Chapter 17

Selection of *Lactobacillus* Species from Intestinal Microbiota of Fish for Their Potential Use as Biopreservatives

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Additional information is available at the end of the chapter

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1. Introduction

Despite recent advances in seafood production, seafood safety is still an important public health issue. It is clear that indigenous bacteria present in marine environment as well as resulting from post contamination during processing are responsible for many cases of illnesses [1-3]. In the last years, traditional processes applied to seafood like salting, smoking and canning have decreased in favor of mild technologies involving lower salt content, lower heating temperature and vacuum (VP) or modified atmosphere packing (MAP, 3-5]. Most of these treatments are usually not sufficient to destroy microorganisms and in some cases psychrotolerant pathogenic such as *Listeria monocytogenes* or spoilage causing bacteria can develop during prolonged shelf-life of these products [2,5,6]. As several of these products are eaten raw, it is therefore essential that adequate precautious and preservation technologies are applied to maintain their safety and quality. Among alternative preservation technologies, particular attention has been paid to biopreservation to extend the shelf-life and to enhance the hygienic quality of perishable food products such as seafood, thereby minimizing the impact on nutritional and organoleptic properties [1,7,8]. In this context, lactic acid bacteria (LAB) possess a major potential in biopreservation strategies, since they are safe to consume, and during storage they naturally dominate the microbiota of many foods [7-11]. Lactic acid bacteria are gram-positive, non-sporulating and catalase negative rods or cocci that ferment various carbohydrates mainly to lactate and acetate [12]. Accordingly, they are commonly associated with nutritious environments like foods, decaying material and the mucosal surfaces of the gastrointestinal and urogenital tract [12- 14], where they enhance the host protection against pathogens [13]. Their antagonistic and inhibitory properties are due to the competition for nutrients and the production of one or more antimicrobially active metabolites such as organic acids (lactic

and acetic acid), hydrogen peroxide, and antimicrobial peptides like bacteriocins [8-11,15-17]. Bacteriocins are ribosomally synthesized peptides that exert their antimicrobial activity against either strains of the same species as the bacteriocin producer (narrow range), or to more distantly related species (broad range) [7,15,18]. An important reason for research on LAB based bacteriocins is due to their activity at nanomolar concentrations against number of bacterial pathogens [1,3,5,6,19,20]. Some bacteriocins even exhibit their activities against multidrug-resistant nosocomial pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci [VRE, 17, 21]. Thus they also may have some big potential in medical and veterinary applications. Fermented food and plant material have been a well-known source for bacteriocin-producing LAB, but isolates from the intestinal of animals and humans has become an increasingly important source for such strains due to an increased awareness of their importance as probiotics. In fish the presence of LAB is meanwhile well documented and the bio-protective potential of some strains and/or their bacteriocin has been highlighted in the last years [4-6,16,18,22-26]. Kvasnikov et al. [12] described the presence of lactic acid bacteria, including *Lactobacillus* in the intestines of various fish species at larval, fry and fingerling stages inhabiting ponds in Ukraine. They give information on the changes in their composition as a function of the season of the year and life-stage of the fish. However, it was discussed that some human activities like artificial feeding in ponds would have had an effect on the bacterial composition and load in some fish, like carp (*Cyprinus carpio*) which showed the highest content of lactic acid bacteria in the intestines. Cai et al. [27] described the lactic acid bacteria in *Cyprinus carpio* collected from the Thajin river in Thailand. They reported the presence of *Enterococcus* spp. and the dominance of *Lactococcus garviae*, an emerging zoonotic pathogen, in *Cyprinus carpio*. Bucio Galindo et al. [23] studied the distribution of lactobacilli in the intestinal content of river fish and reported that various species of lactobacilli were present in relatively high numbers in the intestines of edible freshwater fish from the river, especially in warm season but in low numbers in cold season. There are no reports on the presence of *Lactobacillus* in the intestines of sturgeon fish inhabiting Caspian Sea, whereas other groups of bacteria have been studied in more details. In comparison with other food products of dairy or meat origin, only few bacteriocinogenic LAB strains have been recovered from seafood. The present study focuses on the characterization of antimicrobial compounds produced by the lactobacilli isolates, in addition, their ability to inhibit the growth of relevant food borne pathogens as well as of spoilage bacteria and last but not least, of contaminants in aquaculture.

