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Effect of *Lactobacillus rhamnosus* cells against specific and native fish spoilage bacteria and their spoilage indices on Asian seabass fish chunks

Authors Info

S. Kannappan[1]*, K. Sivakumar[2] and S. Sivagnanam[1]

[1]Nutrition, Genetics and Biotechnology Division, Central Institute of Brackishwater Aquaculture, Chennai - 600 028, India

[2]ICAR – Krishi Vigyan Kendra, Tamil Nadu Veterinary and Animal Sciences University, Kattupakkam - 603 203, India

*Corresponding Author Email : sudalikanna@gmail.com

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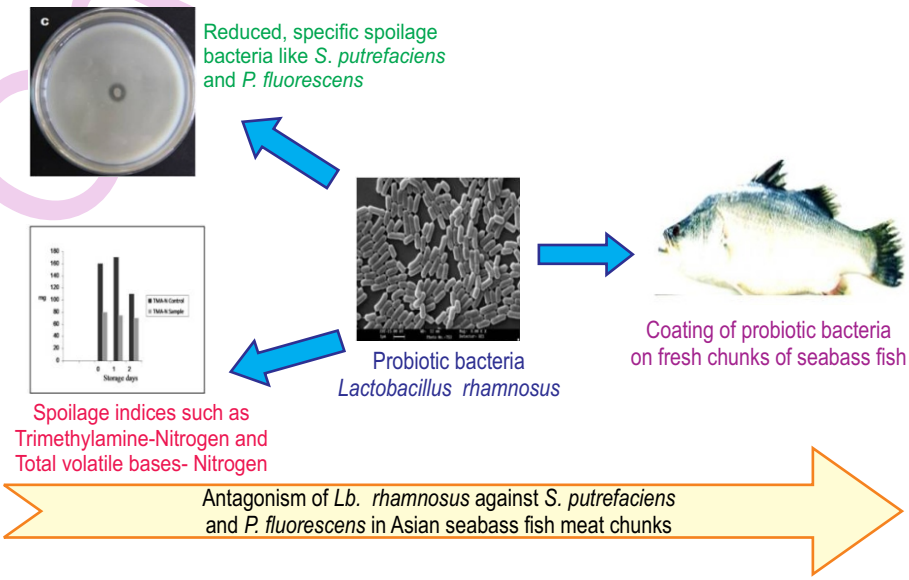
Abstract

Aim : The present study aimed at evaluating probiotic bacterial cells, *Lb. rhamnosus* by coating on seabass fish meat chunks against its spoilage indices, native and specific fish spoilage bacteria.

Methodology : Seabass fish fillets were cut into chunks, washed and taken into petri plates. Scores were made using sterile blade. *Lb. rhamnosus* cells were coated on the chunks using micro pipette and tested for antagonism against fish–borne specific spoilage bacteria such as *Shewanella putrefaciens* and *Pseudomonas fluorescens* through "Deferred antagonism assay". Spent culture of *Lb. rhamnosus* was assayed for antagonism through "Agar-well diffusion assay". The changes were estimated for two days following "Conway micro diffusion" method.

Results : *Lb. rhamnosus* cells when co-cultured with *P. fluorescens* were inhibited on the second day by 1.0 log difference as compared with control. The spent culture of *Lb.rhamnosus,* after treating at 121°C for 10 min showed inhibition against *P. fluorescens*. But the spent culture after treatment at 100°C showed difference of inhibitory zone at 5.0 mm against *S. putrefaciens. Lb. rhamnosus* cells when coated on seabass fish meat chunks, the native proteolytic bacteria was inhibited by *Lb. rhamnosus* on 3rd day by 1.3 log difference as compared to control. *Lb. rhamnosus* cell coat, reduced fish spoilage indices such as Trimethylamine-Nitrogen (TMA-N) and Total volatile bases- Nitrogen (TVB-N) by 80 mg% difference.

Interpretation : The study concluded that coating of probiotic *Lb. rhamnosus* cells on fish meat would contribute antagonism and regulate the growth of specific and native spoilage bacteria with spoilage indices.



