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RESEARCH ARTICLE

Effect of lactic acid bacteria application on shelf life and safety of fish fillet at 6±1°c

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Abstract

Preservation of foods in a sound and safe condition remains an on-going challenge for humans. Application of Lactic Acid Bacteria as food preservative is a novel approach and is an important component of biopreservation technique. The use of LAB and/or their bacteriocins, either alone or in combination with mild physicochemical treatments and low concentrations of traditional and natural chemical preservatives, may help in extending shelf life and food safety. In the present study, *Lactobacillus sakei* ATCC 15521 was used to improve the shelf life of horse mackerel fillet at a temperature of 6±1^{o}C against the pathogen, *Staphylococcus aureus* ATCC 25923, and quantified by microbial and biochemical analyses. The nature of inhibition was bacteriostatic rather than bacteriocidal. The result reveals that, *L. sakei* may be effective as biopreservative of horse mackerel fillet only for a limited period of 15 days.

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Introduction

Preservation of foods in a sound and safe condition remains an on-going challenge for humans. Food fermentation is a traditional preservation technique that was developed using lactic acid bacteria (LAB). Lactic acid bacteria play an important role in food fermentations, causing the characteristic flavor changes and exercising a preservative effect on the fermented product (Chowdhury et al, 2012). The use of LAB and/or their bacteriocins, either alone or in combination with mild physicochemical treatments and low concentrations of traditional and natural chemical preservatives, may help in extending shelf life and food safety. Such application of LAB as food preservative is a novel approach and is an important component of biopreservation technique. Antagonistic properties of lactic acid bacteria (LAB) allied to their safe history of use in traditional food fermented products make them very attractive to be used as biopreservatives. They can be used as natural competitive microbiota or as specific starter cultures under controlled conditions (Cintas et al. 2011). Again, the use of LAB for food preservation is accepted by consumers as 'natural' and 'health promoting'.

In the present study, *Lactobacillus sakei* ATCC 15521 was used to improve the shelf life of horse mackerel fillet at a temperature of 6±1^{o}C. To study the inhibitory effect on pathogens, *Staphylococcus aureus* ATCC 25923 was chosen. *Staphylococcus aureus* is an opportunistic pathogenic Gram positive bacterium known to occupy natural ecological niches such as the nasal cavity and the skin of warm-blooded animals (Kluytmans and Wertheim, 2005). Contamination by *S. aureus* can come from raw material from the processing plant environment (e.g. biofilm on surfaces of processing plant) or from human activity during food preparation and manipulation. Cooked meals and fermented milk products are the most frequent types of food involved in *S. aureus* food poisoning (Le Loir et al. 2009). Certain *S. aureus* strains can produce enterotoxins whose ingestion causes staphylococcal food poisoning. The frequency of *S. aureus* contamination and the impact of staphylococcal food poisoning on public health have justified an early and strong interest in combating this situation by the scientific community and agro food industries.

The choice of the temperature of 6^{o}C for storage is significant considering the fact that 20% of commercial and residential refrigerators maintain temperature >10^{o}C (Van Grade and Woodburn, 1987) and the temperature of

6^oC is the minimum temperature for growth of *S. aureus*. The influence of LAB on shelf life extension of fish fillet was determined based on the changes in microbiological count and bio-chemical characteristics of fillet at 6 ± 1^oC.

Materials and method

Bacteria culture and media

Staphylococcus aureus (ATCC 25923) and *Lactobacillus sakei* (ATCC 15521) was used as test organism for interaction study. Modified de Man Rogosa Sharpe (MRSm) broth [MRS media containing 0.1% (w/v) glucose and supplemented with 50µg/ml of cycloheximide (Hi-media, India) and 100 IU/ml of polymixin B (Hi-media, India)] was used for concentrated broth culture of *L. sakei* (ATCC 15521) (Davidson and Cronin, 1973). Nutrient broth (Hi-media, India) was used for concentrated broth culture of *S. aureus*.

