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INHIBITION OF SEAFOOD–BORNE BACTERIA IN COOKED MACKEREL (*RASTRELLIGER KANAGURTA*) FISH MEAT BY LACTIC ACID BACTERIA

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ABSTRACT

Antagonistic activity of lactic acid bacteria (LAB) namely *Streptococcus faecalis, Pediococcus cerevisiae* and *Lactobacillus casei* was tested against seafood-borne bacteria such as *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Clostridium* perfringens and *Listeria monocytogenes*. Three lactic acid bacteria such as *Streptococcus faecalis, Lactobacillus casei* and *Pediococcus* cerevisiae were coated on cooked mackerel meat, individually and in combination against fish-borne bacteria. *S. faecalis* inhibited *C. perfringens* in individual coat by 3.7 log units as compared to control, whereas *L. casei* did not inhibit *C. perfringens*. *P. cerevisiae* inhibited *S. aureus* by 5 log units. *L. casei,* inhibited *L. monocytogenes* by 3.3 log units on the third day of storage as compared to control. On the other hand *S. aureus* and *B. cereus* were inhibited on the third and second day by 4.9 log and 5.2 log units respectively. *B. cereus, S. aureus, L. monocytogenes* were the most sensitive to all three LAB. *C. perfringens* was the least inhibited among all the seafood-borne bacteria tried. Multiple LAB or LAB strains in combination showed much earlier inhibitory activity on seafood-borne bacteria than single LAB coat.

Keywords: Lactic Acid Bacteria, Antagonism, Seafood-borne bacteria, *C. perfringens, L. monocytogenes,* Mackerel meat.

INTRODUCTION

Lactic acid bacteria (LAB) have long been used as a starter culture as early as 3000 B.C. LAB was used to change the flavour and textural properties of foods such as milk, meat, cereals, vegetable products, pickles and idli batter (McKay and Baldwin, 1990). LAB were applied in dietary items and food fermentation (Spule, 1980; Gibbs, 1987) due to production of extracellular compounds that is effective against food spoilage bacteria and even psychrotrophic pathogens (Gilland and Speck, 1975 and Speck, 1972). Bacteriocin like inhibitory substances (BLIS) are the cationic, ribosomally synthesized and hydrophobic compounds produced by LAB which are indispensable because of their antibacterial activity against wide range of food-borne pathogens (Bibek *et al* 2001, Dahiya and Speck *et al.*, 1967; Tagg *et al.*, 1976). Nisapline, a purified form of BLIS obtained from *Lactococcus lactis* has been used to control seafood-borne bacteria (Kannappan *et al.*, 2004a). *Pediococcus pentasaceous* and *P. acidilactici* were used to prevent the growth of fish-borne bacteria on mackerel fish chunks (Kannappan and Manja,

2004). LAB may be used as bio-preservative bacteria in the food industry. There has been a lot of work done on lactic acid bacterial antagonism in milk and milk-based products but LAB incorporated fish or fish products reports are scanty. Therefore, this work was undertaken with a view to study the antibacterial activities of LAB against seafood-borne bacteria coated on the cooked disintegrated Indian mackerel fish meat.

MATERIALS AND METHODS

Mackerel

Rastrelliger kanagurta (Cuvier) of size 17-19cm (90-125g) were procured from the Chennai fish market, iced at 1:1 ratio and transported to the laboratory.

LAB cultures and seafood-borne bacteria

Streptococcus faecalis NCIM 2607, (National Collection of Industrial Microorganism) *Lactobacillus casei* NCIM 2586, *Pediococcus cerevisiae* NCIM 2171 were obtained from the National Chemical Laboratory, Pune, India. *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Listeria monocytogenes, Clostridium perfringens* and *Vibrio parahaemolyticus* were isolated from spoiled mackerel fish (*Rastrelliger kanagurta*) and identified up to species level following various chemical reactions (Harrigan and McCance 1976 and Swanson et al, 1992). These strains were compared with the standard type strains obtained from National Chemical Laboratory, Pune, India.

