

## Inhibitory Effect of Isolated Lactic Acid Bacteria from *Scomberomorus commerson* Intestines and their Bacteriocin on *Listeria innocua*

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**ABSTRACT-** Lactic acid bacteria are commonly found in the gastrointestinal tract of various endothermic animals and humans, in milk, dairy and seafood products and on some plant surfaces. The aim of this study was to investigate the inhibitory effect of isolated lactic acid bacteria from intestines of narrow-barred Spanish mackerel (*Scomberomorus commerson*) on *Listeria innocua* growth. Five strains of lactic acid bacteria (LAB) were isolated. *Lactobacillus buchneri*, *Lactococcus lactis*, *Lactobacillus acidophilus*, *Lactobacillus fermentum* and *Sterptococcus salivarius* strains were presumptively identified by biochemical and physiological tests. Anti-listerial activities of these isolates and their cell free supernatants (CFS) were evaluated using agar spot tests, agar well diffusion assays and BHI broth. The results revealed that all cultures and their cell free supernatants were able to inhibit *L. innocua* growth. Furthermore, the study showed that wastes of this kind of warm water sea fish, have antagonistic activities against *L. innocua* and can be potentially used as a free source of LAB.

**Keywords:** Fish intestine, Lactic acid bacteria, *Listeria innocua*, Bacteriocin

### INTRODUCTION

Addition of antimicrobial agents is a traditional method of food preservation. For food preservation, many people turn to natural antimicrobial compounds instead of chemical

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antimicrobials such as benzoic acid, nitrates and sorbates. LAB are gram positive, rod or cocci, non-sporogenes, catalase negative and ferment various carbohydrates to lactate and acetate that have generally been regarded as safe. Studies demonstrated that some LAB had antagonism effects towards pathogenic and spoilage organisms. In recent years, systems based on biopreservation, such as bacteriocin producer LAB and their bacteriocins have received increasing attention and development and are considered as new approaches to control pathogenic and spoilage microorganisms (20).

Bacteriocins are ribosomally synthesized polypeptides produced by many Gram-positive and Gram-negative species, but only LAB are of particular interest to the food industry (13). Isolation of bacteriocins involves a screening of LAB from different sources such as cheese and milk (17). It has been revealed previously, that the intestinal microflora of fish is complex and contains several species of LAB (2, 19, 22). Also, it has been reported that fish intestinal LAB flora change parallel to the changes in aqueous environments, i.e. water temperature and season (1, 14, 21). This information can be used to identify the predominant LAB strains.

*Listeria monocytogenes* are pathogenic bacteria involved in several foodborne diseases causing special challenges to food safety due to its psychotrophic and ubiquitous characteristics. These bacteria persist at harsh conditions such as pH as low as 3.6, salt concentration up to 10%, presence of surfactants, sanitizers and several cycles of freezing and thawing (16). The presence of this pathogen in products that are often consumed without reheating is of particular concern for food safety (7, 25).

*Listeria innocua* is one of the six species belonging to the genus *Listeria*. It is widely found in the environment (such as soil) and food sources. It can survive in extreme pH and temperatures, and high salt concentrations. *Listeria innocua* is important because it is very similar to the food-borne pathogen *L. monocytogenes* but non-pathogenic in character (5).

Fish intestinal tract is considered to be valuable waste and a good source for LAB isolation (6). The aim of this study was to evaluate the antagonistic activity of the isolated LAB strains from fish intestines to control the growth of *L. innocua*. This is a first report on the identification of bacteriocin producing LAB isolated from the wastes of warm water sea fish (*Scomberomorus commerson*) active against *L. innocua*.

## MATERIALS AND METHODS

Fresh narrow-barred Spanish mackerel (*Scomberomorus commerson*) caught from the Persian Gulf was purchased from retail markets in Bushehr. The fish was cold stored and transferred in ice to the laboratory of the Department of Food Science and Technology. All media and chemicals were obtained from Sigma, Merck and Difco companies.

