The effects of *lactococcus lactis* subsp. *lactis* and its supernatant on some bacteriological and sensory values in rainbow trout (*Onchorhynchus mykiss*) fillets

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Abstract

Lactic Acid Bacteria (LAB) have a great potential as bio-preservatives. The live cells and supernatant *Lactococcus lactis* subsp. *lactis* induced bacteriological changes in *Onchorhynchus mykiss* fillet by spray and immersion methods was studied during vacuum- packaged storage at 4 °C for 15 days. 40 kg of *O. mykiss* were prepared from a culture farm in Oshnavieh (Northwest Iran) and 112 fillet samples (100g) were prepared by aseptic method. *L. lactis* subsp. *lactis* (PTCC1336) bacteria was cultured in MRS culture medium. Its supernatant (2%, 4%) was extracted and 10^6 $CFUml^{-1}$ dilutions of LAB were prepared and tested on the fillets to enhance their shelf life. All samples were evaluated regarding to growth of psychrotrophic, psychrophilic, mesophilic bacteria, molds and yeasts. Four characteristics including of odor, flavor, texture and color of fillets after and before cooking were evaluated for sensory analysis on days 1, 5, 10 and 15 and compared with control samples. The 4% supernatant and live bacteria were more effective than that of 2% and control ($P<0.05$). The amounts of corrosive bacteria in 4% and live cells in storage time were less than human consumption limits (7log $CFUg^{-1}$), whereas in control and 2% supernatant treatments were more than that limits. The results showed that increasing the percentage of supernatant was more effective on bacteriologic factors and enhanced sensory characteristics of rainbow trout fillets ($P<0.05$).

Introduction

Nowadays, consumers are greatly concerned about on the relationship between food and health. The use of food additives is regarded as unnatural and unsafe methods. However, additives are necessary to preserve foods from spoilage and to improve organoleptic properties. The demand for a reduced use of additives and processing seems contradictory for a market asking for safer and tastier foods. These market demands put the food industry under pressure to search for innovative solutions.[1]

The researchers have claimed that bacteriostatic or bactericidal effects of LAB supernatant was indeed due to the neutralization of organic acids on cytoplasmic membrane, and thus increasing its permeability. This mechanism induces the cell ruptures and eventually kills the bacteria. In contrast, several studies suggested that treatment of neutralized supernatant of *Lactococcus lactis* spp. *lactis* with catalase and NaOH did not alter their inhibitory activity against foodborne pathogens.[2]

Annually, from 100 million tons of harvested fishes all over the world, 70 million tons of them are recognized to be consumed by human. Around 25% of this amount is consumed as fresh flesh and the remained are processed using meat preserving techniques.[3] Various techniques are used to preserve and enhance the shelf life of foods and bacterial security including freezing, use of chemical preservatives, salting and fumigation.[4,5]

Flesh spoilage is usually occurred due to changes from chemical reactions such as lipid oxidation, activities of fish enzymes and metabolic activities of micro-organisms. These activities decrease shelf life of fish and other fisheries products.[6] Chemical and microbial spoilage is the causative agent for loss of 25% of agriculture and fisheries products, annually. About 4-5 tons of harvested fish and shrimp are destroyed by enzymatic and microbial spoilage.[7]

Most of the processing methods cannot sufficiently eradicate microorganisms and sometimes psychrotrophic bacteria such as *listeria monocytogenes* or spoilage bacteria grow during fisheries products storage. Since some products are consumed as raw material, the use of bio-preservatives is essential to increase the shelf life of fishery products. In this regard, lactic acid bacteria (LAB) are more attentive.[8] LABs are a group of gram-positive bacteria including of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* spp. Their general characteristics are included: gram-positive, non-spore, cocci or rod shaped bacteria that produce lactic acid during carbohydrates fermentation.[9] LABs have a great potential as bio-preservatives, since they have a great potential to increase the duration of food storage.[10] Also, they naturally form microflora of many foods. Their greatest benefit is in the development of bio-preservatives that supports the health of consumer and environment and preserves some of the properties of the food, such as being natural and valid, without any side effects.[11] In recent years, side effects of chemical preservatives during storage are the most important concern of the consumers. These concerns are necessary for a continuous food storage research for increasing the shelf life with minimal adverse effects. Bio-preservation is a strategy for increasing the shelf life of foods that are widely used by microorganisms or metabolites.[12]

