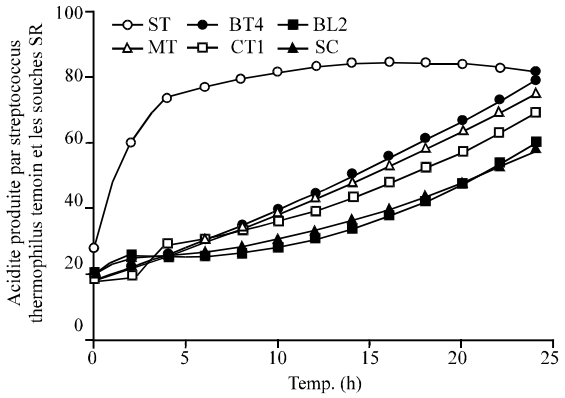

Fig. 3: Acidification activity of LAB strains isolated from intestinal tracts of marine fish

Acidification activity: According to the results, it is shown that the control strain ST produces a high amount of acidity during the first hours of growth (47°C after 4 h of culture) and then stabilizes at this value during the fermentation (Fig. 3). This stability is due to inhibition of the growth rate which is due to the high acidity of the medium. Bacterial isolates display a low power acidifier compared to the ST but constant throughout the fermentation. BT4 and MT strains produce lactic acid after fermentation similar to that produced by the control strain ST.

Proteoltic and lipolytic activity: All strains of LAB screened for their antagonistic activity showed proteolytic activities (showing >2 mm hydrolysis zone in milk agar plate). However, none of them was able to hydrolyze tributyrin.

Safety properties: According to the results shown in the Table 2, researchers note that no strain was hemolytic and sensitive to chloramphenicol, tetracycline, amicacin and rifamycin. Isolates of LAB were screened also for their ability to produce biogenic amines. The results obtained show that the majority of them produce biogenic amines in the applied method, except for *L. lactis diacetylactis* BL10.

DISCUSSION

The main aim of this study was the isolation and characterization of biopreservatives properties of some lactic acid bacteria isolated from intestinal tracts of marine fish. Sixteen strains out of a total of 67 LAB were screened for their antagonistic activitie against indicators microorganisms (*S. aureus*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *B. cereus*, *Salmonella* sp., *E. faecalis*, *Fusarium oxysporum* and *Aspergillus* sp.). LAB screened for their antimicrobial activity were identified as *L. lactis* ssp. *lactis*, *L. lactis diacetylactis Leuconostoc* sp. *Enterococcus faecium* and *Lactobacillus plantarum*. However, this identification is based on phenotypic characters and the API system, authentic identity of species should be confirmed by molecular identification. 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 (Ringo and Gatesoupe, 1998; Campos *et al*., 2006; Balcazar *et al*., 2008; Itoi *et al*., 2008; Valenzuela *et al*., 2010). The presence of these bacteria in the intestinal tract of marine fish may result from the high adaptability to varied environments.

L. lactis ssp. *lactis* MT and *L. lactis diacetylactis* BT4 showed the broadest spectrum by inhibiting target strains (gram positive and negative). This antimicrobial activity would be based on the secretion of bacteriocins. This result is comparable with those obtained by Campos *et al*. (2006) and Ayad *et al*. (2002) who found that 41% of wild lactococci produced bacteriocins. Balcazar *et al*. (2008) showed 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 found that strains of *L. plantarum* had inhibitory activity against *S. aureus* and not against *P. aeuroginosa*, *E. coli* and *Salmonella* sp. According to the study of Valenzuela *et al*. (2010), *Enterococcus faecium* isolates from seafoods were able to inhibit *S. aureus* and other enterococci but no antibacterial activity was detected towards *Bacillus cereus* or *E. coli*. However in the 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*., 1996; Lasagno *et al*., 2002; Valenzuela *et al*., 2010).

The detected antifongic activity against *Fusarium oxysporum* and *Aspergillus* sp. can be due to organic acids, produce hydrogen peroxide (H_2O_2), proteinaceous compounds or reuterin (Schnurer and Magnusson, 2005). Several researchers have reported that the antifungal activity of LAB is lost after treatment with proteolytic enzymes. Thus, Batish *et al*. (1989) suggested that the nature of antifungal substance produced by LAB isolate was of proteinaceous, since activity disappeared with

proteinase treatment. All the sixteen strains could hydrolyze casein however, none strain was lipolytic. These results partially confirm those of Thapa *et al*. (2006) who found that *Enterococcus 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).

Now-a-days, 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 mainly implicated 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 the study, only *L. lactis* ssp. *diacetylactis* BL10 does not produce biogenic amines.

Hence, these results enable us to exploit the bacteriocin produced by *L. lactis* ssp. *lactis* MT and *L. lactis diacetylactis* BT4. On the other hand, the inability of *L. lactis* ssp. *diacetylactis* BL10 to produce biogenic amines is a good indication of its acceptability in the possible development of starter cultures.

CONCLUSION

The study concluded that LAB of gastrointestinal tract of marine fish may be exploited as protective cultures (such as those strains lacking undesirable traits) or as sources of bacteriocin for food safety and preservation providing future scope for application as food preservative. In future, researchers prospect to characterize the bacteriocin and determining the nature of the antifungal agent as well as study of their safety and sensory acceptability for a food application.

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