Agar spot method

Deman Rogosa Sharpe agar (MRS) plates were prepared and allowed to solidify overnight (Deman *et al,* 1960). *P.cerevisiae, S. faecalis and L. casei* (1.0 ml of 24 h old) were inoculated separately into 25.0 ml of tryptic glucose yeast extract broth (Hi-media, Mumbai) and incubated at 37^{0}C for 24 h (Schillinger and Luke, 1989). The cells were harvested by centrifugation using a super speed RC5B Refrigerated centrifuge (10,000 rpm /10 min, 46274g force) and suspended in sterile saline. After washing thrice in sterile saline, the cells of 20 l (at 10^{8} cfu/ml level) were spotted onto MRS agar plates. The plates were allowed to set for 30 min at room temperature and incubated overnight at 37^{0}C without disturbing the spots. Seafood-borne bacteria (24 h) of 100 l (175 cfu) from 10^{1} dilution were transferred into 8.0 ml of BHI (brain heart infusion) soft agar (1.0 %). After vortexing, this was overlaid onto the MRS plates without disturbing LAB spots. After overnight incubation at 37^{0}C, the diameters of the zones of inhibition, excluding the spots were measured in mm (Annear and Hudson, 1970).

Coating of LAB and seafood-borne bacteria on fish

Fresh Indian mackerel (100g) was dressed, cut into chunks and washed in 10 ppm chlorinated water. Later the residual chlorine was eliminated to "0" level by washing in sterile distilled water thrice. The chunks were then cooked in boiling water for 15 min. The meat (40g) was disintegrated and transferred to the sterile petriplate. Seafood–borne bacteria such as *L. monocytogenes, C. perfringens* and *V. parahaemolyticus* 0.5 ml (10^{5}-10^{6} cfu/ml) each

(total 1.5 ml, but the level of individual fish spoilage bacteria varied within the range as mentioned in the table) were mixed together, coated uniformly using sterile pipette and allowed to remain for 5 min at room temperature. Later, 5.0 ml each of LAB cells such as *P.cerevisiae, L. casei and S. faecalis* (10^8 cfu/ml) was mixed together with 1 ml of 1% glucose as growth initiator for LAB, (as fish does not have adequate carbohydrate) and coated using sterile pipette as mentioned earlier. The petri plate was then wrapped on the sides using polyfilm and stored at 37^0C. Similarly other seafood-borne bacteria such as *S. aureus, B. cereus* and *E.coli* were coated with the same level along with the LAB cells such as *P.cerevisiae, L. casei* and *S. faecalis*. The meat was then mixed together and the petri plates were sealed using polyfilm and incubated at 37^0C. Combinations of *L. monocytogenes, C. perfringens* and *V. parahaemolyticus* and *S. aureus, B. cereus* and *E. coli* has been coated on fish meat and studied their growth pattern at 37^0 C for control.

Interaction of multiple LAB against seafood-borne bacteria on cooked mackerel meat

Forty gm of cooked mackerel meat was taken in a sterile petriplate. Seafood-borne bacteria of 0.5ml (10^5-10^6cfu/ml) each (total 1.5 ml, but the level of individual fish spoilage bacteria varied within the above range as mentioned in the table) were coated on cooked disintegrated mackerel meat using sterile pipette. Followed by active LAB cells, five ml each (10^8cfu, total 15ml) such as *S. faecalis, L. casei* and *P. cerevisiae* were mixed together with 1 ml of 1% glucose as growth initiator for LAB and coated on 40g cooked mackerel meat using sterile pipette and incubated at 37^0. (Since the combination three LAB mixed together and applied can be called multiple LAB treatment). LAB growth has been studied as combined and separately with 1 ml of 1 % glucose solution on fish meat for control.

pH

The pH was measured using a combination electrode pH meter after estimating the total viable count.

Variation in spoilage bacteria on the mackerel meat after boiling for 15 min

The dressed mackerel fish chunks (50g) were cooked in boiling water for 15min. The meat was then disintegrated aseptically. The total viable counts, *Staph. aureus* and thermophilic spore formers were estimated. The purpose of cooking mackerel meat in boiling water is to reduce the microbial flora, which interrupt with the LAB antagonism. Similarly all seafood-borne bacterial loads were estimated in fresh mackerel fish. Seafood–borne bacterial load were estimated in fresh mackerel to ascertain their initial load.

Statistical Analysis

Student "t" test was conducted between the pairs of bacteria, of three replicates which were not inhibited by LAB and the significant growth difference was reported (Edwin, 1986).