Bacteriocins produced by LAB are a heterogeneous group of peptides and proteins. The latest classification scheme divides them into two main categories: the lanthionine-containing lantibiotics (class I)

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and the nonlanthionine- containing bacteriocins (class II); while the large, heat-labile hydrolases (formerly class III bacteriocins) constitute a separate group called bacteriolysins. The high heat stability and broad pH range of lacticin 3147 makes it attractive for use in the food industry. The preservative ability of LAB in foods is attributed to the production of antimicrobial metabolites including organic acids and bacteriocins. Acid production as a result of carbohydrate catabolism is a common feature among LAB, although not all LAB can produce antimicrobial peptides during growth. Since numerous bacteriocins have been isolated over the past three decades, the production of these antagonistic substances seems to be a common phenotype among LABs. They vary in size from small (<3 kDa), heavily post-translationally modified peptides to large heat labile proteins.[13]

The strategies for the application of LABs and/or bacteriocins in food are diverse: i) Inoculation of food with LAB (starter cultures or protective cultures) where bacteriocins are produced *in situ*; ii) Use of food previously fermented with the bacteriocin-producing strains as an ingredient in the food processing (NisaplinTM, MicrogardTM, and AltaTM 2341); iii) Addition of purified or semi purified bacteriocins. The purified bacteriocins are considered additives and always require express authorization for their use.

Although nisin is the only commercially exploited lantibiotic has been used to date, efforts are being made to develop applications for other lantibiotics. Lacticin 3147, a two-peptide lantibiotic produced by *L. lactis* subsp. *lactis* DPC3147 isolated from Irish kefir grains, exhibits a bactericidal mode of action against food spoilage and pathogenic bacteria.

Nisin is the most important commercial bacteriocin produced by *L. lactis* and is used as a food preservative.[12-21] The aim of this study was to use *L. lactis* subsp. *lactis* (PTCC1336) as LAB and its supernatant as a preservative to enhance the microbial and organoleptic quality of rainbow trout fillets using two methods including immersion and spray at 4°C refrigerated temperature and vacuum condition.

Materials and Methods

40 kg of *O. mykiss* with average weight of 600±5g were obtained from a Rainbow trout farm in Oshnavieh (Northwest Iran) and transferred near ice bags to the laboratory of National Artemia Research Center. The samples were prepared according to protocol 1803929 – Ministry of Health and Medical Education, Iran. All fishes were beheaded and gutted immediately and after washing with tab water, were prepared as 100g fillets.

L. lactis subsp. *lactis* was provided from Iranian Scientific and Industrial Research Organization.

Bacterial preparation and supernatants extraction

Lyophilized bacteria (*Lactococcus lactis* spp. *Lactis* PTCC1336), were cultured on MRS, made by Micro Media Co. E.U. batch NO. 14260604, broth medium for 72 hours and incubated at in room temperature (30°C) for restoring to life, and then cultured on MRS agar in room temperature during 48 hours according to the protocol of the bacteria producer. The bacteria were cultured on MRS broth medium again and serial dilutions (1×10^{-1} up to 1×10^{-6}) were prepared and pour plated on solid MRS agar culture medium. The initial solution was stored at 4°C in refrigerator to avoid growth of bacteria. Colonies were counted after 48 hours and the initial concentration of solution were obtained after adding distilled water. 10^{6}CFUml^{-1} concentration of bacteria was obtained.

The supernatant was extracted by centrifuge (6000g for 15 min) according to Scillinger *et al*. (1989) and filtered by cellulose acetate filter with 0.2 µl mesh size to obtain cell free supernatant.

pH of extracted supernatant was adjusted to 6.5 using sodium hydroxide 1N to neutralize the pH effects. The obtained supernatant concentrate was considered as 100%. 2%, 4% solutions of this supernatant were prepared using distilled water.[13-21]

Pure bacteria of lactic acid bacteria preparation

Serial dilutions were prepared and then pour plated in solid nutrient broth culture, made by Merck Co. Germany, medium and incubated in 30°C to count the grown colonies after 48 hours and stored in the refrigerator to avoid the further growth of bacteria during the colony counting and the initial concentration of our solution were obtained. Concentration of 10^{6}ml^{-1} were prepared that in this work we call it live cells or pure bacteria of *L. lactis* subsp. *lactis*.