Preparation of fish samples

Fresh Horse Mackeral (*Megalaspis cordyla*) with an average weight of 500g was purchased from the fish market in South Kolkata and were transferred to the laboratory in iced condition, decapitated and filleted by hand. Cubes were cut from the fillets such that the final weight of each piece was approximately 10g. The interaction study was conducted by applying LAB culture on *Staphylococcus aureus* ATCC 25923 inoculated fillet of horse mackerel. The study was conducted as a completely randomized design, with two treatments, all stored at 6^oC and six sampling intervals overtime. The treatments were (i) fillets treated with only *S. aureus* ATCC 25923 (ii) fillets treated with both *S. aureus* ATCC 25923 and *L. sakei* ATCC 15521 and fillets treated with both *S. aureus* ATCC 25923 and *L. sakei* ATCC 15521 packed in vacuum.

An overnight culture of *S. aureus* ATCC 25923 was diluted approximately in sterile peptone buffer to obtain a viable cell population of approximately 10^6 CFU.ml^{-1}. The LAB strain was grown in MRSm broth, under anaerobic conditions in order to avoid H_2O_2 formation, up to stationary phase (48 h at 30^0C) and re-suspended into sterile distilled water. Surface inoculation of the cubes of fillets with *S. aureus* was achieved by aseptically dipping the samples into the previously prepared diluted mixture of the pathogen for 7 minutes.

Control sample (C) was prepared by packaging the fillets with no LAB cells added. The cubes of treatments (T_1 and T_2) were surface inoculated with a fresh culture of *L. sakei* ATCC 15521 at a level of approximately 1 x 10^7 CFU.ml^{-1} of rinsate. Treatment T_1 was packed similar to C while T_2 samples were vacuum packaged using INDVAC vacuum packaging machine. All samples were stored at 6 ± 1^0C for 15 days as described by Amezquita and Brashears, 2002. All steps involved in the preparation of this culture were done in food grade laboratories. The packaging material used was film bag of 15X20 cm vacuum package bags having low gas permeability at 23^0C (oxygen: 10 cm^3.m^{-2}daybar, nitrogen: 6 cm^3.m^{-2}daybar, carbondioxide: 35 cm^3.m^{-2} day bar and water vapor: <2g.m^{-2} day).

Microbial and biochemical analyses

The fillets were subjected in triplicate to microbial, biochemical and sensory analyses during the storage periods. Samples stored were collected on days 0, 3, 6, 9, 12, 15 and analyzed for total number of *S. aureus* ATCC 25923 by pour plating appropriate dilutions onto Baird Parker medium. Total volatile base nitrogen (TVB-N) was determined by the method recommended by Export Inspection Council (1995). Peroxide value (PV) and Free fatty acid (FFA) of the lipid was determined using the method as described by Jacobs (1958). The pH of the sample was determined by the method described by Suzuki (1981).

Statistical analysis

All of the data were checked for normal distributions with normality plots prior to analysis of variance (ANOVA), to determine significant differences among means at $\alpha = 0.05$ level, DMRT was done.

Results

The changes in *S. aureus* ATCC 25923 count (log cfu.g^{-1}) on horse mackerel fillets in the presence of *L. sakei* ATCC 15521 under different packaging conditions at 6^oC are given in table1. In the control sample C (only *S. aureus* ATCC 25923, under aerobic condition) the population of *S. aureus* ATCC 25923 ranged between 5.43±0.15 log cfu.g^{-1} to 9.35±0.06 log cfu.g^{-1} over a period of 15 days at 6^oC. In case of T_1 (*S. aureus* ATCC 25923 and *L. sakei* ATCC 15521, under aerobic condition), the final count of *S. aureus* was recorded to be 8.4±0.26 log cfu.g^{-1} at the end of 15 days at 6^oC. Addition of *L. sakei* ATCC 15521 on to the *S. aureus* inoculated fillets (T_1), thus resulted in more than 1.0 log cycle reduction in *S. aureus* count. Similar trend was observed in case of T_2 samples, with almost 1.2 log cycles reduction in final count of *S. aureus*, finally reaching a count of 8.15±0.14 log cfu.g^{-1}.