Introduction

Fish is a highly perishable food which spoils faster than any other meat owing to the presence of unsaturated fatty acids in it, therefore fish has to be preserved rapidly soon after harvest. The shelf life of fish is always assessed based on the freshness and spoilage level (Ghaly *et al.,* 2010). *Shewanella putrefaciens* and *Pseudomonas fluorescens* are the foremost specific spoilage bacteria found among the marine fish (Aberoumand, 2010). *P. fluorescens* is morphologically a Gram-negative rod that causes hemorrhage in fish skin, fins, oral cavity and muscles (Tsai *et al*., 2008). Specific spoilage level is responsible for the conversion of Trimethylamine oxide to trimethylamine in fish. Under specific spoilage bacteria, *S. putrefaciens* causes common clinical manifestations such as otitis, soft tissue infection, bacteraemia and infection among human beings. *S. putrefaciens* also produce off-flavour in fish at high (10^8cfu g^{-1}) cell density (Tsai *et al*., 2008).

Lactic acid bacteria has long been recognized as bio-preservative bacteria due to their inhibition potential against bacterial pathogens by producing organic acids and bacteriocin like inhibitory substances, etc (Zaheer *et al.,* 2010). Under Lactic acid bacteria the use of probiotic bacteria is gaining recognition to control aquatic bacterial pathogens (Lara-Flores, 2011) in aquaculture. Probiotics bacteria such as ammonia oxidizing and nitrite oxidizing bacteria have been reported to be effective in monitoring microbiological and environmental parameters in *Litopenaeus vannamei* grow-out practices (Patil *et al.,* 2016).

Supplementation of Lactic acid bacteria in broiler as probiotic bacteria improves the meat quality (Kabir, 2009). Antimicrobial activity of *Lactobacillus rhamnosus* has been well documented for controlling food spoilage bacteria (Magdalena *et al.,*2009) has been used to control trimethylamine nitrogen and spoilage indices in fresh Indian Mackerel fish (Kannappan and Manja, 2011). The efficacy of lactic acid bacteria in controlling human pathogenic bacteria and fish-borne spoilage microbiota were studied on fresh mackerel fish chunks (Kannappan and Manja, 2012). Chemicals cannot be used as fish preservative agent because of the disagreeable contamination caused to the contemporary consumers. Further, chemical preservatives are not much encouraging in combating fish-borne spoilage bacteria. Thus, fish preservation using Lactic acid bacteria as bio-preservative agent would increase the shelf life. *Lb. rhamnosus* GG, human gut-borne lactic bacteria, has been well proved as antagonistic agent against pathogenic and food spoilage microbes (Sarika *et al.,* 2010). *Lb. rhamnosus* has been used as feed supplement cum probiotic bacteria during the grow-out practices of rainbow trout fish (Balcazer *et al.,* 2006). *Lb. rhamnosus* has different immune-stimulative effects with reduced intestinal damage when challenged against pathogenic *Aeromonas* bacteria (Ngamkala *et al.,* 2010). In India, the application of Lactic acid bacteria is well propagated in milk based products. But, yet to disseminate in fish based products. *Lb. rhamnosus*, probiotic bacteria has specially been selected for humans since it has been exposed to be harmless for humans. However, application of *Lb. rhamnosus* for fish preservation through controlling the fish-borne spoilage bacteria and their spoilage indices is scarce. Therefore, the present study was undertaken to study the antagonistic efficacy of *Lb. rhamnosus* against, specific spoilage bacteria such as *S. putrefaciens* and *P. flourescens* present in the food. They are typically present in very low numbers with their spoilage indices and other native spoilage bacteria in chunks of seabass fish *(Lates calcarifer),* which is one of the candidate species in the brackishwater aquaculture practices in India.