Fish fillets inoculation

For inoculation of fillets, 2 methods including represented by immersion and spray were used. In immersion method, 50cc of initial *L. lactis* subsp. *Lactis* supernatant was considered for every 5kg fish fillet. Fillets of *O. mykiss* 100g fillets were immersed in 2%, 4% supernatant containers and live cells and were left for 15 minutes.[16]

For spray method, the inoculum was sprayed from 10 cm-distances to both sides of each fillet and fillets were left for 15 minutes for inoculums attachments,[16] later in both methods of inoculation (spray and immersion) the inoculated samples were vacuumed packed in nylon bags aseptically using Multivac-Germany apparatus at room temperature and stored at 4° C in refrigerator.

Samplings were carried out in days 1, 5, 10 and 15. The samples were analyzed for bacteriological tests (counting of psychrophilic, psychrotrophic, mesophilic bacteria & molds and yeasts) and evaluation of sensory properties.

Microbial tests

To count mesophilic bacteria, 25g rainbow trout fillet was ere added to 225 ml normal saline and stirred up using stomacher®400 circulator, UK for 1 minutes to be homogenized. The samples were mixed with peptone water made by Merck Germany (0.1% w/v) for 2 minutes and serial dilutions were prepared and incubated on Plate Count Agar (PCA) medium, made by Merck Co. Germany, and incubated in 35º C for 48 hours and colonies were counted and reported based on CFUg^{-1} fish weight.[17] To count the psychrotrophic bacteria the Iranian Industrial standard protocol No. 2629 was used. For this purpose, 10g rainbow trout fillet were added to 90 ml normal saline and stirred up using stomacher®400 circulator, UK for 1 minutes to homogenize and then mixed with peptone water (0.1% w/v) for 2 minutes, serial dilutions of each homogenate were carried out with the same diluents (1:10 by Vol.) up to 10^{-6}. For conventional psychrophilic bacteria enumeration, 0.1ml samples of serial dilution were spread on the surface of dry media (selective nutrient agar). Psychrophilic bacteria were counted after incubation for 10 days at 7º C and colonies were counted and reported based on CFUg^{-1} fish weight.

Molds and yeasts

25 g of each sample was weighted and put into narrow neck flask which contained 225 mL sterile distilled water. This was shaken to get the 1:10 dilution; they were mixed by a bag-mixer for 2 min, to get the 1:10 dilution. 1mL of this solution was pipetted into a test tube contained 9 mL of sterile distilled water. Another 1 mL was aseptic pipetted to repeat pipetting until getting a 1:100 dilutions. This was repeated to prepare series of 10× dilution.[18]

According to the estimation of sample pollution situation, 2 appropriate dilutions were selected and while 10 times dilutions

are respectively conducted, 1 mL sample solution per every dilution was pipetted and put into a sterilized flat plate. 1 mL of sample solution was pipetted and put into a sterilized flat plate as blank control. The potato dextrose agar (PDA), made by Merck Co. Germany, culture medium was cooled down to 46 °C. Promptly 15mL was poured into the flat plate, rotated the medium to disperse evenly. The yeasts and molds were counted according to their appearance. After the agar was gelling, the flat plate was put upside down and incubated 5 days at 28°C ±1°C. Results were recorded.[19]

Sensory evaluation

The organoleptic analysis was performed by evaluating four characteristics (odour, texture, flavor and color) of fillets before and after cooking according to 3-10 points – scale panel of five trained persons in seafood evaluation. Questionnaires were designed according to Ndaw *et al*. (2007) and Codex guidelines for the sensory evaluation of fish and shellfish in laboratories (1999). In this test, the double blind method was used, so that the specimens were uncertain for both the assessors and investigator. The information for each sample was encoded on the sample. The samples of fish fillets were cooked at 270°C for 20 minutes in edible liquid oil and a constant amount of salt. Samples of cooked fillets were consumed by oral consumption and the opinions were taken by the assessor.

Statistical analysis

Results of the microbial and sensory analysis were reported as Mean ± SE. Data were analyzed with SPSS software version 18 and ANOVA test. The Least Significant Differences (LSD) procedure was used to test for differences between means at the 0.05 significance level.