2. Materials and methods

2.1. Fish intestine samples

Two species of Persian sturgeon (*Acipenser persicus*) and Beluga (*Huso huso*) were collected from the south coast of Caspian Sea in Iran. Twenty two individuals of these fish in adult stage were selected. The weight and length of the fish were measured before dissection. The fish were sacrificed by physical destruction of the brain, and the number of incidental organisms was reduced by washing the fish skin with 70% ethanol. Then, the ventral surface

was opened with sterile scissors. After dissecting the fish, 1 g of the intestinal tract content of each fish was removed under aseptic condition and placed into previously weighed flasks containing storage medium.

2.2. Media and culture condition

Intestinal content was homogenized in a storage medium using a vortex mixer. One milliliter was transferred to reduced neutralized bacterial peptone (NBP, Oxoid L34, Hampshire, England) 0.5 g/L, NaCl 8 g/L, cysteine.HCl 0.5 g/L, pH adjusted to 6.7 [29]. Afterwards serial dilutions were spread on plates of selective media and incubated at the following conditions. Columbia blood agar (CAB, Oxoid CM 331) was used as a selective medium to make an estimation of the cultivable total anaerobic counts [29]. All the inoculated plates were incubated anaerobically at 30°C for 48 h. The following two media were used to isolate lactic acid bacteria (LAB). MRS (MRS, Merck, Darmstadt, Germany) with 1.5% agar (M641, HiMedia, Mumbai, India) and pH adjusted to 4.2 (MRS 4.2) and incubated anaerobically at 30°C for 96 h was used as a selective medium for lactic acid bacteria. MRS is an inhibitory medium for *Carnobacterium*. Anaerobic MRS with Vancomycin and Bromocresol green (LAMVAB), incubated at 30°C for 96 h was used as an elective and selective medium for *Lactobacillus* spp. [30]. Anaerobic incubation of the three media was made in an anaerobic Gas-Pack system (LE002, HiMedia, Mumbai, India) with a mixture of 80% N_2, 10% H_2 and 10% CO_2. Colonies were selected either randomly, or in case of less than 10 colonies per each plate, all the samples were counted according to the method described by Thapa et al. [31]. Purity of the isolates was checked again by streaking them onto fresh agar plates of the isolation media, followed by microscopic examinations. Identified strains of lactobacilli were kept in MRS broth with 15% (v/v) glycerol at -20°C.

2.3. Characterization procedures for lactic acid bacteria

Eighty four strains were randomly selected for identification procedures based on the phenotypical characteristics. Cell morphology and motility of all isolates were observed using a phase contrast microscope (CH3-BH-PC, Olympus, Japan). Isolates were gram-stained and tested for catalase production test. Preliminary identification and grouping was based on the cell morphology and phenotypic properties such as CO_2 production from glucose, hydrolysis of arginine, growth at different temperatures (10, 15 and 45°C), and at different pH (3.9 and 9.6). As well as the ability to grow in different concentrations of NaCl (6.5% (w/v), 10% (w/v) and 18% (w/v)) in MRS broth was checked as well. The configuration of lactic acid produced from glucose was determined enzymatically using d-lactate and l-lactate dehydrogenase test kits (Roche Diagnostic, France). The presence of diaminopimelic acid (DAP) in the cell walls of LAB was determined using thin-chromatography on cellulose plates. Fermentation of carbohydrates was determined using API 50 CHL (API 50 CH is a standardized system, associating 50 biochemical tests for the study of carbohydrate metabolism in microorganisms. API 50 CH is used in conjunction with API 50 CHL Medium for the identification of *Lactobacillus* and related genera) strips according to the

manufacturer's instructions (Biomerieux, Marcy l' Etoile, France). The APILAB PLUS database identification software (bioMe´rieux, France) was used to interpret the results. Identification was undertaken according to the method described by Kandler and Weiss [12] and Hammes and Vogel [32].

2.4. Statistical analysis

Statistical analysis using Student's t-test (SPSS, Version 11.0) was performed to find significant difference on lactobacilli count between LAMVAB and MRS 4.2. Pearson's correlation coefficient was used to investigate the correlation of lactobacilli count between LAMVAB and MRS 4.2 (SPSS Inc., Version 11.0, Chicago, USA). A significance level of $p<0.05$ was used.