Materials and Methods

Bacterial strain, *Lb. rhamnosus* was procured from the American Type Culture Collection Centre (ATCC 53103) and revitalized in MRS agar (Deman Rogosa Sharpe-Hi-Media, India, Deman *et al.,* 1960) medium at 37°C for 24 hrs aerobically. *Lb. rhamnosus* cells (1.0 ml) were inoculated into 10 ml MRS broth and shaker incubated at 37°C, 150 rpm for 24 hrs. Five ml of this inoculum was transferred into 25 ml MRS broth and shaker incubated at 37°C, 150 rpm for 24 hrs (Polak-Berecka *et al*., 2010). Later, *Lb. rhamnosus* cells were harvested by refrigerated centrifuge (Sorval RC5B @ 47432 g/45 min/5°C). Further, cells were washed in normal saline (0.85 % NaCl (w/v) thrice and quantified as 10^6cfu ml^{-1} by total plate counting.

Isolation of specific spoilage bacteria (SSB) : Specific spoilage bacteria Were isolated by taking 10 g of partially putrefied seabass meat and homogenized with 90 ml normal saline (0.85% NaCl). One ml of this suspension was inoculated into 25 ml of Brain Heart Infusion Broth (BHI broth) and shaker incubated at 25°C / 24 hrs (150 rpm). One ml of BHI inoculum was serially diluted in normal saline and transferred into petri plates with replications. Iron agar was transferred into the petri plates and incubated at 37°C/24 hrs for the growth of *P. fluorescens* and at 25°C/24 hrs for *S. putrefaciens*. The composition of medium was as follows: 20 g peptone:, 3.0 g meat extract:, 3.0 g yeast extract:, 0.3 g ferric citrate:, 0.3 g sodium thiosulphate:, 5.0 g sodium chloride:, 0.6 g L-cysteine:, 15.0 g agar:, pH: 7.40±0.2. For *S. putrefaciens,* after the agar was solidified, iron agar (2mm level) was over laid to create anaerobic condition. This step is important to minimize the possibility of oxidation of the ferrous sulphate which would cause instability of black precipitate). After incubation, *S. putrefaciens,* appeared as black colonies, whereas *P. fluorescens* as white. Iron agar contains two sources of sulphur, *i.e.,* thiosulphate and L-Cysteine. Bacteria that are able to produce hydrogen sulphide from either sulphur source appeared as black colonies due to precipitation of ferrous sulphide in the agar. Non-hydrogen sulphide producing bacteria appeared as white colonies (Shewan *et al.,* 1960). The isolates of *S. putrefaciens* and *P. fluorescens* were maintained in BHI broth (Hi-Media, India) containing 20% glycerol at –80°C. Successive specific spoilage bacterial cultures were inoculated into BHI broth and revitalized before the experiments.

Confirmation of specific spoilage bacteria : *S.putrefaciens* and *P. fluorescens* were characterized using tests such as Gram-reaction (KOH), shape and motility (Phase contrast Microscopy), cytochrome oxidase, catalase formation (H_2O_2, 3% v/v), glucose metabolism (O/F test), reduction of TMAO–TMA, H_2S production (Gram *et al.,* 1987), production of DNAase, decarboxlyase and ornithine (Shewan *et al.,* 1960). Specific spoilage bacteria such as *S. putrefaciens* ATCC 8071 and *P. fluorescens* ATCC 1352 were procured from the American Culture Type Collection (ATCC) and their cultural characteristics were compared with the specific spoilage bacterial isolates (Stenstrom and Molin, 1990) used in this study.

Deferred antagonism assay : Production of antimicrobial substances from *Lb. rhamnosus* was detected by "Deferred antagonism assay" (Barefoot and Klaenhammer, 1983). Cells of *Lb. rhamnosus* (Producer strain) were harvested then washed with sterile distilled water. Twenty µl of these cells (±10^7cfu ml^{-1}) were spotted on the surface of MRS agar plates and incubated for 16 hrs at 30°C. Approximately 50 µl (±10^4cfu ml^{-1}) of *S. putrefaciens* and *P. flurorescens* were inoculated separately into 8.0 ml of BHI soft agar (0.8%), after uniform mixing, this was over laid on the plates without disturbing the producer strain. After incubation at 30°C/24 hrs, the spot from producer strain was examined for zone of inhibition against the indicator bacteria. Inhibition was recorded positive if the width of clear zones around the colonies of the producer strain was greater than 2.0 mm.