Results

Psychrophilic bacteria

The results showed that psychrophilic bacteria in immersion technique of live cells, 2%, 4% supernatant and control, on day 1, were 1.70±0.15, 1.37±0.4, 1.31±0.015 and 1.51 ±0.015 $CFUg^{-1}$, respectively. After 15 days vacuumed storage at 4ºC of refrigerator, these values reached to 4.25±0.063, 7.31±0.038, 5.36±0.092 and 7.51±0.22$CFUg^{-1}$. In fact, after 15 days of storage of rainbow trout fillet the number of psychrophilic bacteria was significantly higher in test treatment than that of the other treatments and also it was higher than human consumption limit (7 log $CFUg^{-1}$, P<0.05) (Figure 1).

In contrast, in spray technique, psychrophilic bacteria on day 1, of rainbow fillet vacuumed storage, in live cells, 2%, 4% supernatant treatments and control were 1.57±0.066, 1.70±0.15, 1.61±0.058 and 1.42±0.005 $CFUg^{-1}$. After 15 days vacuumed storage at 4ºC refrigerator, these values reached to 4.34±0.08, 7.73±0.037, 3.48±0.07 and 7.90±0.06$CFUg^{-1}$, respectively. This showed that after 15 days of storage, the number of psychrophilic bacteria at test treatment was significantly higher than that of all other treatments (P<0.05) (Figure 2).

Mesophilic bacteria

During rainbow trout fillet storage in vacuumed storage at 4ºC (refrigerator conditions) mesophilic bacteria in immersion technique of live cells, 2%, 4% supernatant and control, on day 1, were 2.27±0.09, 2.41±0.3, 2.15±0.02 and 2.21±0.07 $CFUg^{-1}$, respectively. After 15 days of test, these values changed to 4.89±0.06, 8.32±0.9, 4.56±0.09 and 8.39±0.06$CFUg^{-1}$, respectively. The results showed that the use of 4% was significantly more effective in mesophilic bacterial load decrease compared to 2% supernatant (P<0.05) (Figure 3). In comparison, in spray technique, the number of mesophilic bacteria on day 1, were 2.37±0.07, 2.77±0.5, 2.19±0.01 and 2.16±0.03 $CFUg^{-1}$, respectively, which reached to 5.25±0.01, 9.2±0.40, 5.52±0.06 and 7.82±0.06 $CFUg^{-1}$, respectively at the end of the storage period (P<0.05) (Figure 4).

Psychrotrophic bacteria

The results of psychrotrophic bacteria in immersion technique for all 4 treatments on day 1 to day 15 of storage in refrigerator indicated a significant decrease in the number of bacteria (P<0.05). As in day 1, the number of bacteria in live cells, 2%, 4% supernatant and control treatments, were

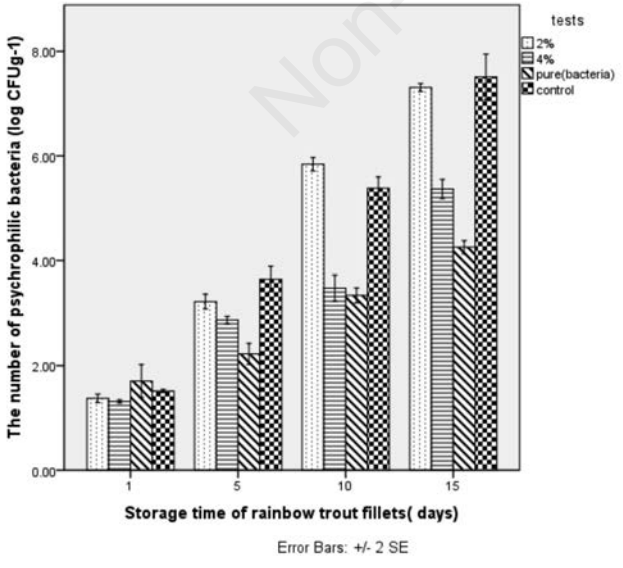

Figure 1. The comparative changes of psychrophilic bacteria (log mean ±SE) during storage period at 4°C in refrigerator in different treatments using of Immersion.