Analysis of variance reveals that treatments T_1 and T_2 resulted in significant lowering ($p<0.05$) of *S. aureus* count when compared to control samples.

Table 2 shows the effect of *L. sakei* ATCC 15521 on the changes in TVB-N value at 6±1^oC temperature. The values of TVBN show an increasing trend in all samples during the 15 days storage periods. Highest value of TVBN was encountered for the control samples C (32.91±1.29mg% after 15 days). In case of the fillet treated with *L. sakei* ATCC 15521 (T_1), under aerobic condition, the TVBN value was recorded significantly lower ($p<0.05$), compared to control. The lowest value of TVB-N (24.65±0.15mg%) was encountered in *L. sakei* ATCC 15521 treated sample suggesting a significant ($p<0.05$) effect in reducing TVB-N content when compared to control.

The control fillets (C) crossed the limit of acceptance of PV i.e. 20 meq $O_2.kg^{-1}$ fat on day 9 of storage at 6^oC (table 3). For the fillets treated with *L. sakei* ATCC 15521, packed in vacuum (T_2) the values of PV crossed the limit of acceptability on 9thday of storage, finally reaching values of 28.27±0.34meq $O_2.kg^{-1}$ fat at the end of day 15 of storage study. Combination treatment (T_2) of LAB and vacuum thus seems to have positive effect ($p<0.05$) in reducing the PV in comparison to the control samples (table3).

The FFA values of the control ranged from 1.6±0.21 to 3.68±0.40% of oleic acid (table 4).The FFA value of the fillet, treated with *L. sakei* ATCC 15521, under aerobic condition (T_1) has values ranging from 1.6±0.29% of to 2.93±0.31% of oleic acid, whereas higher value 3.25±0.28% of oleic acid is observed in case of the fillet, treated with *L. sakei* ATCC 15521, under vacuum packaging condition (T_2), after 15 days storage period at 6±1^oC. An increasing trend of FFA in all samples was observed during the 15 days storage periods with significantly lower values ($p<0.05$) recorded for all treatments as compared to control.

The pH value of the control ranged from 7.8 to 7.58 (table 5). Lowering of pH was observed in all treatments with significantly low values of pH observed in *L. sakei* ATCC 15521 treated samples.

Discussion

For the purpose of interaction study *S. aureus* was chosen as an indicator strain because it is considered a food borne pathogen and has been incriminated in incidents involving a wide range of food vehicles including seafood, meat, dairy, cream-filled bakery, poultry and egg products, salads and canned mushrooms. *S. aureus* occurrence is very frequent on seafood due to cross contamination during handling. Up to 40% of humans carry *S. aureus* in their nose as a part of the normal microflora. Once a product such as fish fillet gets contaminated, *S. aureus* will multiply and may produce toxin, especially in the absence of other competitive microflora. Especially there is a chance of encountering this species in cooked fish products. In the present study, *L. sakei* ATCC 15521 was used as a biopreservative agent on *S. aureus* inoculated horse mackerel fillet under aerobic and vacuum packaged (anaerobic) condition at 6^oC temperature. The temperature of 6^oC was chosen because; most of the commercial and domestic refrigerators suffer from temperature abuse. Instead of 4^oC they maintain temperature of 6 to 10^oC.