*Lactococcus lactis* subsp. *lactis* Persian Type Culture Collection (PTCC) 1336, was purchased from the Iranian Research Organization for Science and Technology (IROST) and used as an anti-listerial reference strain in this study. *L. innocua* was obtained from the non-pathogenic American Type Culture Collection (ATCC) 33090, and used instead of pathogenic *L. monocytogenes*.

## **Isolation and Characterization of Bacterial Strains**

The fish intestinal tract samples were shaken in saline solution, serially diluted and pour plated on MRS (de Man, Rogosa and Sharpe medium) plates and incubated at 37°C for 72 h. Farther isolation of microorganisms was performed using trypticase glucose yeast extract broth (TGE) containing 1% trypticase, 1% glucose, 1% yeast extract, 0.2% Tween 80, 0.05% di-potassium phosphate, 0.1% sodium acetate, 0.02 mM MgSO<sub>4</sub> and 0.033 mM MnSO<sub>4</sub> (pH=6.5). Well isolated colonies (pure white, small size 2-3 mm diameter) were picked from each plate and identified according to their morphological, cultural and physiological and biochemical characteristics (4, 11). Bacterial identification was carried out by Gram staining, production of catalase, oxidase and hydrogen peroxide, growth at 15°C and 45°C in one week, acid production from carbohydrates (1%w/v) arabinose, cellobiose, galactose, glucose, maltose, melezitose, melebiose, mannitol, mannose, raffinose, sorbitol and xylose in phenol red broth medium base, production of gas from 1 % glucose, methyl red and Voges-Proskauer tests in MR-VP (Methyl Red-Voges Proskauer) medium, production of ammonia from arginine, nitrate reduction in nitrate broth and indole production in tryptone broth tests.

## **Anti-Listerial Activity of LAB Isolates**

Antimicrobial activities of the LAB isolates were investigated using two methods: an agar spot test based on Tome et al. (24) except for the culture media which was BHI (Brain Heart Infusion) broth and measurement of the optical density (OD<sub>620</sub>) of the culture at 0, 48 and 96 h after inoculation against *L. innocua* (24).

The OD<sub>620</sub> of the overnight culture (37°C) of *L. innocua* in the BHI broth reached between 0.2 and 0.3 (ca. 10<sup>6</sup> cfu/ml). Later, 100 µl of the culture was spread on the plate count agar medium with a swab. Drops (100 µl) of each LAB culture, previously grown in TGE (Trypton Glucose East extract) broth at 30°C for 48 h, were then inoculated. Plates were incubated at 30°C for 48 h. Clear zones of inhibition around the LAB culture spot were measured. LAB cultures showing defined inhibition zones of >8 mm width, were selected. To inoculate cultures in BHI broth tubes, ca. 10<sup>6</sup> cfu/ml of each isolated strain and *L. innocua* were added individually. In one treatment, the mix of five isolated strains was inoculated with *L. innocua* at 30°C for 96 hrs.

Cell free culture supernatants were examined by two methods; one in an agar well diffusion assay (500 µl) and another in a BHI tube similar to the experiment as mentioned previously. The pHs of 48 h TGE culture broths were adjusted to 6.5, heated at 80°C for 10 min to destroy the cells, and centrifuged at 10,000 g for 15 min to harvest the cells. To avoid antagonism by hydrogen peroxide, catalase was added to the culture medium. In order to determine the nature of the inhibitors, the CFS were treated with catalase (Sigma; 500 IU/ml) and trypsin (Sigma; 0.1 mg/ml) at 25°C and all cultures were incubated at 30°C.

### Statistical Analyses

Data were subjected to analyses of variance (ANOVA) at a 95% confidence level. All the analyses were carried out using the statistical software SPSS13. Means were separated using Duncan's multiple range tests ( $P<0.05$ ).