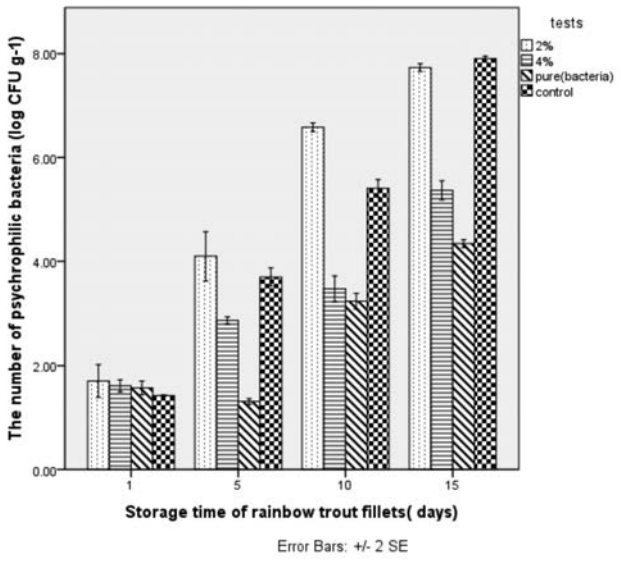

Figure 2. The comparative changes of psychrophilic bacteria (log mean ±SE) during storage period at 4°C in refrigerator in different treatments using of Spray.

1.50±0.05, 1.27±0.3, 1.11±0.01 and 1.27±0.09 $CFUg^{-1}$, respectively. After day 15 of storage, psychrotrophic bacteria enhanced to 3.48±0.06, 6.30±0.1, 3.21±0.04 and 7.56±0.03 $CFUg^{-1}$, respectively. In all treatments the number of psychrotrophic bacteria was less than that of control samples as control sample was exceeded from permissible human limit (P<0.05) (Figure 5). Comparing to it, psychrotrophic bacteria counts in spray method enhanced to 1.17±0.09, 1.50±0.20, 1.16±0.06 and 1.26±0.06 $CFUg^{-1}$, respectively. After 15 days vacuumed storage at 4ºC, refrigerator, the number of bacteria enhanced to 4.63±0.02, 6.39±0.37, 3.83±0.07 and 7.24±0.06 $CFUg^{-1}$, respectively. In all treatments the number of psychrotrophic bacteria was significantly less than that of control samples (P<0.05) (Figure 6).

Mold and yeast

On day 1, the number of mold and yeast in immersion technique in live cells, 2%, 4% supernatant treatments and control were 1.42±0.07, 1.26±0.05, 1.16±0.06 and 1.17±0.04 $CFUg^{-1}$, respectively. After 15-day vacuum storage at 4ºC, these values increased to 4.44±0.02, 8.14±0.03, 4.06±0.03 and 7.50±0.02 $CFUg^{-1}$, respectively (Figure 7). While, molds and yeasts

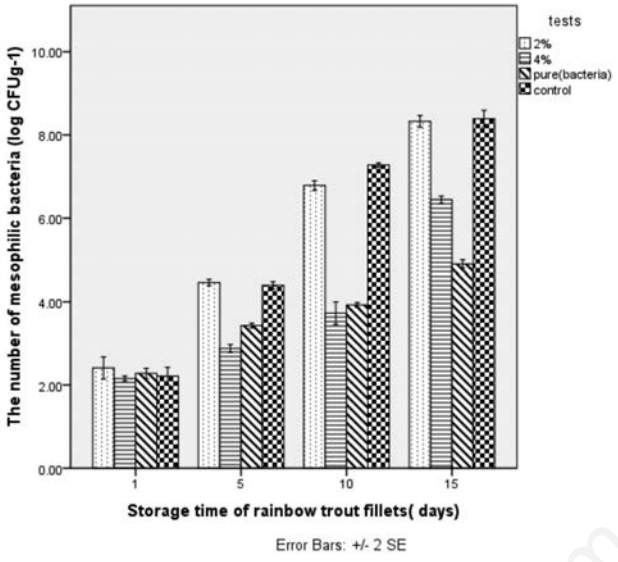

Figure 3. The comparative changes of mesophilic bacteria (log mean ±SE) during storage period at 4°C in refrigerator in different treatments using of Immersion.