RESULTS AND DISCUSSION

Antibacterial activities of LAB against seafood-borne bacteria: *S. faecalis, P.*

cerevisiae and *L. casei* inhibited all the Gram-positive bacteria examined, while the Gram-negative bacteria such as *V. parahaemolyticus* (0.5mm) and *E. coli* (1mm) were not inhibited. *S. facealis* showed identical inhibitions on *S. aureus* and *L. monocytogenes* (20 mm) than *P. cerevisiae* and *L. casei* (Table 1). In the control, *V. parahaemolyticus, L. monocytogenes* and *C. perfringens* were grown well on cooked fish meat changing of the pH to alkaline condition (6.7 - 8.5). Similarly, *B.cereus, S. aureus* and *E.coli* were grown together in another combination where the pH got changed from 6.3 – 8.6 (Table 2). This was appropriate for the growth of fish-borne bacteria.

Growth pattern of multiple and single LAB cells on mackerel meat

Multiple LAB cells were grown on the fish meat. The pH reduced to 5.10 in multiple LAB, whereas it was 6.1 in single LAB coat (Table 3).

Variation among native bacteria of mackerel cooked in boiling water for 15 min

After cooking in boiling water for 15 min, the viable count of TPC, *S. aureus* and thermophilic spore formers were estimated as log 2, log 1.1 and log 1 respectively. However, the load of these bacteria in fresh fish was observed almost thrice higher to these values. Therefore, it was evident that cooking in boiling water for 15 min reduces only 85 % of the bacteria present in it.

S. faecalis inhibited *L. monocytogenes* and *C. perfringens* on the third day by 3.5 and 3.7 log units difference as compared to control (Table 4). *B.cereus* was inhibited on the second day by 5.3 log units whereas, *S. aureus* on the third day by 1.3 log. Daly et al. (1972) explained that *S. faecalis* in association with *S. diacetylactis* as multiple LAB were capable of inhibiting many associative pathogens. In the present study none of the Gram-negative bacteria were inhibited by *S. faecalis*, but their load reduced considerably. The pH reduced to 6.0 from 6.2 by the combination of *Vibrio, Clostridium* and *Listeria species* and from 6.1 to 5.2 in *S. aureus, B.cereus* and *E. coli* combination. The *Vibrio* and *Clostridium* were highly putrefactive in nature than *S. aureus, B. cereus* and *E. coli* combination. Significant growth difference was observed between *V. parahaemolyticus* and *S. faecalis* (P: 0.023, T:-3.02), but not between other pairs of bacteria.

Table 1. Inhibitory activity of lactic acid bacteria on seafood-borne bacteria

Seafood-borne bacteria	*Strep.faecalis*	*P. cerevisiae*	*Lb.casei*
Listeria monocytogenes	20 ± 0.20	10 ± 0.22	19.5 ± 0.50
Bacillus cereus	14 ± 0.51	10 ± 0.31	9.5 ± 0.41
Escherichia coli	1 ± 0.01	0.5 ± 0.10	Nil
Clostridium perfringens	10 ± 0.20	11 ± 0.32	12 ± 0.31
Staphylococcus aureus	20 ± 0.12	9.5 ± 0.51	8.5 ± 0.20
Vibrio parahaemolyticus	0.5 ± 0.10	0.5 ± 0.10	0.6 ± 0.10

Mean ± SD (n = 3), values are in mm

Table 2. Growth pattern of various seafood–borne bacteria on fish meat as control

Combination of *Vp, Cp* and *Lmc* cells as control on fish meat with pH					Combination of *Bc, Sa* and *E.coli* cells as control on fish meat with pH			
Storage period in days	*Vp*	*Cp*	*Lmc*	pH	*Bc*	*Sa*	*Ec*	pH
0	5.3	5.2	5.2	6.7	6.1	6.1	6.3	6.3
1	8.2	6.7	6.2	6.9	6.0	7.0	7.0	7.6
2	8.0	5.9	5.9	8.1	7.4	7.3	8.0	8.1
3	7.0	5.7	5.6	8.5	7.1	7.0	8.4	8.6

