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Inhibition of Fish Bacterial Flora by Bacteriocin of Lactic Acid Bacteria

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Seven strains of lactic acid bacteria (LAB) were tested for the production of bacteriocins or bacteriocin-like substances by the agar spot method. By excluding inhibition due to acid and confirming their proteinaceous nature, the inhibitors were confirmed as bacteriocins. The ability of four bacteriocin producing strains, viz., *Lactobacillus plantarum* 89, *L. plantarum* 8014, *L. helveticus*, and *Pediococcus pentosaceus* 25445, to inhibit thirty-five strains of bacteria isolated from fish and fishery products were screened. Strains of *Enterococcus* spp. and *Flavobacterium/Cytophaga* were inhibited by bacteriocins of LAB. *Vibrio parahaemolyticus* was inhibited slightly by bacteriocins of *L. plantarum* 89 and *P. pentosaceus* 25445. *Aeromonas hydrophila, Salmonella* sp. and *V. cholerae* were not inhibited. The bacteriocins of LAB are bactericidal to 42 to 54% of the fish microflora tested.

Key words : Lactic acid bacteria, bacteriocin, fish bacteria inhibition.

Lactic acid bacteria (LAB) produce a number of antimicrobial agents such as hydrogen peroxide, lactoperoxidase and diacetyl apart from organic acids (Daschel, 1989) and bacteriocins (Klaenhammer, 1988). Among the LAB, the bacteriocins have been extensively studied in lactococci. Because bacteriocins are microbially produced, the potential exists for their use as natural food preservatives. Nisin, a bacteriocin, produced by certain strains of *Lactococcus lactis* sub sp. *lactis* was affirmed as GRAS by the FDA (FDA, 1988) specially for use in certain pasteurised cheese spreads to inhibit the growth of *Clostridium botulinum* spores. Nisin is the only bacteriocin that has been approved for use in foods. Further research on other bacteriocins from the LAB may lead to their successful implementation as food preservatives. The objective of this study was to screen the lactic cultures for the production of bactriocins or bacteriocin-like substances and to test the specific bacteriocin mediated inhibition of the bacterial flora of fish.

Materials and Methods

The LAB strains, listed in Table 1, were obtained from National Dairy Research Institute, Karnal (India). *Aeromonas hydrophila, Salmonella* sp., *Vibrio cholerae* and *V. parahaemolyticus* were obtained from Department of Fishery Microbiology, College of Fisheries, Mangalore (India). Other bacterial strains were isolated from Indian goat fish, *Parupeneus indicus*, on seawater agar plates, purified and identified up to generic level as per Surendran & Gopakumar (1981). DeMan, Rogosa, Sharpe (MRS) medium and tryptic soy medium were used for the growth and maintenance of LAB strains and other strains, respectively.

The LAB were screened for bacteriocin production by agar spot method (Spelhaug & Harlander, 1989) on MRS agar, MRS agar with reduced glucose level (MRS 0.2% Glu agar), brain heart infusion agar (BHIA) and tryptic soy agar (TSA). In this screening assay, bacteriocin sensitive

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strain of lactic acid bacterium, *Pediococcus pentosaceus*-X, was used as the indicator organism. MRS soft agar (MRS broth + 0.8% agar) was used to seed the indicator organism on MRS agar and MRS 0.2% Glu agar plates. BHI soft agar (BHI broth + 0.8% agar) was used for BHIA and TSA plates.

The sensitivity of bacteriocins to proteolytic and other enzymes was tested by using a modified spot-on-the-lawn method (Okereke & Montiville, 1991) on BHIA plates. BHI soft agar was used to seed the indicator organism, *P. pentosaceus*-X. The Agar spot method of Spelhaug & Harlander (1989) was followed for the *in vitro* assay on the inhibition of bacterial flora of fish by four strains of LAB, viz., *L. plantarum* 89, *L. plantarum* 8014, *L. helveticus* and *P. pentosaceus* 25445 on BHIA plates. BHI soft agar was used to seed the fish isolates.

Results and Discussion

The results of the screening of LAB for bacteriocin production are presented in Table 1. Larger inhibitory zones indicating non-specific inhibition due to acid production were observed with MRS agar. No zones were observed on TSA. Bacteriocin inhibition was clear on BHIA and MRS 0.2% Glu agar, except for the strain of *Lactococcus lactis* sub sp. *diacetylactis*. The inhibition due to acid on MRS agar was excluded by the use of reduced glucose level. Likewise, Schillinger & Lucke (1989) and Okereke & Montville (1991) have demonstrated the production of bacteriocins in a medium with reduced glucose level by excluding the inhibition due to acid. It has been reported that numerous LAB produce hydrogen peroxide (Raccach & Baker, 1978) and in some cases, in sufficient quantity to inhibit microorganisms (Dahiya & Speck, 1968). However, experiments of Spelhaug & Harlander, (1989) and Ahn & Stiles (1990) demonstrated that hydrogen peroxide was not responsible for the observed inhibition by the agar spot method. Although 6 out of 7 strains of LAB were bacteriocin producers (Bac+), variation in zone size was observed on different media. For example, the zones were smaller on BHIA than on MRS 0.2% Glu agar. This may probably be due to the influence of certain growth factors and growth conditions in MRS 0.2% Glu agar, as this medium is specific for LAB.

Table 1. Screening of lactic acid bacteria (LAB) for bacteriocin production

Test organisms (LAB producer strains)	Zone of inhibition (mm) on MRS agar	MRS 0.2% Glu agar	BHI agar
Lactobacillus plantarum 89	4.00	2.00	1.00
Lactobacillus plantarum 8014	5.50	2.00	1.50
Lactobacillus casei 300	5.00	2.00	< 1.00
Lactobacillus acidophilus R	5.00	2.50	2.00
Lactobacillus helveticus	5.00	2.50	1.50
Pediococcus pentosaceus 25445	6.00	2.00	1.00
Lactococcus lactis sub sp. *diacetylactis*	5.00	ND	ND

A bacteriocin sensitive *Pediococcus pentosaceus* x was used as the indicator strain.; Zone in mm indicate the distance from the border of the producer spot to the edge of the clear zone.