2.5. Screening of *Lactobacillus* strains for their inhibitory potential

In a first test series, the ability of each of the *Lactobacillus* isolates to exert an antibacterial effect against *Listeria monocytogenes* ATCC 19115 and *Salmonella* Typhimurium PTCC 1186 were examined by using three methods: the spot-on-lawn method, standardized agar disk diffusion method and the well diffusion method as described by Schillinger and Lucke [33], Benkerroum et al. [34] and Tagg & Mc Given [35]. Throughout, cell-free supernatants (CFS) of strains were obtained by centrifugation at 10,000 ×g for 20 min and then adjusted to pH 6.5 by applying NaOH (to exclude the effect of organic acid) before sterilization by filter (0.2 μm, Sigma, UK). Based on the screening tests, the inhibitory spectrum of potential bacteriocin-producing isolates was assessed against 42 indicator strains using a standardized agar disk diffusion test. The strains were kept frozen in 20% (v/v) glycerol at -20°C. For this purpose, an aliquot of 20 ml CFS was applied on disks (6 mm) and set on agar plates previously inoculated with each individual indicator strain suspension, which corresponded to a 10^5 CFU/ml. Plates were incubated 24 h at optimum temperatures of the test organism. Antimicrobial activity was detected as a translucent halo in the bacterial lawn surrounding the disks.

2.6. Characterization of the inhibitory effect

In order to determine the biological nature of the antimicrobial activity of bacteria, CFS (pH 6.0) of 24-h lactobacilli cultures of two selected isolates (*Lactobacillus casei* AP8 & *Lactobacillus plantarum* H5) incubated at 30°C, were tested for their sensitivity to the proteolytic enzymes. One ml of CFS was treated for 2 h with 1 mg ml^{-1} final concentration of the following enzymes: papain, trypsin, proteinase K, pronase E and α-amylase (Sigma, London). To clarify whether the antimicrobial activity detected derives from the production of hydrogen peroxide, 2600 IU/ml of catalase (Sigma, London) were added to 1 ml portions of extracellular extracts of LAB exhibiting antimicrobial activity and incubated for 24 h at ambient temperature. Chemicals were added to the CFS and the samples incubated for 5 h before being tested for antimicrobial activity. To determine the sensitivity of potential bacteriocin activities to the temperatures, samples of CFS were incubated under defined conditions. The effect of pH on bacteriocin

activity was determined by adjusting the pH of the CFS (cell free supernatant (pH 6.5) of 24-h lactobacilli cultures incubated at 30°C) with diluted appropriate volumes of HCl and NaOH (Table 3). After incubating for 2 h, the pH of the samples was readjusted to 6.5 followed by sterilization (0.2 μm, Sigma, UK). In all cases, the remaining bacteriocin activity was assessed exemplarily by using strain *L. monocytogenes* ATCC 19115 as the indicator bacterium and by applying the agar disk diffusion plate bioassay. Untreated cell-free supernatants were used as controls and experiments were performed in duplicate.

2.7. Growth dynamics and antimicrobial compounds production

The time course of inhibitory substance production was performed by inoculating 10 mL of an overnight culture of selected *Lactobacillus* isolates into 100 mL of MRS broth followed by incubation at 30°C. Cells were subsequently removed by centrifugation at 10,000 ×g for 20 min. At appropriate intervals, changes in pH and optical density (600 nm) of the cultures were measured to monitor bacterial growth using a spectrophotometer (Hitachi U 1100, Tokyo, Japan). Antibacterial activity was evaluated every hour by using serial twofold dilutions of each culture used as a neutralized cell-free supernatant (CFS) tested against *L. monocytogenes* ATCC 19115 based on the agar disk diffusion plate bioassay. In a separate experiment, the inhibitory effect of CFSs of lactobacilli strains on target cells in liquid medium was also examined against *L. monocytogenes* ATCC 19115 as indicator strain. For this purpose, 20 mL of each filter-sterilized bacteriocin-containing cell-free supernatant were added to a 100 mL culture of the indicator organism at early exponential phase (4 h old). These experiments were also repeated with stationary-phase cells. The optical density at 600 nm and viable cell count were determined every hour during an observation period of 20 h. Indicator cells without CFSs were used as control.

2.8. Adsorption of bacteriocin to producer cells

Bacteriocin-producing cells were cultured for 18 h at 30 °C. The pH of the cultures was adjusted to 6.0 with 1 M NaOH to allow maximal adsorption of the bacteriocin to the producer cells, according to the method described by Yang et al. [36]. The cells were then harvested (10,000 ×g 20 min, 4 °C) and washed with sterile 0.1 M phosphate buffer (pH 6.5). The pellet was re-suspended in 10 ml of 100 mM NaCl (pH 2.0) and stirred slowly for 1 h at 4 °C. The suspension was then centrifuged (10,000 ×g 20 min, 4 °C), the CFS was neutralized to pH 7.0 with sterile 1 M NaOH followed by testing the bacteriocin activity as described above.