Production of antimicrobial substances from *Lb. rhamnosus* : In order to evaluate the production of antimicrobial substances, 25 ml of the spent culture from *Lb. rhamnosus* was treated with 40% ammonium sulphate (w/v) and allowed to remain at 4°C/30 min. This filtrate was then centrifuged and passed through 0.42 µm pore-size filters. Half of each filtrate was neutralized with 5M NaOH (pH 6.8) and evaporated through vacuum to 5.0 ml. The resultant filtrate was subjected to heat treatments at 100°C and 121°C for 10 min, respectively, (Silva *et al.,* 1987) and tested for antagonism against specific spoilage bacteria.

Agar-well diffusion assay : Spent culture of *Lb. rhamnosus* was assayed for antagonism through Agar-well diffusion assay (Tagg *et al.,* 1971) specific spoilage bacteria. (50 µl of 10^4cfu ml^{-1} of a log-phase) like *S. putrefaciens* and *P. fluorescens* were transferred separately into sterile petri plates. Iron agar was dispensed into the petri plate. Wells of 7.0 mm diameter was made into the plate and then sealed using 15 µl of (0.8%) soft agar. The concentrated and heat inactivated spent culture of two fold dilution (200 µl) was transferred into the well. The plates were then allowed to remain at room temperature for 30 min and then incubated at 37°C/24 hrs. The zone of inhibition were measured excluding the wells.

Associate growth of *Lactobacillus rhamosus* against *S. putrefaciens* and *P. fluorescens* : *S. putrefaciens, P. flourescens* and *Lactobacillus rhamnosus* were separately sub-cultured in BHI and MRS broths, respectively, and incubated at 30°C/12 hrs on a shaker incubator (100 rpm). SSB were then inoculated (1.0 ml) separately into 100 ml of BHI broth to provide an initial cell density of approximately 10^3cfu ml^{-1}.The initial level of *Lb. rhamnosus* cells inoculated (3.0 ml) into BHI broth was approximately 10^7cfu ml^{-1}. The flasks were incubated at 30°C/12 hr in shaker incubator (100 rpm). All the experiments were repeated thrice. One ml of inoculum from the co-culture broth was drawn every day for estimation of bacterial cell density. The cell counts were assessed by spread plating using 1.0 ml aliquots, serially diluted and plated on iron agar and MRS agar plates. The plates were incubated for 24hr/30°C and colonies were counted and expressed as cfu ml^{-1}.

Preparation of seabass fish meat chunks : Fresh seabass fish fillets of 40 g were cut into chunks (4.0 cm thickness) and taken into the petri plates. Scores of 0.5 cm deep were made on four places of each chunks using sterile blade. *Lb. rhamnosus* cells, 5.0 ml (10^7cfu ml^{-1}) were coated on the chunks using micro pipette. The plates were sealed externally using parafilm to avoid cross contamination and incubated at 37°C/2 days (Kannappan *et al.,* 2004).

Estimation of native fish spoilage bacteria with spoilage indices : Proteolytic and lipolytic bacteria, total plate counts, coliforms, *E.coli*, mesophilic and thermophilic spore forming bacteria were determined over a period of 3 days susing media such as Milk agar, Tributryn agar, Plate count agar, VRBA, (Violet Red bile agar) EMB (Eosin Methylene blue) agar, etc. (Hi-Media, India). All these bacteria were re-confirmed through various biochemical tests (Stenstrom and Molin, 1990). For estimating the changes in fish spoilage indices, *Lb. rhamnosus* cells were coated on seabass fish meat chunks as described above. However, the chunks were allowed to remain with the cell suspension for 2 days. TMA-N, TVB-N and pH were estimated for 2 days. The changes of TMA-N and TVB-N were determined through Conway micro diffusion method (AOAC, 1980). Uncoated chunks of *Lb. rhamnosus* were considered as control. The pH was measured using a combination electrode attached to a pH meter (Electronic Corporation of India) in the fish meat after estimating the native spoilage bacteria and *Lb. rhamnosus*.