## RESULTS

### Phenotypic LAB Characterization

Results of determinative tests showed that the isolates were Gram positive cocci or rod. According to carbohydrate fermentation patterns, their strains were tentatively identified as *Lactobacillus buchneri*, *Lactococcus lactis*, *Lactobacillus acidophilus*, *Lactobacillus fermentum* and *Streptococcus salivarius* (Table 1). These microorganisms were previously isolated from cold smoked salmon and other fish and fish products (3, 8, 9, 18, 19). Distribution of different genera of LAB in fresh fish was 60 % *Lactobacillus* and 20 % *Streptococcus* species (18).

**Table 1. Characteristics and presumptive identification of the LAB isolates**

	LAB strains				
	<i>Lactococcus lactis</i>	<i>Streptococcus salivarius</i>	<i>Lactobacillus buchneri</i>	<i>Lactobacillus fermentum</i>	<i>Lactobacillus acidophilus</i>
Cell morphology	Cocci	Cocci	Rod	Rod	Rod
Catalase/oxidase	—	—	—	—	—
Grams strain	+	+	+	+	+
Growth at 15°C/45°C	+/-	-/+	+/-	-/+	-/+
production of gas from 1 % glucose	+	—	+	+	—
NH <sub>3</sub> from arginine	+	—	+	+	—
Acid production from:					
Arabinose	—	—	+	—	—
Cellobiose	+	+	+	+	+
Galactose	+	+	+	+	+
Glucose	+	+	+	+	+
Maltose	+	+	+	+	+
Mannitol	+	—	—	—	—
Mannose	+	+	+	+	+
Melibiose	+	+	+	+	—
Melezitose	+	+	+	—	+
Raffinose	—	+	+	+	+
Xylose	—	—	+	+	—
Sorbitol	—	—	—	—	—

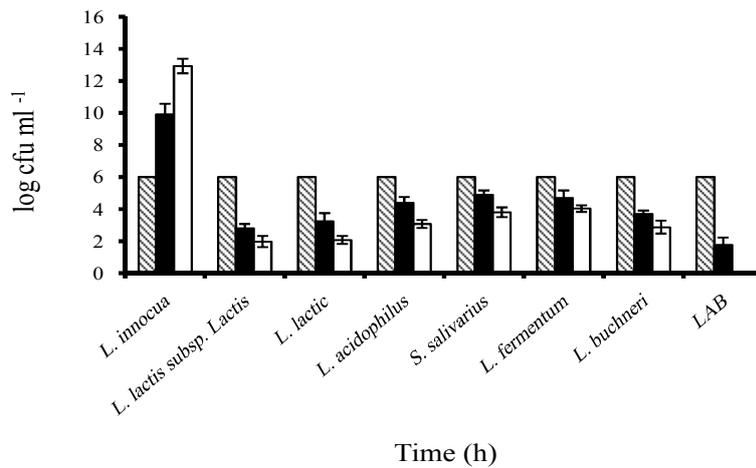
### Anti- Listerial Activity

The five isolates, individually or in co-culture, together with their CFS inhibited *L. innocua* growth and expressed different levels of inhibitory activity. Among isolated

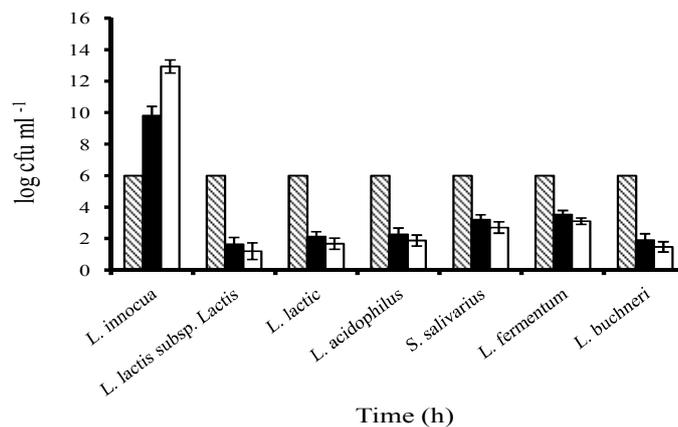
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bacteria and their CFS, the *L. lactis* and *L. buchneri* strains had the most and *L. fermentum* had the least inhibitory effects (Figs. 1 and 2).