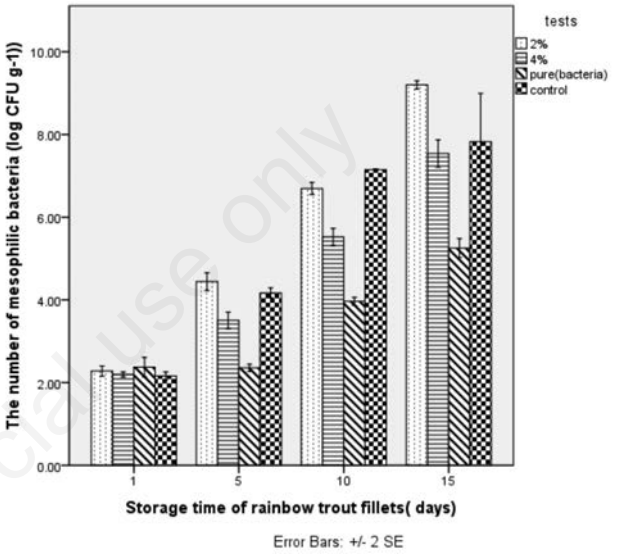

Figure 4. The comparative changes of mesophilic bacteria (log mean ±se) during storage period at 4°C in refrigerator in different treatments using of Spray.

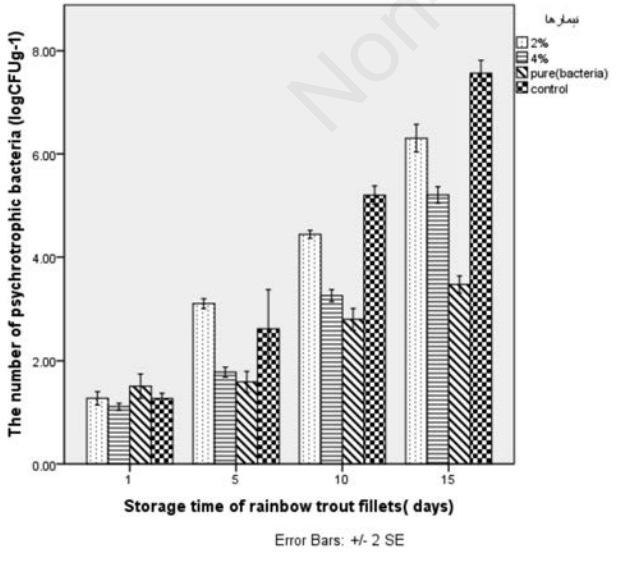

Figure 5. The comparative changes of psychrotrophic bacteria (log mean ±SE) during storage period at 4°C in refrigerator in different treatments using of Immersion.

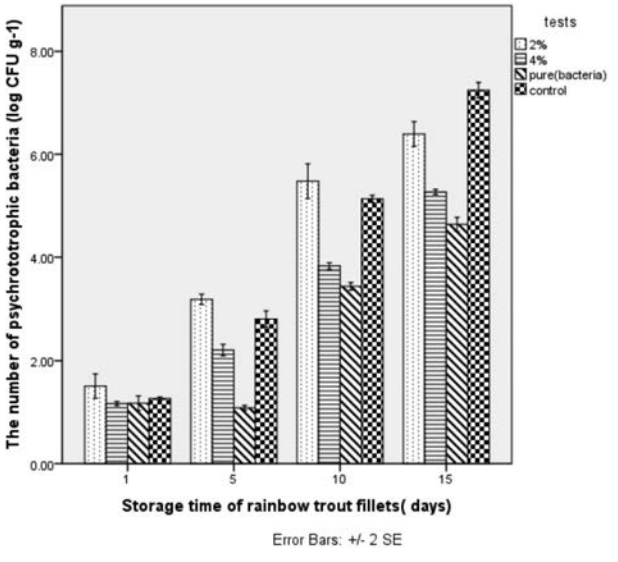

Figure 6. The comparative changes of psychrotrophic (log mean ±SE) during storage period at 4°C in refrigerator in different treatments using of Spray.

counts in spray method were 1.38±0.08, 1.42±0.90, 1.26±0.05 and 1.27±0.02 CFUg^{-1}, respectively. After 15 days vacuumed storage at 4ºC, the values enhanced to 4.67±0.01, 8.19±0.02, 4.99±0.09 and 7.13±0.06 CFUg^{-1}, respectively (P<0.05) (Figure 8).

Sensory tests

The results indicated no significant differences between spray and immersion methods (p>0.05) but there were significant differences within groups of treatments (p<0.05).

