Antibacterial Activity of *Lactobacillus* species against Pathogens of Seafood Origin

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Lactic acid bacteria (LAB) were isolated from seafoods. Among the LAB, *Lactobacillus* was the predominant genus isolated. The *Lactobacillus* species identified were *Lactobacillus plantarum, L. gasseri, L. divergens, L. brevis, L. viridescens, L. casei rhamnosus, L. farciminis, L. buchneri, L. alimentarius, L. fermentum, L. acidophilus, L. animalis* and *L. reuteri*. *L. plantarum* was the predominant *Lactobacillus* species in the seafoods tested. The biochemical and antibacterial activity of selected cultures were studied. The antibacterial activity of *Lactobacillus* cultures were tested against pathogenic bacteria like *Listeria monocytogenes, Salmonella typhimurium, Bacillus cereus, Escherichia coli, Staphylococcus aureus, Vibrio cholerae and V. vulnificus*. The *Lactobacillus* cultures showed highest antibacterial activity against *L. monocytogenes* and *B. cereus*. The bacteriocins from LAB cultures were active against other LAB cultures. The characterization of the antibacterial factor confirmed the presence of bacteriocins in *Lactobacillus* cultures from seafood.

Key words: Antibacterial activity, lactic acid bacteria, bacteriocin

Lactic acid bacteria (LAB) were primarily used for the preservation of highly perishable foods. LAB exerts a strong antagonistic activity against many microorganisms, including food spoilage organisms and pathogens. The inhibition may be due to (i) the production of organic acids such as lactic and acetic acids that inhibit the growth of many bacteria (Gilliland & Speck, 1975), (ii) the production of hydrogen peroxide that inhibits the growth of pathogens through its strong oxidizing effect on the bacterial cells or through the destruction of basic molecular structures of nucleic acids and cell proteins and (iii) the production of specific proteins or protein complexes called bacteriocins (Tagg *et al.*, 1976; Klaenhammer, 1988) that inhibits some gram-positive bacteria, mainly homologous species, and some sphaeroplasts of Gram-negative bacteria (De Vuyst).

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Although it was believed earlier that LAB are not inherent in the marine environment, these organisms are now reported to be present in freshwater fishes (Stiles & Holzapfel, 1997; Gonzalez *et al.*, 2000), and several processed fish products (Ross & Toth, 1974; Blood, 1975; Schroder *et al.*, 1979; Cone, 1982; Valdimarsson & Gudbjornsdottir, 1984; Okafor & Nzeako, 1985; Mauguin & Novel, 1994; Pilet *et al.*, 1995). However, there are no reports on the isolation of LAB from tropical fresh and frozen fishes. There are a few reports on the isolation of bacteriocin producing LAB from fish (Schroder *et al.*, 1979; Stoffels *et al.*, 1992; Fricourt *et al.*, 1994 and Pilet *et al.*, 1995).

The objective of this study was to isolate LAB from seafood and study their antibacterial activity against seafood pathogens.

Materials and Methods

Bacterial strains and culture conditions

Listeria monocytogenes (ATCC 19111), *Salmonella typhimurium* (ATCC 14028) and *Vibrio vulnificus* (ATCC 2046) were obtained from the American Type Culture Collection (Maryland, USA). *Bacillus cereus* (B3/3), *Vibrio cholerae* (Vc-7) *Staphylococcus aureus* (SA3B) and *Escherichia coli* (Ec101) were from the National Collection of Aquatic and Fish Bacteria (NCAFB) of CIFT,Cochin. All other cultures of LAB used for analysis were isolated from various tropical fresh and frozen fish/prawn as a part of the study and identified by standard methods. (Kandler & Weiss, 1986; USFDA, 1995).

de Man Rogosa and Sharpe (MRS) medium was used to isolate and propagate LAB. The MRS plates were incubated in 5% CO_2 incubator (NAPCO automatic CO_2 incubator, Precision Scientific, USA) at 37°C for 48-72 h. Brain Heart Infusion (BHI) broth was used to culture *Listeria monocytogenes* and *Salmonella typhimurium*. Nutrient broth was used to culture all the other pathogens used in the study. For plating, 1.5% agar was added to the broth media described above.