Table 3. Growth pattern of multiple and single LAB on fish meat as control

Combined LAB as control (*S. faecalis, L.casei* & *P.cerevisiae*) with 1 ml of 1 % glucose solution on fish with pH			**Growth of single LAB (*P. cerevisiae, S. faecalis* and *L. casei*) as control with 1 ml of 1 % glucosee solution on fish with pH**					
Storage period (in days)	Growth of combined LAB		*P.cerevisiae*		*S.faecalis*		*L. casei*	
	load	pH	load	pH	load	pH	load	pH
0	8.0	6.1	8.0	6.5	8.0	6.6	8.0	6.8
1	8.9	6.0	8.3	6.3	8.9	6.4	8.4	6.6
2	8.7	6.1	8.5	6.1	8.6	6.3	8.0	6.5
3	8.5	6.0	8.0	6.7	8.1	6.0	8.1	6.5

Vp: *V. parahaemolyticus, Cp : C. perfringens , Lmc : L . monocytogenes. Bc: B. cereus, Sa: Staphylococcus aureus,* Values are $\log_{10}$ cfu/ml, Mean ± SD (n = 3)

Table 4. Associative growth of different LAB on seafood-borne bacteria on fish meat.

Growth of *S. faecalis* with Vp, Cp and *Lmc* cells with 1 ml of 1% glucose solution on fish with pH *Streptococcus faecalis*						Growth of *S. faecalis* with Bc, Sa and *Ec* cells with 1 ml of 1% glucose solution on fish with pH				
Storage period in days	Sf	Cp	*Lmc*	Vp	pH	Sf	Bc	Sa	*Ec*	pH
0	8.0	5.1	5.2	5.3	6.2	8.0	6.1	6.1	6.3	6.1
1	6.5	5.8	5.2	6.1	5.5	8.1	2.1	6.00	6.9	5.5
2	8.5	2.0	2.1	6.0	5.8	8.0	ND	2.00	5.2	5.5
3	8.0	ND	ND	5.9	6.0	7.7	ND	ND	5.0	5.2

Lactobacillus casei

	Lbc	Cp	*Lmc*	Vp	pH	Lbc	Bc	Sa	*Ec*	pH
0	8.0	5.2	5.1	5.4	6.1	8.0	6.0	6.1	6.2	6.1
1	8.6	5.5	5.2	7.2	6.5	7.1	2.2	4.0	7.6	5.6
2	8.3	5.0	2.3	7.4	7.4	8.2	ND	2.1	6.7	5.0
3	8.2	4.0	ND	7.3	7.8	7.3	ND	ND	6.6	5.0

Pediococcus cerevisiae

	Pc	Cp	Lmc	Vp	pH	Pc	Bc	Sa	*Ec*	pH
0	8.0	5.1	5 .0	5.1	6.2	8.0	6.1	6.2	6.2	6.7
1	8.5	4.6	4. 2	5.1	5.0	8.3	4.2	3.8	6.2	6.9
2	8.6	2.0	2. 2	6.1	5.2	8.5	2.1	2.0	5.9	5.6
3	8.3	ND	ND	6.1	5.3	8.0	ND	ND	5.6	5.1

Combined growth of Sf, Pc, Lbc on Vp, Cp & Lmc cells with 1 ml of 1% glucose solution on fish with pH					Combined growth of Sf, Pc, Lbc on Bc, Sa & Lmc cells with 1 ml of 1% glucose solution on fish with pH				
	Multiple LAB	Cp	*Lmc*	pH	Vp	Multiple LAB	Bc	Sa	*Ec*	pH
0	8.0	5.3	5.1	5.9	6.3	8.0	6.0	6.1	6.2	6.0
1	8.4	2.0	2.0	4.8	6.4	8.2	2.1	2.0	6.0	5.0
2	8.3	ND	ND	5.0	5.0	8.1	ND	ND	5.9	4.9
3	8.5	ND	ND	4.9	3.0	8.4	*ND*	ND	4.0	5.3

NB: Vp: *V. parahaemolyticus*, Cp: *Cl. perfringens*, Lmc : *L.monocytogenes*. Bc: *B. cereus*, Sa : *Staph.aureus*, Ec: *E. coli*, Sf : *Streptococcus faecalis*, Pc : *Pediococcus cerevisiae* , *Lactobacillus casei*
Values are log $_{10}$ cfu/ ml, Mean ± SD (n = 3), ND: Not detected (indicate bacterial inhibition).