Discussion

The use of chemical preservatives during storage of food and their potential side effects have created consumer concerns in recent years. This concern has induced necessity of a search for an alternative strategy for food preservation with the sole objective of extending the shelf-life of the food with least undesirable effects. Bio-preservation, the strategy to extent the shelf-life of food using microorganisms and/or their metabolites, has received wide research interest and acceptance. The use of LAB as safe organisms with potential to produce the antimicrobial protein *bacteriocin*, could be considered as a suitable candidate for bio-preservation.[17] Psychrophilic bacteria limitation in fish fillet is 7 logCFUg^{-1}.[19]

According to results of day 15, in 2% treatment of immersion method and control until day 15 the number of psychrophilic bacteria exceeded from 7log CFUg^{-1} that were 7.31±0.03, 7.51±0.22 logCFUg^{-1}, respectively (Figure 1). In samples with 4% supernatant and live cells with concentration of 6log CFUg^{-1}, the number of psychrophilic bacteria on 15th day were 5.36±0.09, 4.25±0.06 logCFUg^{-1}, respectively (Figure 1) that were less than human consumption limit.[21-23]

Although, adding 2% supernatant to rainbow trout fillet was effective to reduce psychrophilic bacteria, but adding 4% supernatant had better results that showed the advantage of adding 4% supernatant in immersion method Also, the results showed that using live bacteria and 4% supernatant induced significant difference (p<0.05). However, the use of supernatant had a relative advantage due to lower psychrophilic bacteria density during rainbow trout fillet storage compared to live cells (Figures 1 and 2) (p<0.05).

Our results showed that using live cells during storage time decreased the rate of mesophilic bacteria than that of 2% supernatant treatment. Also, comparing it with 4% supernatant, the latter had relatively better effects in decreasing the rate of mesophilic bacteria during storage period (p<0.05). Furthermore, comparing it with 4% supernatant, showed that the latter had relatively better effects in decreasing the rate of mesophilic bacteria during storage period (Figures 3 and 4) (p> 0.05). Comparing the means growth of psychrotrophic bacteria in both immersion and spray methods showed that 4% supernatant was more effective than 2% supernatant and control. Besides, live cells were more effective than 2% and control in psychrotrophic bacterial growth inhibition (Figures 5 and 6) (p<0.05). In addition, during the study, the use of 4% supernatant reduced the mold and yeast numbers compared to 2 % one especially at the start and the end of the study period (Figures 7 and 8) (p<0.05). The 2% supernatant treatment had fewer less effects on bacterial growth than the 4% supernatant and the live bacteria treatments (p<0.05). Similar results were obtained by Diop.[24] It can be concluded that the more the supernatant concentration, the less the growth of molds and yeasts.

Several studies have been conducted on the effects of LAB specially *L. lactis* and its metabolites on pathogenic microorganisms and shelf life of fish and its fillets.[25] The results of this study in the field of adding supernatant to enhance the shelf life of rainbow trout, was in agreement with that of Behnam,[21] who studied the effects of nisin as a bio-preservative agent on quality and shelf life of vacuum packaged rainbow trout (*O. mykiss*) stored at 4 °C.[21] The results of this study showed that the psychrotrophic bacteria after adding of the supernatant of *L. lactis* until day-15 were acceptable.

Maintenance of treatments with 4% supernatant in both immersion and spray methods had lower effects on odor quality on day 5; but on day 10, the quality of the odor was improved (Tables 1 and 2) and the same improvement in food features, like

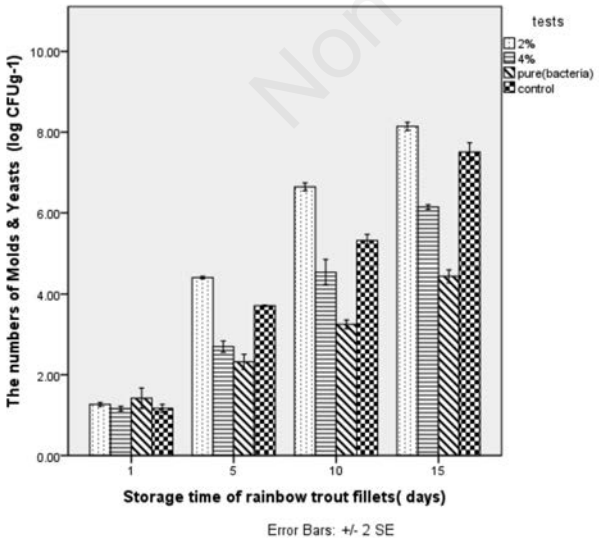

Figure 7. The comparative changes of molds and yeasts (log mean ±SE) during storage period at 4°C in refrigerator in different treatments using of Immersion.