Detection of antibacterial activity

Agar well diffusion method as described by Itoh *et al.* (1995) was used. BHIA, TSA or MRS agar (Oxoid) plates were overlaid with a 10 ml BHI, TS or MRS soft agar (0.75%) containing an indicator bacterial strain. The indicator lawns were prepared by adding 0.20 ml of a 10^{-1} dilution from

overnight cultures of bacteria other than lactobacilli to soft agar. Three numbers of wells of 8 mm diameter were cut into the agar in each plate by using a cork borer and 100 µl of culture supernatant of test lactobacilli strains were placed into the first well. The culture filtrate was neutralized with sterile 2N NaOH to pH 6.5±0.2 and placed in the second well. The neutralized culture filtrate was treated with catalase (5mg.ml^{-1} culture) and placed in the third well in the same plate. The plates were incubated at 37°C for 24 h and examined for zones of inhibition.

Preparation of crude bacteriocin

The test *Lactobacillus* culture was inoculated to 200 ml of MRS broth and incubated at 37°C in a CO_2 incubator (5% CO_2) for 24-48 h. The bacteriocins were precipitated by addition of ammonium sulphate (40%-60% w/v). The precipitate was dissolved in 1 ml of glycine-NaOH buffer (pH 7.5) and stored at 4°C. The crude preparation was tested for antibacterial activity against pathogenic bacteria by agar well diffusion method.

Characterization of bacteriocin

For determining the heat stability of the bacteriocins, the cell-free supernatants adjusted to pH 6.5 were heated for 30 min at 80°C, 10 min at 100°C and 10 min at 121°C and tested for bacteriocin activity. For determining the proteinaceous nature of bacteriocins, proteolytic enzymes proteinase K in Tris HCl 20 mM pH 7, Trypsin in 20 mM sodium phosphate pH 8.2, a-Chymotrypsin in 20 mM Tris HCl pH 8 (Sigma) prepared in buffers were added to supernatant samples to a final concentration of 0.1mg.ml^{-1}. After incubation for 1 h at 37°C, bacteriocin activity was tested. Controls were enzymes in buffer.

Results and Discussion

Lactobacillus cultures isolated from fresh and frozen seafood were identified by their morphological and biochemical characters (Sharpe *et al.*, 1979; Kandler & Weiss, 1986). The *Lactobacillus* species were identified as *Lactobacillus plantarum, L. gasseri, L. divergens, L. brevis, L. viridescens, L. casei rhamnosus, L. farciminis, L. buchneri, L. alimentarius, L. fermentum, L. acidophilus, L. animalis* and *L. reuteri*. *L. plantarum* was the predominant species in fresh and frozen fish and prawn.

Table 1 shows the antibacterial activity of *Lactobacillus* cultures against *Listeria monocytogenes, Salmonella typhimurium, Bacillus cereus, Escherichia*

coli, *Staphylococcus aureus*, *Vibrio cholerae* and *V. vulnificus*. All the cultures tested inhibited *L. monocytogenes*, *B. cereus* and *S. typhimurium*. The inhibition was maximum against *L. monocytogenes* and *B. cereus*. The two strains of *L. divergens* tested could not inhibit the *Staphylococcus aureus* and *E. coli* strains tested. However, all the other cultures could inhibit *Staphylococcus aureus* and *Escherichia coli* to varying degrees. *Salmonella typhimurium* was inhibited mainly by *L. plantarum* strains.

Table 1. Antibacterial activity of *Lactobacillus* cultures against seafood pathogens measured as diameter of zone of inhibition (in mm)

Lactobacillus strains	Inhibition zone diameter, mm.				
	Listeria monocytogenes (ATCC 19111)	*Bacillus cereus* (B3/3)	*Staphylococcus aureus* (SA3B)	*Escherichia coli* (Ec 101)	*Salmonella typhimurium* (ATCC 14028)
L. divergens	20	16	NZD	NZD	12
L. divergens	18	16	NZD	NZD	14
L. gasseri	18	18	12	12	14
L. gasseri	16	20	16	14	12
L. plantarum	24	22	16	16	18
L. plantarum	18	24	16	14	20
L. reuteri	22	20	16	14	18
L. animalis	26	22	18	14	16
L. brevis	22	24	12	16	18
L. fermentum	20	22	16	18	18