Statistical analysis : All the tests were repeated thrice; average and standard deviations were calculated. Repeated measures of ANOVA were followed to evaluate the effect of bacterial inhibition and reduction of amines between the control and *Lb. rhamnosus* cells coated on fish chunks.

Results and Discussion

Total length of seabass fish was 54 ± 0.2 cm, total width: 14 ± 0.2 cm, weight of whole fish: 1.8 ± 0.2 kg. Total plate count (TPC) level was 8×10^5cfu g^{-1}, specific spoilage bacteria was

20×10^3cfu g^{-1}, proteolytic bacteria 20×10^4cfu g^{-1}, lipolytic bacteria were 15×10^3cfu g^{-1}, whereas mesophilic spore formers 4×10^2 cfu g^{-1}, coliforms 20×10^3cfu $g^{-1}$and thermophilic spore formers were 2×10^1cfu g^{-1}, respectively. All these values would vary in accordance with the size, feeding habits, migration and sex of fish. However, the values are agreeable as compared with other seabass fish (Kocatepe and Turan, 2012). In agar spot method, the highest inhibitory zone (Table 1) was observed against *S. putrefaciens* (20 mm) followed by *P. fluroescens* (18 mm).

Spent culture of *Lb. rhamnosus* inhibited the growth of *Bacillus brevis*, *B. pumilus*, *B. subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Vibrio harveyi*, *Acinetobacter* sp. and *Arthrobacter* sp. (Sarika *et al.,* 2010). Usually majority of LAB may not inhibit Gram–negative bacteria, if so it gives narrow inhibition. Much data was not available to directly support antagonism against *S. putrefaciens* and *P. fluroescens.* But the spent culture of *Lb. rhamnosus* treated at 100°C showed highest zone of inhibition against *S. putrefaciens* (28 mm), whereas spent culture of *Lb. rhamnosus,* treated at 121°C showed lesser zone of inhibition against *P. fluorescens* (25.0 mm) and *S. putrefaciens* (23.0 mm), respectively. The culture filtrate and extracellular protein concentrate of *Lb. rhamnosus* possessed antimicrobial substances which were active against *P. aeruginosa*, *E. coli*, *Enterobacter aerogenes*, *S. aureus*, *Salmonella* sp., *Helicobacter pylori*, *Campylobacter jejuni*, *Bacillus cereus*, *B. megaterium* and *L. monocytogenes* (Ambalam *et al*., 2009). The spent culture of *Lb. rhamnosus* has proved to contribute antioxidant, whitening, moisture-retention properties in cosmetic applications (Tsai *et al.,* 2013). *Lb. rhamnosus* strains exhibit antimicrobial activity against food spoilage bacteria, gastrointestinal pathogens, as well as *Candida* and *Aspergillus* sp. (Pithva *et al*., 2014).

Here, the zone of inhibition was moderately higher throughout the treatment at 100°C. *Lb. rhamnosus* cells were controlled *Edward siellatarda* infection and the cumulative mortality caused by *E. tarda* in *Tilapia* (*Oreochromis niloticus*) fish (Pirarat *et al.,* 2006). Therefore, it was evident that the concentrated filtrate (spent culture) of *Lb. rhamnosus* will be considerably more effective at 100°C than at 121°C in significant level. Although, the inhibitory mechanism of spent culture was not characterized in this study. However, the source of antimicrobial activity cannot be attributed to the acidity of spent culture, since spent culture was neutralized to pH 6.8.

It was evident that the inhibitory effects might be due to the secondary metabolites such as organic acids, hydrogen peroxide and bacteriocins etc., produced by LAB. Nisin obtained from LAB such *Lactococcus lactis* has been commonly accepted as bacterial inhibitor (GRAS status) in milk that also inhibits various fish-borne bacteria (Kannappan *et al.,* 2004). Consequently, the spent culture of *Lb. rhamnosus* can be effectively used against fish spoilage bacteria. During co-culture, *Lb. rhamnosus* showed inhibition of *S. putrefaciens* on 2nd day by 2.40 $\log_{10}$ difference as compare to control whereas *P. fluorescens* got inhibited on the 2nd day by 2.28 $\log_{10}$ difference (Table 2).