The treatment involving the combination of five live cell isolates caused maximum antimicrobial activity and no growth was observed after 96 h incubation. *L. fermentum* and *S. salivarius* Strains in live cells and CFS tests and other strains in the CFS test in the BHI broth had statistically similar antagonistic effects. Inhibitory activity was greater with live cells than CFS. Statistical analysis of the data showed that all strains significantly decreased the *L. innocua* count after 96 h incubation.



**Fig. 1.** Effect of isolated LAB alone and in combination against *L. innocua* in BHI broth at 30°C. AB: combination of five isolated LAB. Time (h): (■) 0, (▨) 48 and (□) 96



**Fig. 2.** Effect of cell free supernatant of isolated LAB against *L. innocua* in BHI broth at 30°C. Time (h) (▨) 0 (■) 48 and (□) 96

Inhibition zones against *L. innocua* (>8 mm diameter) in the spot test and the well diffusion assay were observed for all five isolates and their CFS (Table 2). Anti-listerial activity did not disappear in CFS after adjusting their pH to 6.5 and treatment with catalase. These findings suggest that the antagonistic activity was not only caused by organic acids or hydrogen peroxide production, and was eliminated by adding trypsin enzyme (Fig. 3). Inhibitory zones were more extended for live cells (data not shown). While preserving *L. lactis* plates from spot tests in the refrigerator at 4°C, it was observed that inhibitory zones increased after one week.

Table 2. Antagonistic activity of LAB against *L. innocua* determined by spot assay and well diffusion test on plate count agar

	Microorganism				
	Live cell	Cell free supernatant	Cell free supernatant <sup>a</sup>	Cell free supernatant <sup>a</sup> treated with catalase <sup>b</sup>	Cell free supernatant <sup>a</sup> treated with catalase <sup>b</sup> and trypsin <sup>c</sup>
1	+	+	+	+	-
2	+	+	+	+	-
3	+	+	+	+	-
4	+	+	+	+	-
5	+	+	+	+	-
<i>L. lactis</i>	+	+	+	+	-
<i>subsp. lactis</i>	+	+	+	+	-

<sup>a</sup>Adjusted to pH 6.5. <sup>b</sup>500 IU ml<sup>-1</sup> <sup>c</sup>0.1 mg ml<sup>-1</sup>; + inhibition zone; - no inhibition zone



Fig. 3. Zone of inhibition of *L. innocua* growth by agar well diffusion assay in PCA; A. cell free Supernatant treated with trypsin 500 IU ml<sup>-1</sup>; B. cell free supernatant of *L. lactis*

## DISCUSSIONS

Fish are continuously exposed to a wide range of microorganisms present in the environment and their microbiota has been the subject of several reviews. The review by Ringo and Gatesoupe (19) evaluated lactic acid bacteria in fish and focused on several investigations which demonstrated that *Streptococcus*, *Leuconostoc*, *Lactobacillus*, and

*Carnobacterium* genera belonged to the normal microbiota of the gastrointestinal tract in healthy fish (19). However, it is well known that the population level of lactic acid bacteria associated with the digestive tract is affected by nutritional and environmental factors such as dietary polyunsaturated fatty acids, chromic oxide, stress and salinity. Pathogenic lactic acid bacteria such as some species of *Streptococcus*, *Enterococcus*, *Lactobacillus*, *Carnobacterium* and *Lactococcus* have been detected from ascites, kidney, liver, heart and spleen. It has also been reported that some lactic acid bacteria isolated from the gastrointestinal tract of fish can act as probiotics. These candidates are able to colonize the gut, and act antagonistic against Gram-negative fish pathogens. These harmless bacteriocin-producing strains may reduce the need to use antibiotics in future aquaculture (19).