NZD – No zone detected

Table 2 shows the characterization of antibacterial activity of selected *Lactobacillus* cultures against *L. monocytogenes*. During the screening of *Lactobacillus* cultures for antibacterial activity against chosen pathogenic bacteria, *L. monocytogenes* was found to be the most susceptible strain. The neutralization of culture filtrate with alkali also neutralizes the antibacterial activity due to organic acids. Catalase treatment deactivates H_2O_2 formed in the culture media. Heat treatment in 80°C, 100°C and 121°C had been done with the specific purpose of denaturing proteins other than bacteriocins. Bacteriocins withstand heating to a considerable degree at 100°C for 10 min and are generally denatured at 121°C in 10 min. Treatment with four different proteolytic enzymes confirmed the protein nature of the bacteriocin.

The antibacterial activity of *Lactobacillus* cultures is due to organic acids and bacteriocins produced. The bacteriocins produced by LAB were

Table 2. Characterization of antibacterial activity of *Lactobacillus* cultures against *Listeria monocytogenes*

Lactobacillus cultures	BCN	BCN +catalase	Heat treatment			Proteinase treatment			
			80°C 30 min	100°C 10 min	121°C 10 min	Protease	Trypsin	Chymo trypsin	Pepsin
L. divergens	18	18	18	15	NZD	NZD	NZD	14	14
L. gasseri	18	18	18	18	16	NZD	NZD	NZD	NZD
L. brevis	22	22	22	22	NZD	NZD	NZD	NZD	NZD
L. fermentum	20	20	18	18	NZD	NZD	12	12	NZD
L. plantarum	22	22	22	15	NZD	NZD	NZD	NZD	10

BCN – Bacteriocin concentrate neutralized ; NZD – No zone detected

effective against other closely related *Lactobacillus* cultures. Further, this is a confirmation of the bacteriocins produced by LAB. The antibacterial activities of the 10 chosen *Lactobacillus* cultures are presented in Table 3. It can be seen from the table that bacteriocins from most of the cultures were inhibitory to the other six closely related *Lactobacillus* with a few exceptions. Bacteriocins from two *L. plantarum* cultures and one *L. animalis* culture showed antagonistic activity against all the test *Lactobacillus* cultures. These observations confirmed the nature of bacteriocins produced by the 10 *Lactobacillus* cultures included in the study.

Table 3. Antibacterial activity of bacteriocin concentrate against other *Lactobacillus* cultures

Lactobacillus strains	Inhibition zone diameter, mm					
	L. plantarum	*L. brevis*	*L. fermentum*	*L. lactis*	*L. casei*	*L. acidophilus*
L. divergens	NZD	NZD	10	10	10	10
L. divergens	NZD	NZD	12	10	12	NZD
L. gasseri	16	NZD	NZD	NZD	NZD	NZD
L. gasseri	NZD	NZD	12	12	10	10
L. plantarum	14	16	10	10	10	10
L. plantarum	12	12	10	10	10	12
L. reuteri	12	14	10	NZD	NZD	NZD
L. animalis	12	12	10	10	10	10
L. brevis	12	NZD	NZD	NZD	10	10
L. fermentum	12	NZD	NZD	NZD	10	10

NZD – No zone detected

Halami *et al.* (1999) had reported the isolation of LAB from fish intestine, which were identified and characterized with reference to the production of bacteriocins. One isolate of *L. casei* ssp. *casei* (C-40) was found to be a potent bacteriocin producer active against other LAB cultures. Toba *et al.* (1991) isolated *L. gasseri* from infant faeces, which inhibited several other *Lactobacillus* cultures. The bacteriocins from *L. gasseri* inhibited *L. acidophilus, L. casei* and *L. brevis* cultures. *L. gasseri* cultures did not inhibit any of the test *L. plantarum* cultures. These results agree with our findings, that the *L. gasseri* cultures were inhibitory to *L. acidophilus* and *L. casei.* However, the *L. gasseri* isolated in this study also inhibited *L. plantarum* cultures. None of the bacteriocins produced by the 20 *Lactobacillus* cultures showed any antibacterial activity against *V. vulnificus* and *V. cholerae.* However, it was observed that the organic acids produced by the *Lactobacillus* cultures inhibited the vibrios effectively. It can be concluded that *Lactobacillus* cultures and their bacteriocins are effective against seafood pathogens.

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