Table 1 : Antagonism of *Lb. rhamnosus* cells and its heat activated spent culture against *S. putrefaciens* and *P. fluorescens* at 37°C

Producer strain	Indicator bacteria	Zone of inhibition
Cells of *Lb. rhamnosus*	*S. putrefaciens*	20.0 ± 0.2
Cells of *Lb. rhamnosus*	*P. fluorescens*	18. 0 ± 0.2
Spent culture of (200 µl) *Lb. rhamnosus* (7.0 pH) sterilized at 121°C	*S. putrefaciens*	23.0 ± 0.5
Spent culture of (200 µl) *Lb. rhamnosus* (7.0 pH) sterilized at 121°C	*P. fluorescens*	25.0 ± 0.5
Spent culture (200 µl) of *Lb. rhamnosus* (7.0 pH) sterilized at 100°C	*S. putrefaciens*	28.0 ± 1.0
Spent culture (200 µl) of *Lb. rhamnosus* (7.0 pH) sterilized at 100°C	*P. fluorescens*	29.0 ± 0.5

Values are mean of three replicate ± SD

Table 2 : Antagonism of *Lb. rhamnosus* cells against the growth of *S. putrefaciens* and *P. fluorescens* in BHI broth through co-culture at 37°C for three days

Storage period in days	*Lactobacillus rhamnosus*	pH	*S. fluorescens*	*P. putrefaciens*	*S. putrefaciens*	pH	*P. fluorescens*	pH
Initial	5.90± 0.2	7.30± 0.3	2.00 ± 0.0	2.00 ± 0.0	2.00 ± 0.0	7.20 ± 0.3	2.00 ± 0.0	7.20± 0.3
I	7.04± 0.1	7.00 ± 0.2	2.47 ± 0.1	2.47 ± 0.1	4.90 ± 0.2	7.80 ± 0.2	4.75± 0.2	8.10 ± 0.3
II	6.82± 0.2	5.80± 0.2	ND	ND	3.00 ± 0.1	8.10 ± 0.3	3.00 ± 0.1	8.40± 0.2
III	4.60 ± 0.1	5.60± 0.1	ND	ND	2.50 ± 0.1	8.20 ± 0.3	2.30 ± 0.1	8.50 ± 0.1

Values are $\log_{10}$ cfu ml^{-1}. Values are mean of three replicate ± SD

Lb. rhamnosus was able to inhibit 2.40 log load of both bacteria grew in BHI broth. *Lb. rhamnosus,* grew in increasing and decreasing trends (4.6 log loads) on 3rd day. Considerable level of *S. putrefaciens* and *P. fluoresences* survived at 5.6 pH. The pH was significantly reduced to 5.6 with *Lb. rhamnosus*. But, in control the pH increased to 8.50. In general, *Lb. rhamnosus* cells have strong efficacy to antagonize *Aeromonas salmonicida* in rain bow trout fish which cause Furunculosis disease (Nikoskelainen *et al.,* 2001). Repeated measures of ANOVA showed that inhibition of *S. putrefaciens* and *P. fluorescens* was significantly different from control ($F_{1,4}$ = 23290.7, P<0.001). There was a significant difference between the storage days in inhibition of *S. putrefaciens* ($F_{3,12}$ = 400.21, P<0.001). But there was no significant difference between the control and co-culture of *Lb. rhamnosus* with *P. fluoresences* ($F_{1,4}$ = 4.62, P = 0.09). The storage days did not vary significantly in inhibiting *P. fluorescens*. ($F_{3,12}$ = 1.41, P = 0.28). *Lb. rhamnosus* cell coat inhibited *E. coli* on 3rd day of storage by 2.0 log differences as compared to control. This case was also same with specific spoilage bacteria.