ND : Not detected

The sensitivity of bacteriocins to enzymes are presented in Table 2. The antimicrobial activity was lost after treatment with proteolytic enzymes. Treatment with lysozyme did not cause any loss in antimicrobial activity of bacteriocins. The loss in antimicrobial activity following treatment with proteolytic enzymes indicate that the active components secreted extracellularly by LAB strains are proteinaceous. These results are in agreement with Bhunia *et al.* (1988), Ahn & Stiles (1990) and Okereke & Montville (1991). However, trypsin 2500 NFU mg^{-1} and pancreatin did not degrade the bacteriocins of *L. helveticus*. Treatment with protease type XXVII did not cause any loss in activity of the bacteriocins produced by *L. plantarum* 89 and *L. plantarum* 8014. This could be attributed to the probable presence of inhibitory substance other than bacteriocin as reported by Daeschel (1989).

Table 2. Sensitivity of bacteriocins to enzyme

Enzyme treatment	Lp 89	Lp 8014	Lh	Pp 25445
Trypsin, 2000 u g^{-1}	+	+	+	+
Trypsin, 2500 NFU mg^{-1}	+	+	-	+
Pancreatin, NF	+	+	-	+
Papain	+	+	+	+
Protease, Type XXVII	-	-	+	+
Lysozyme	-	-	-	-
Control	-	-	-	-

+ = sensitive; - = resistant; Lp 89 : *Lactobacillus plantarum* 89; Lp 8014: *L. plantarum* 8014, Lh : *L. helveticus*; Pp 25445 : *Pediococcus pentosaceus* 25445

The *in vitro* inhibition of thirty five bacterial isolates from fish by four strains of LAB are presented in Table 3. *Enterococcus* spp. and *Flavobacterium/Cytophaga* were found to be inhibited by all Bac+ LAB. *Vibrio parahaemolyticus* was inhibited slightly by *L. plantarum* 89 and *P. pentosaceus* 25445. *A. hydrophila, Salmonella* sp. and *V. cholerae* were not inhibited by any of the

Table 3. Sensitivity of bacterial isolates from fish to bacteriocin positive lactic acid bacteria

Fish isolate	Isolates inhibited/isolates tested			
	Lp 89	Lp 8014	Lh	Pp 25445
Aeromonas hydrophila	0/1	0/1	0/1	0/1
Bacillus spp.	1/9	1/9	2/9	1/9
Enterobacteriaceae	1/4	1/4	1/4	1/4
Enterococcus spp.	2/2	2/2	2/2	2/2
Flavobacterium/Cytophaga	1/1	1/1	1/1	1/1
Micrococcus sp.	1/1	0/1	1/1	0/1
Pseudomonas sp.	1/2	1/2	1/2	1/2
Salmonella sp.	0/1	0/1	0/1	0/1
Staphylococcus sp.	0/3	1/3	1/3	0/3
Staphylococcus aureus	2/3	3/3	3/3	3/3
Vibrio cholerae	0/1	0/1	0/1	0/1
Vibrio parahaemolyticus	1/1	0/1	0/1	1/1
Vibrio spp.	5/6	6/6	6/6	6/6
Total	15/35 (42.8)	16/35 (45.7)	19/35 (54.3)	16/35 (45.7)
Gram positives	6/18 (33.3)	7/18 (38.9)	9/18 (50.0)	6/18 (33.3)
Gram negatives	9/17 (52.9)	9/17 (52.9)	10/17 (58.8)	10/17 (58.8)

Lp 89 : *Lactobacillus plantarum* 89 ; Lp 8014 : *L. plantarum* 8014 Lh : *L. helveticus*; Pp 25445 : *Pediococcus pentosaceus* 25445. Values in parenthesis are percent isolates inhibited

Bac+ LAB tested. About 42 to 54% of fish bacterial flora tested in this study were inhibited by the Bac+ LAB. These Bac+ LAB strains were, in general, bactericidal to 53 to 59% of gram negative and 33 to 50% of gram positive bacteria tested. Among the Bac+ LAB, *L. helveticus* was the most potent inhibitor, followed by *P. pentosaceus* 25445 and *L. plantarum* 8014. The bactericidal action of antibacterial substance produced by *L. plantarum* on a mixed catalase positive bacterial population isolated from fish (*Gadus virans*) and on *Vibrio* sp. have been observed (Schroeda *et al.*, 1980). Inhibition of the *Pseudomonas* strains by LAB on agar lawns have also been reported (Moon *et al.*, 1981). It has been demonstrated that the bacteriocins of food grade LAB are bactericidal to many gram positive bacteria (Bhunia *et al.*, 1988; Klaenhammer, 1988). Spelhaug & Harlander (1989) have

shown that the bacteriocins of *Lactococcus lactis* sub sp. *lactis* 11454 was capable of slightly inhibiting several gram negative organisms including *A. hydrophila, V. cholerae* and *V. parahemolyticus.*

The present study suggests that the use of Bac+ strains of LAB may provide a natural means of preservation of fish in terms of inhibiting the growth of spoilage microflora. Although the Bac+ strains of dairy cultures have been shown to be antagonistic to bacterial flora of fish, Bac+ LAB from fish could probably be the best candidate for improving the microbiological safety of fish, because they would be better adapted and therefore more competitive than Bac+ LAB from other sources.

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