2.9. Partial purification and characterization of the bacteriocin

Bacteriocin producer strains were grown in MRS broth, and incubated without agitation for 18 h at 30°C. The cells were harvested (10,000 ×g, 20 min, 4 °C) and the bacteriocin precipitated from the CFS with 60% saturated ammonium sulphate [45]. The precipitate in the pellet and floating on the surface were collected and re-suspended in one-tenth volume 25 mM ammonium acetate buffer (pH 6.5). The sample was stored at -20 °C for one week and activity tests were performed as described above. For the determination of the molecular size of the bacteriocins, precipitated

peptides re-suspended in 25 mM ammonium acetate buffer (pH 6.5) were separated by Tricine-SDS-PAGE, according to Schägger and Von Jagow [38]. Low molecular weight markers, ranging from 2.5 to 45 kDa (Pharmacia, Sweden) were used. One half of the gel containing the molecular marker was fixed for 20 min in 5% (v/v) formaldehyde, then rinsed with water and stained with Coomassie Brilliant Blue R250 (Bio-Rad) overnight. The other half of the gel (not stained and extensively pre-washed with sterile distilled water) was overlaid with a culture of 10^6 cfu/ml *L. monocytogenes* ATCC 19115 embedded in BHI agar. The position of the active bacteriocin was visualized by an inhibition zone around the active protein band [39].

3. Results

3.1. Isolation of lactobacilli

Intestinal content of 22 fish were analysed for the presence of lactobacilli. To determine the most appropriate medium for isolating lactobacilli from fish intestines, two media (MRS agar, LAMVAB) were used. LAMVAB was highly selective to quantify lactobacilli, as 99% of 143 randomly picked colonies and purified isolates were identified as *Lactobacillus* spp. and confirmed according to [12] (Table 1). Counts of intestinal lactobacilli for Persian sturgeon and beluga were detected at the range of approximately $10^{5.3}$ to $10^{6.4}$ cfu/g, respectively. The physiological and biochemical characterization of *Lactobacillus* isolates and the presumptive *Lactobacillus* species found in two fish species are shown in Table 2. From 84 isolates, 2 metabolic groups of *Lactobacillus* were recovered: facultative and obligate heterofermentatives. *L. sakei* and *L. plantarum* were the most often found isolates (Table 2). MRS 4.2 was suitable to quantify lactobacilli. As 30 randomly picked colonies on the highest dilution were identified as lactobacilli and coccoid forms were not found. Means of counts of 90 samples were not statistically different to LAMVAB counts in the Student's t-test ($P=0.29$) and were correlated with LAMVAB counts ($r = 0.85$; $P<0.001$). The correlation of counts on MRS 4.2 with those on LAMVAB and the absence of coccoids suggests that lactobacilli were the most important acidophilic lactic acid bacteria in the samples analysed. Facultative anaerobic flora recovered in CAB medium provided the highest counts in the samples analysed (Table 1).

Fish species	No.	CAB (cfu/g)	LAMVAB (cfu/g)	MRS 4.2 (cfu/g)
Acipenser persicus	12	7.84	5.32	4.85
Huso huso	10	8.21	6.45	5.64

CAB: Columbia blood agar; LAMVAB: *Lactobacillus* spp. Anaerobic MRS with Vancomycin and Bromocresol green; MRS 4.2: deMan, Rogosa and Sharp

Table 1. Average bacterial counts of intestinal bacteria (Log cfu/g of intestinal content) for Persian sturgeon and beluga in different media

3.2. Screening of *Lactobacilli* strains for antimicrobial activity and bacteriocin production

Eighty four lactobacilli strains previously isolated from two species of Sturgeon fish identified and their cell free supernatant extracts were assayed for antimicrobial activity and