Proteolytic bacteria was inhibited by *Lb. rhamnosus* on 3rd day of storage by 1.30 log difference (Table 3). It has been proved that Lb. rhamnosus and its spent culture inhibited food-borne bacteria such as *V. parahaemolyticus, E. coli, B. cereus* and *S. aureus* (Leela *et al.,* 2005). *Lb. rhamnosus* did not inhibit LLB, however there might have been inhibition if the storage period was extended further. *Lb. rhamnosus* inhibited MSF on 3rd day of storage by 1.0 log difference as compared to control. *Lb. rhamnosus* also inhibited TSF on the 2nd day of storage by 0.50 log difference. *Lb. rhamnosus* cells coating on fish chunks inhibited *E. coli* (Susanna *et al.,* 2000) and the overall *coliform* load reached to 2.0 log in the fish. In control, the *coliforms* load attained 2.90 log. *Lb. rhamnosus* cells with spent culture, inhibited human pathogenic bacteria such as enterotoxigenic *E. coli,* entero pathogenic *E.coli, Klebsiella pneumoniae, Shigella flexneri, Salmonella typhimurium, Enterobacter cloacae, Pseudomonas aeruginosa, Enterococcus faecalis* and *Clostridium difficile* through in-vitro experiments (Forestier *et al.,* 2001).

In the present study also proteolytic bacteria such as *Pseudomonas* species was inhibited by *Lb. rhamnosus.* Sarika *et al.,* (2010) reported that *Lb. rhamnosus* spent culture inhibited various spoilage bacteria. Further, research is needed to elucidate the possible muscle protein changes on fish by coating *Lb. rhamnosus.* Repeated measures of ANOVA showed that there was significant difference among the various group of bacteria inhibited by *Lb. rhamnosus* ($F_{5,12}$ = 322.868, P = < 0.01).

Table 3 : Effect of coating *Lb. rhamnosus* cells against growth changes in various fish borne spoilage bacterial cells in seabass fish chunks for three days at 37°C

Storage period in days	TPC	Coli	*E. coli*	SSB	PLB	LLB	*Lb. rhamnosus*	MSF	TSF	pH
Seabass fish chunks coated without *Lb. rhamnosus* (control)										
Initial day	4.69 ± 0.2	4.69 ± 0.2	4.00 ± 0.1	4.00 ± 0.1	3.77 ± 0.1	3.69± 0.1	ND	3.30 ± 0.1	1.00 ± 0.0	6.70 ± 0.3
I	5.77 ± 0.2	5.00± 0.2	5.90 ± 0.1	5.36 ± 0.1	4.90 ± 0.2	4.84 ± 0.2		4.60 ± 0.2	1.47 ± 0.0	7.60 ± 0.3
II	4.39 ± 0.1	4.90 ± 0.2	4.00 ± 0.1	4.00 ± 0.1	3.90 ± 0.0	3.54 ± 0.1		3.00 ± 0.1	1.00 ± 0.0	8.70 ± 0.4
Seabass fish chunks coated with *Lb. rhamnosus*										
Initial day	4.69 ± 0.1	4.69 ± 0.2	4.00 ± 0.1	4.00 ± 0.1	3.77 ± 0.1	3.69 ± 0.1	7.00 ± 0.3	3.30 ± 0.1	1.00 ± 0.0	6.60 ± 0.2
I	4.17 ± 0.1	2.47 ± 0.1	2.00 ± 0.0	2.47 ± 0.1	2.60 ± 0.1	3.39 ± 0.0	8.00 ± 0.2	2.00 ± 0.0	ND	6.00 ± 0.1
II	3.69 ± 0.1	2.00 ± 0.0	ND	ND	ND	3.00 ± 0.1	6.00 ± 0.1	ND	ND	5.90 ± 0.2

Values are mean of three replicate ± SD and are expressed in $\log_{10}$cfuml^{-1}, TPC - total plate count, coli-coliforms, PLB - proteolytic bacteria, LLB - lipolytic bacteria, MSF - Mesophilic spore formers, TSF - thermophilic spore formers, ND : Not detected

Table 4 : Changes in seabass fish quality indices against coating *Lb. rhamnosus* cells