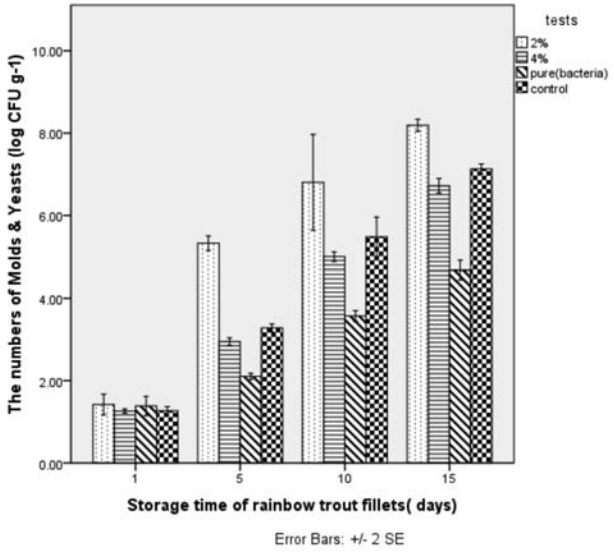

Figure 8. The comparative changes of molds and yeasts (log mean ±SE) during storage period at 4°C in refrigerator in different treatments using of Spray.

Table 1. The comparative changes of sensory evaluation (Mean ±SE) at the end of storage period in refrigerator in different treatments using of immersion.

Range	Treatments	Odor	Flavor	Texture	Color
1	2%	3.7±0.47[a]	3.7±0.25[a]	4.2±0.47[a]	4±0.40[a]
2	4%	8±0.00[b]	6.7±0.25[b]	7.75±0.25[b]	8.25±0.25[b]
3	Pure bacteria (10^6CFUml^{-1})	4.25±0.25[c]	7±0.40[c]	7.75±0.28[c]	8.5±0.28[c]
4	Control	3±0.00[d]	3±0.00[d]	3±0.00[d]	3±0.00[d]

Columns that have no common small and capital letters, have significant differences between treatments ($p<0.05$).

Table 2. The comparative changes of sensory evaluation (Mean ±SE) at the end of storage period in refrigerator in different treatments using of spray.

Range	Treatments	Odor	Flavor	Texture	Color
1	2%	3.7±0.47[a]	4±0.00[a]	4 ±0.40[a]	4±0.48[a]
2	4%	7.75±0.25[b]	7.5±0. 50[b]	7.75±0.25[b]	8 ±0.40[b]
3	Pure bacteria (10^6CFUml^{-1})	4.75±0.47[c]	5±0.00[c]	8±0.00[c]	8.5±0.28[c]
4	Control	3±0.00[d]	3±0.00[d]	3±0.00[d]	3±0.00[d]

Columns that have no common small and capital letters, have significant differences between treatments ($p<0.05$).

improving the sensory properties (flavor and texture) and safety, has been mentioned by Taous *et al.*,[26] probably due to chemical changes that occurred in the tissue and induced to create a better odorant material that has affected other unpleasant smells. By comparing all treatments in both spray and immersion methods (2%, 4% and pure bacteria), only 4% supernatant treatments were in better quality than the control sample, because the quality of odor until day 15 is held at an acceptable level (above the 5). But other treatments were unacceptable (less than 5). After consumption of treatments containing 2% supernatant and control, one of the to the assessor's presented nausea signs thus further storage of the samples in the coming days was prevented. Similar results were obtained by Reham.[27]

Conclusions

Sensory analysis revealed that using pure bacteria, as well as 4% supernatant were more acceptable. Also, treatments containing 4% supernatant and pure bacteria had shown better fillets texture quality than other ones. Among these two treatments pure bacteria had more acceptable tissue, because the filaments in them had brittleness and the score was above 5 (*i.e.* 8). Comparing of two methods in both control treatments and 2% supernatant, placed them in an unacceptable condition of score 5.

Examination of color of fish fillets in both spray and immersion methods prior to baking showed that they were unpleasant, were kept unpleasant until 15 days in control treatments and 2% supernatants.

According to sensory tests, it is recommended that fish fillets in treatments of control and 2% supernatant should be avoided to be consumed.

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