From the result of the present study it is evident that application of *L. sakei* ATCC 15521 and packaging of horse mackerel fillet in vacuum (T_2) resulted in significant reduction ($p<0.05$) in the final count of *S. aureus* ATCC 15923 after 15 days at 6^oC. During the period of study the growth of *S. aureus* was encountered in all samples but with varying rates suggesting a bacteriostatic rather than bacteriocidal effect. Soccol et al. (2005), reported a growth from $5.3X10^1$cfu.g^{-1} to $3.1x10^2$cfu.g^{-1} for *S. aureus* during the first seven days followed by a decrease to $8.7x10^1$ cfu.g^{-1} on 13th day at 1±1^oC. Passy et al. (1983) observed similar increase in *S. aureus* growth from $10^1 – 10^2$cfu.g^{-1} to $10^3 – 10^4$log cfu.g^{-1} in prawn (*M. rosenbergii*) stored under CO_2 after 12 days storage. Plaatjies et al. (2004) reported an approximately 60% lower *Staphylococcus* growth at 5^oC under vacuum which in the present study was 1.20 log cfu.g^{-1} less in T_2 than C. The combination of *L. sakei* ATCC 15521 and vacuum (T_2) yield the best inhibitory effect on *S. aureus* ($p<0.05$, table 1). Ananou et al, 2007 reported that hurdle technology approach is influential in inhibiting the growth of bacteria. The principal hurdles applied in treatment T_2 are vacuum, low temperature and competitive microflora (lactic acid bacteria). The combination effect of these hurdles probably resulted in almost 1.2 log cycles reduction in *S. aureus* count when compared to control (C).

Hisar et al. (2005) reported a decrease in *Staphylococcus* count on application of the lactic culture while studying the effect of *L. sakei* Lb 706 – B a non –bacteriocin producing strain on the growth of *L. monocytogens*. This may be because of the production of the lactic acid by the LAB species. According to Charlier et al. (2009), the optimal pH for growth of *S. aureus* is close to neutrality and medium acidification resulting from lactic fermentation by LAB seems to be one of the main factors involved in the inhibition of *S. aureus* growth. In the present study medium acidification was observed in treatments T_1 where pH values below neutral was observed (table 5).

The effect of growth of *L. sakei* ATCC 15521 on changes in TVB –N of the horse mackerel fillet was analyzed anaerobically (under vacuum) and aerobically at 6^oC. Table 2 shows the changes in TVB-N content of horse mackerel fillet at 6^oC. For the control sample (C) the TVB-N values was within the acceptable limit of 35

mg% till 15 days (32.91±1.29mg%) as suggested by EIC (1995). In case of samples treated with LAB culture and stored anaerobically at 6°C (T_2) the changes were significantly lower ($p<0.05$) when compared to the control samples. In case of control samples, the TVB-N increase was gradual till day 6 (15.47±1.33mg%) followed by a sharp increase on day 9 (21.31±1.45 mg%) onwards. This result is in agreement with the findings of Aubourg (2001), who reported a sharp increase in TVB-N content after 9-10 days of storage. He suggested that this may be as a result of the end of lag phase of microorganisms. For treatment samples the values of TVB-N were less which may be because of extended lag phase of the spoilage micro-organisms as a result for competitive inhibition by *L. sakei* ATCC 15521 and at the same time effect of *L. sakei* acidification. Alak et al. (2010) reported that on day 9 of storage, the TVB-N of vacuum packed bonito fillets were above 25mg%. Ibrahim and Salha, 2009 reported that the combined coating of lactic acid bacteria in Tilapia fillets had decreased TVB-N values. Sudalayandi and Manja, 2011, reported that, out of seven LAB tested for quality indices reduction, *Lb. helveticus*, *Lc. lactis* and *Pediococcus acidilactici* successfully controlled TVB-N. From the result of the present study it may be concluded that application of *L. sakei* ATCC 15521to horse mackerel fillet controlled the TVB—N values ($p <0.05$) when compared to control samples.