Storage period in days	Growth of *Lb. rhamnosus* (log)	TMA-N mg %	TVB-N mg %	pH	TMA-N mg % as control	TVB-N mg % as control	pH Control
Initial	8.50	80 ± 1.0	70 ± 0.5	6.8 ± 0.2	160 ± 0.8	120 ± 0.9	6.8 ±0.1
I	9.50	75 ± 0.5	65 ± 0.9	6.0 ± 0.1	170 ± 0.8	110 ± 0.8	7.8 ± 0.2
II	8.00	70 ± 1.0	60 ± 0.7	5.7 ± 0.2	110 ± 0.9	105 ± 0.8	8.4 ± 0.1

Values are mean of three replicate ± SD, TMA-N: Trimethylamine nitrogen, TVB-N: Total volatile base nitrogen

The bacterial inhibition was also significantly different between storage days ($F_{2,24}$ =1165.46, P< = 0.01). Pairwise comparison among various groups of bacteria showed that the effect of inhibition on *E. coli,* Proteolytic bacteria, specific spoilage bacteria, were similar. Rest of them were significantly different from one another. Normally, TMA-O was known to react with the fish fats and produce the typical spoilage odor (Anthony *et al.,* 1990). In the present study, it was found that 10^3 to 10^4cfu g^{-1} of *S. putrefaciens* was required for producing 160 mg g^{-1} of TMA-N in control. TMA-N, 10 to15 mg for 100 g of fish is usually regarded a limit beyond which the fish would be considered slightly (Table 4) spoiled (Saito *et al.,* 1959). In control TVB-N values changed from 120 to 105 mg %, whereas TMAO-N was 160 – 110 mg %. Therefore, it was obvious that *Lb. rhamnosus* cells were able to reduce TMA-N to 95 mg % as compared to control, where as TVB-N was 45 to 50 mg % respectively. The use of *Lactobacillus plantarum* and *Lactobacillus pentosus* cells prevented the formation of total volatile nitrogen contents such as TVA and TMA in vacuum packed fish *Dicentrarchus labrax* (Bassi *et al.,* 2009).

The rate of reduction was higher in case of TMA-N as compared to TVB-N. However, higher level of NPN in control showed more multiplication of bacteria that would produce amines. The reduction of TMA-N and TVB-N might be due to the antagonism excerted by *Lb. rhamnosus* cells or their bacteriocin like inhibitory substances against amines producing specific spoilage bacteria present in the fish. Lactic acid bacteria such as *Lb. helveticus, Lactococcus lactis* and *Pediococcus acidilactici* proved to be the best probiotic bacteria for reducing TMA-N and TVB-N in fresh Mackerel fish chunks (Kannappan and Manja, 2011). *Lb. rhamnosus* incorporated probiotic diet has facilitated growth and enzyme change in *L. vannamei* (Kumar *et al.,* 2013).

Kannappan and Manja (2011) documented that coating of various lactic acid bacterial cells such as *Lb. helveticus, Lactococcus lactis* and *Pediococcus acidilactici* were able to reduce TMA-N and TVB-N in fresh mackerel fish meat. As per repeated measures ANOVA, there was a significant difference between TMA-N in the treated sample and control ($F_{1,4}$ = 5716.6, P<0.001). There was also significant difference between the storage days on the reduction of TMA-N ($F_{2, 8}$ = 351.16, P<0.001) and TVB-N in the treated fish and control ($F_{1,4}$ = 691.7, P<0.001). There was also significant difference between the storage days on the reduction of TVB-N ($F_{2,8}$ =203.5, P<0.001). TMA-N reduction in fish would reduce the characteristic fishy odor. Coating of cells of LAB such as *Carnobacterium* and *Lactobacillus plantarum* species reduced the fishy smell on Salmon fish caused by amines (Francoise *et al.,* 1996).

The present study suggests that probiotic *Lb. rhamnosus* bacteria inhibited most of the spoilage bacteria in fish chunks. This step would also control the formation of amines by controlling spoilage bacteria and preserve fish.

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