L. casei inhibited *L. monocytogenes* on the third day by 3.3 log units as compared to control, on the other hand *Staph.aureus* and *B.cereus* were inhibited on the second and third day by 5.2 and 4.9 log units respectively (Table 4). *Vescovo et al.* (1996) observed inhibition on *S. aureus, L. monocytogenes* and *Aeromonas hydrophila* by *L. casei* IMPCLC 34. In this study, *V. parahaemolyticus* load was not reduced by *L. casei.* Nancy *et al.* (1992) reported inhibitory effect of *L. casei* on *V.parahaemolyticus* and *E. coli.* Vescovo *et al.* (1996) also observed inhibition on *Salmonella typhimurium* by *Lb. casei* in combination with Pediococcus spp. at 37^{0}C. *L. casei* did not inhibit *C. perfringens*, but *P. cerevisiae* and *S. faecalis* inhibited on *C. perfringens.*

In single LAB coating, *P. cerevisiae* and *S. faecalis* equally inhibited *L. monocytogenes* and *C. perfringens* but not *L. casei.* It indicates that the efficacy of inhibition of seafood-borne bacteria by LAB varies from genus level. Significant growth difference was observed between *V. parahaemolyticus* and *C. perfringens* (P: 0.0008. T:-6.28), *C. perfringens* and *L. casei* (P: 0.0008, T:-6.20) and *E. coli* and *L. casei* (P: 0.0001, T:-10.13) but not between other pairs of bacteria.

P. cerevisiae did not inhibit the growth of *E. coli* whereas, *S. aureus* and *B. cereus* were inhibited by *P. cerevisiae* on the third day by 5 log units. Gilland and Speck (1975) reported inhibition on *Pseudomonas fragi* by coating LAB strains such as *L. bulgaricus* and *P. cerevisiae* together. Raccach *et al.* (1979) observed that *P. cerevisiae* cells in combination with *L. plantarum* inhibited *S. typhimurium* and *S. aureus* in poultry meat. But during this study, *P.cerevisiae* alone inhibited *S. aureus*. The inhibition may be due to *P.cerevisiae* cells or its ribosomally synthesized compounds such as lactic acid, hydrogen peroxide and bacteriocin like inhibitory substances (BLIS). It has been reported that *P. cerevisiae* and *L. rhamnosus* GG ATCC 53013 inhibited various food-borne pathogens (Leela et al 2005). The pH changed from 6.7-5.1 in *E. coli, S. aureus* and *B. cereus* combination. This might be due to the action of LAB and sucrose. *L. monocytogenes* was inhibited by *P. cerevisae* on the third day by 3.4 log units as compared to control, whereas *C. perfringens* was inhibited on the third day of storage by 3.7 log units. This difference does not seem to be very high. Significant growth difference was observed between *V. parahaemolyticus* and *P. cereviasae* (P: 0.0000. T:- 10. 66), but not between other pairs of bacteria.

Interaction of multiple LAB against seafood-borne bacteria on cooked mackerel meat

Coating with multiple LAB strains inhibited *C. perfringens* and *L. monocytogenes* on the second day by 3.8 and 3.9 log units as compared to control. Similarly *B. cereus* and *S. aureus* were inhibited by 5.2 and 6 logs units (Table 4). Significant growth difference was found between *E. coli* and multiple LAB (P: 0.0000, T:-14.36), but not between other pairs of bacteria. Multiple LAB did not inhibit Gram-negative bacteria. Therefore, it was concluded that multiple LAB coat was not effective to inhibit Gram-negative bacteria. Probably coating LAB cells with chelating agent such as EDTA and lysozyme would inhibit Gram-negative bacteria (Kannappan *et al.*, 2004b). Very low pH was observed in multiple LAB

strain coating due to LAB growth as compared to single strain coat.

CONCLUSION

Most of the LAB showed inhibitory activity against seafood-borne bacteria and coating multiple LAB further showed much earlier inhibitory activity on seafood-borne bacteria than single LAB coat. Hence, LAB can be incorporated to the fish in order to reduce undesirable bacteria. On cooked fish, LAB coat may be used for extending their shelf life prior to consumption.

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