Oxidative rancidity is one of the most important factors that determine the acceptability of the fish during processing and storage. Lakshmanan (2000) recommended a level of PV in seafoods is 10 – 20 meq $O_2.kg^{-1}$ of fat as a limit of acceptability. In the present study, the control fillets crossed the limit of acceptance of PV i.e. 20 meq $O_2.kg^{-1}$ on day 9 of storage (table 3) at 6°C. In the presence of *L. sakei* ATCC 15521 under vacuum, the sample T_2 crossed the limit of acceptability for PV on day 12. Schillinger and Holzapfel (2003) reported that, in spite of *L. sakei* being an important starter culture for the production of fermented meat products; it may dominate the spoilage in association of vacuum packaged processed meat products. In the present study, the highest reduction in PV was observed in *L. sakei* ATCC 15521 treated samples under vaccum (T_2), reaching a final value of 28.27±0.34meq $O_2.kg^{-1}$ of fat as compared to 37.38±0.29meq $O_2.kg^{-1}$ of fat for control. Sudalayandi and Manja, 2011, reported that, *P. pentosaceous* reduced PV content from 3.15 to 3.03 millieq $fat.kg^{-1}$ of fat, *P. acidilactici* reduced PV from 15.5 to 1.20 millieq $fat.kg^{-1}$ of fat and *L. plantarum* reduced PV from 40 to 6.5 millieq $fat.kg^{-1}$ of fat respectively in Indian Mackerel chunks during 2 days storage at 37°C.

The trend in changes in FFA values of Horse Mackerel Fillet showed that a combination treatment of *L. sakei* ATCC 15521 and vacuum at 6°C (T_2) yielded the best result. From table 4, its evident that, the final FFA value for treatments C, $T_1$and T_2 samples were 3.68±0.40, 2.93±0.31 and 3.25±0.28 % of Oleic Acid respectively, with significant lowering of FFA values ($p<0.05$) in the treated samples than control. FFA content is considered as the most popular measure of the lipolysis in the fish. FFA contributes to off flavor and causes textural alteration by complexing with muscle proteins. Aubourg, 2001 reported a similar gradual increase in FFA values in fish fillet over a period of 19 days. Sudalayandi and Manja, 2011, reported that, *Lb. plantarum* reduced FFA from 9.4 to 6.4 % of oleic acid in Indian Mackerel chunks during 2 days storage at 37°C.

In the presence of *L. sakei* ATCC 15521 in aerobic or anaerobic (vacuum packaged) condition all the samples exhibited a lowering of pH values from the initial level 7.8. However, pH values did exhibit significant lowering ($p<0.05$) in both the LAB treated samples (T_1 and T_2). The final value of pH recorded for T_1 and T_2 were 6.49±0.35 and 6.94±0.05 respectively. Such medium acidification may have contributed to the inhibition of *S. aureus*.

Conclusion

From the results of the present study it may thus be concluded that LAB application in the form of *L. sakei* ATCC 15521 may be used for inhibition of pathogenic organisms like *S. aureus*, although complete elimination may not be possible. The nature of inhibition was bacteriostatic rather than bacteriocidal. Biochemical studies of the horse mackerel fillet suggest that LAB has some role in reducing TVB-N, FFA, PV and pH. Therefore, *L. sakei* may be effective as biopreservative of horse mackerel fillet only for a limited period of 15 days beyond which the changes in biochemical characteristics of the fillet is expected to occur.

Table 1.Inhibition of *Staphylococcus aureus* (ATCC 25923) at 6°C by lactic culture

Days	Vacuum (log cfu/g)		
	C	T1	T2
0	5.43±0.15[a]	5.43±0.18[a]	5.43±0.29[a]
3	7.12±0.35[a]	6.58±0.05[a]	6.06±0.12[a]
6	8.5±0.12[a]	7.74±0.29[a]	7.11±0.37[a]
9	9±0.57[a]	7.98±0.13[a]	8.02±0.15[a]

12	9.5 ± 0.16^{a}	8.47 ± 0.19^{a}	8.42 ± 0.05^{a}
15	9.35 ± 0.06^{a}	8.4 ± 0.26^{a}	8.15 ± 0.14^{a}

Results are average value of three determinants (n=3) with standard deviation.
Values with same subscripts are not significantly different.
Here, C: Control, only *S. aureus* , aerobic
T1: *S. aureus* + *L. sakei*, aerobic
T2: *S. aureus* + *L.sakei*, vaccum

Table 2: Changes in TVB-N of Horse mackerel Fillet treated with Lactic culture at 6°C