Although all isolated LAB showed different degrees of inhibitory activity, it is possible that these bacteria and their antimicrobial substances had a synergistic effect when presented in association, thereby enhancing their antimicrobial effect against *L. innocua* (Fig. 2). Lyver et al. (15) and Jamuna et al. (12) observed this phenomenon for *Bacillus* and *Lactobacillus* isolates (12, 15). Hanlin et al. (10) demonstrated the synergistic effect of pediocin AcH and nisin against spoilage and pathogenic bacteria in a liquid medium (10). A greater anti listerial effect has been observed when a combination of nisin with other bacteriocins was employed, while a mixture of three bacteriocins was found to be effective in preventing the spontaneous emergence of a bacteriocin-resistant *Listeria* population in broth and meat systems (26).

In the broth medium experiment, *L. lactis* subsp. *lactis* and the isolated *L. lactis* were the most effective strains, while *L. fermentum* and *S. salivarius* showed the least inhibitory effect and were not statistically different. This may be due to the production of many Streptococcal bacteriocins such as inhibitory substances which were poor in liquid media (23).

The results showed that the antagonistic mechanism of the isolates could be due to the presence and action of peptides or proteins. The sensitivity of these substances to trypsin and their lack of antimicrobial activity indicated their proteinaceous nature and suggested their inhibitory activity to be caused by bacteriocins. However, the production of lactic acid, H<sub>2</sub>O<sub>2</sub> and other LAB metabolites may be responsible for the inhibition of *L. innocua* growth in treatments by live cells. Increasing the inhibitory effect of *L. lactis* at 4°C (is not shown) may be due to the production of antimicrobial compounds such as diacetyl, acetaldehyde, CO<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> that are less volatile at this temperature.

In conclusion, fish waste, presenting the potential for use as a free source of bacteriocin producing LAB, showed significant antagonistic activity against *L. innocua* in solid and liquid media.

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# اثر بازدارندگی باکتری های لاکتیک اسید ایزوله شده از روده ماهی *Scomberomorus commerson* و باکتریوسین آنها بر لیستریا اینوکوا

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چکیده- باکتری های اسید لاکتیک، معمولاً در مجرای دستگاه گوارش (روده بزرگ) انسان ها، حیوانات، شیر، محصولات دریایی، لبنی و در بسیاری از گیاهان یافت می شوند. هدف از این مطالعه بررسی اثر بازدارندگی باکتری های اسید لاکتیک ایزوله شده از روده ماهی شیر بندر (*Scomberomorus commerson*) بر رشد باکتری لیستریا اینوکوا می باشد. برای این منظور پنج گونه باکتری اسید لاکتیک ایزوله شدند. گونه های لاکتوباسیلوس بوشنری، لاکتوکوکوس لاکتیس، لاکتوباسیلوس اسیدوفیلوس، لاکتوباسیلوس فرماتوم و استرپتوکوکوس سالیواریوس بوسیله تست های فیزیولوژیکی و بیوشیمیایی به صورت احتمالی شناسایی شدند. فعالیت ضد لیستریایی باکتری های ایزوله شده و نیز مایع فوقانی بدون سلول حاصل از این باکتری ها با استفاده از تست های نقطه گذاری روی آگار، انتشار در چاهک های قرار داده شده در آگار و در محیط کشت BHI بررسی شد. نتایج نشان داد که تمام باکتری های ایزوله شده و مایع فوقانی بدون سلول آنها قادر به ممانعت رشد لیستریا اینوکوا هستند. این تحقیق نشان می دهد که ضایعات این نوع ماهی دریای آب گرم قابلیت بالقوه ای بعنوان منبع باکتری های اسید لاکتیک و دارای فعالیت بازدارندگی بر روی باکتری لیستریا اینوکوا می باشند.

واژه های کلیدی: باکتری های لاکتیک اسید، باکتریوسین، روده ماهی، لیستریا اینوکوا

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