Days	C	T1	T2
0	9.757 ± 1.29^{d}	9.76 ± 0.12^{d}	9.76 ± 0.36^{d}
3	13.75 ± 1.21^{bcd}	12.08 ± 0.26^{d}	11.03 ± 0.12^{d}
6	15.47 ± 1.33^{bcd}	13.95 ± 0.27^{bcd}	12.72 ± 0.04^{cd}
9	21.31 ± 1.45^{abcd}	16.75 ± 0.28^{bcd}	23.35 ± 0.20^{abcd}
12	27.75 ± 1.67^{abc}	23.06 ± 0.27^{abcd}	24.85 ± 0.36^{ab}
15	32.91 ± 1.29^{a}	24.65 ± 0.15^{abcd}	25.21 ± 0.21^{abcd}

Results are average value of three determinants (n=3) with standard deviation.
Values with same subscripts are not significantly different.
Here, C: Control, only *S. aureus* , aerobic
T1: *S. aureus* + *L. sakei*, aerobic
T2: *S. aureus* + *L.sakei*, vaccum

Table 3: Changes in PV of Horse mackerel Fillet treated with Lactic culture at 6°C

Days	C	T1	T2
0	3.23 ± 0.07^{i}	3.23 ± 0.33^{i}	3.23 ± 0.14^{i}
3	7.87 ± 0.12^{hi}	4.7 ± 0.11^{hi}	5.91 ± 0.12^{hi}
6	14.11 ± 0.37^{efghi}	10.77 ± 0.26^{ghi}	12.62 ± 0.14^{fghi}
9	26.95 ± 0.15^{bcdef}	34.14 ± 0.26^{abc}	18.78 ± 0.10^{defgh}
12	35.95 ± 0.25^{bc}	41.34 ± 0.22^{ab}	27.6 ± 0.21^{bcde}
15	37.38 ± 0.29^{abc}	43.91 ± 0.27^{a}	28.27 ± 0.34^{bcde}

Results are average value of three determinants (n=3) with standard deviation.
Values with same subscripts are not significantly different.
Here, C: Control, only *S. aureus* , aerobic
T1: *S. aureus* + *L. sakei*, aerobic
T2: *S. aureus* + *L.sakei*, vaccum

Table 4: Changes in FFA value of Horse mackerel Fillet treated with Lactic culture at 6°C

Days	C	T1	T2
0	1.6±0.21a	1.6±0.29a	1.6 ± 0.20^{a}
3	2.199 ± 0.34^{a}	1.95 ± 0.17^{a}	1.79 ± 0.35^{a}
6	2.78 ± 0.18^{a}	2.58 ± 0.22^{a}	2.36 ± 0.27^{a}
9	3.1 ± 0.07^{a}	2.73 ± 0.25^{a}	2.94±0.26a
12	3.43 ± 0.46^{a}	3.05 ± 0.10^{a}	3.2 ± 0.27^{a}
15	3.68 ± 0.40^{a}	2.93 ± 0.31^{a}	3.25 ± 0.28^{a}

Results are average value of three determinants (n=3) with standard deviation.
Values with same subscripts are not significantly different.
Here, C: Control, only *S. aureus* , aerobic
T1: *S. aureus* + *L. sakei*, aerobic
T2: *S. aureus* + *L.sakei*, vaccum

Table 5: Changes in pH value of Horse mackerel Fillet treated with Lactic culture at 6°C

Days	C	T1	T2

0	7.8±0.39a	7.8±0.31[a]	7.8±0.15[a]
3	7.69±0.10a	6.75±0.15[a]	7.224±0.29[a]
6	7.68±0.29a	6.69±0.25[a]	7.17±0.04[a]
9	7.675±0.18a	6.68±0.08[a]	7.13±0.24[a]
12	7.64±0.05a	6.5±0.08[a]	6.98±0.11[a]
15	7.58±0.17a	6.49±0.35[a]	6.94±0.05[a]

Results are average value of three determinants (n=3) with standard deviation.
Values with same subscripts are not significantly different.
Here, C: Control, only *S. aureus* , aerobic
T1: *S. aureus* + *L. sakei*, aerobic
T2: *S. aureus* + *L.sakei*, vaccum

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