Presumptive *Lactobacillus* species	*L. sakei*	*L. plantarum*	*L. coryneformis*	*L. alimentarius*	*L. brevis*	*L. casei*	*L. oris*
No. of isolates	30	18	12	10	7	5	*2*
Diaminopimelic acid	ND	+	ND	ND	ND	ND	*ND*
CO_2 from glucose	-	-	-	-	+	-	+
NH_3 from arginine	-	-	-	-	+	-	+
10°C	+	+	+	+	+	+	+
15°C	+	+	+	+	+	+	+
45°C	-	-	-	2	-	-	-
Glycerol	-	+	-	1	-	+	-
L-Arabinose	+	+	-	2	2	-	+
Ribose	+	-	-	+	+	+	+
D-Xylose	26	-	-	-	-	-	+
Galactose	29	-	-	-	-	+	-
Rhamnose	-	-	+	-	2	+	-
Inositol	-	+	-	-	-	+	+
Mannitol	-	+	5	-	+	+	-
Sorbitol	-	+	-	-	-	+	-
1-Methyl-D-mannoside	-	+	-	-	-	-	+
1-Methyl-D-glucoside	-	+	-	7	+	-	+
N-Acetyl glucosamine	28	+	+	+	+	+	+
Amygdaline	10	+	-	+	-	+	+
Arbutine	1	+	-	+	-	+	+
Esculine	+	+	+	+	1	+	+
Salicin	+	+	-	+	-	+	+
Cellobiose	27	+	-	+	-	+	+
Maltose	19	+	-	+	+	+	+
Lactose	26	+	-	+	-	+	+
Melibiose	+	+	+	2	+	-	+
Sucrose	+	+	+	8	+	+	+
Trehalose	+	+	-	+	-	+	-
Melezitose	-	+	-	-	+	+	+
D-Raffinose	29	-	-	2	+	-	-
Starch	-	-	-	-	-	+	-
Xylitol	-	+	3	-	-	-	+
2-Gentiobiose	+	+	-	+	-	+	+
D-Turanose	-	-	-	-	+	-	-
D-Tagatose	1	-	-	+	-	+	-
D-Arabitol	-	+	5	-	-	-	+
Gluconate	+	-	-	+	+	+	+
2-keto-gluconate	-	-	1	2	-	-	+
5-keto-gluconate	-	-	-	1	-	+	+
Lactic acid configuration	*DL*	*DL*	*DL*	*DL*	*DL*	*DL*	*D*

† +: Positive reaction of all the isolates. Numbers are the positive isolates. All isolates fermented D-Glucose, D-Fructose, D-Mannose, however they didn't ferment erythrol, D-Arabinose, L-Xylose, Adonitol, 2-Methyl-xyloside, L-Sorbose, Dulcitol, Inulin, Glycogen, D-Fucose, L-Fucose, L-Arabitol. ND: Not data

Table 2. Biochemical characteristics of *Lactobacillus* species isolated from the intestines of Persian sturgeon and beluga

bacteriocin production against *Listeria monocytogenes* ATCC 19115 and *Salmonella* Typhimurium PTCC 1186 by using spot-on-lawn method, standardized agar disk diffusion

method and well diffusion method. In each instance, diameters of inhibition were quantified. Fifteen strains (18%) exhibited inhibitory activity against both indicator organisms. Consequently, all candidate isolates (Inhibition zone> 8mm) subjected to different tests such as growth at different temperatures, pH, salt content, antibiotic resistance, etc. Based on the result of aforementioned tests, two strains *Lactobacillus* casei AP8 and *Lactobacillus plantarum* H5, isolated from Persian sturgeon and beluga respectively, were chosen as active strains and were subjected to further examinations.

Presumptive *Lactobacillus* species	*L. sakei*	*L. plantarum*	*L. coryneformis*	*L. alimentarius*	*L. brevis*	*L. casei*	*L. oris*
Acipenser persicus	**	**	*	**	-	**	*
Huso huso	**	*	-	*	**	*	*

* = Presence of lactobacilli. ** = High number of lactobacilli presence

Table 3. *Lactobacillus* species isolated from the intestines of sturgeon fish

3.3. Inhibitory spectrum of bacteriocin

As the results in screening test showed that greater inhibition was observed by agar disk diffusion tests of cell-free supernatant extracts, so this method was selected as the best technique for examining the antibacterial activity of *L. casei* AP8 and *L. plantarum* H5 CFSs against forty two Gram-positive and Gram-negative bacteria. The CFS preparations from both strains showed a broad inhibitory spectrum against a wide range of LAB of different species and some food-borne pathogens and spoilage bacteria including *Listeria innocua*, *L. monocytogenes*, *Staphylococcus aureus*, *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Bacillus cereus*, *Bacillus pumilus*, *Bacillus subtilis*, *Brochotrix thermosphacta*, Gram-negative *E. coli*, *Salmonella* and *Pseudomonas*, *Clostridium perfringens* and *Vibrio parahaemolyticus* (Table 4). Result showed that the Gram-positive bacteria tested were more sensitive to the bacteriocin produced by the isolates than Gram-negative bacteria. The largest spectrum of inhibition was showed by *L. casei* AP8 bacteriocin, which inhibited 33 out of 42 indicator strains.

3.4. Characterization of inhibitory effect

Table 5 and table 6 depict the stability of inhibitory substances at different physic-chemical conditions. To determine the biological nature of the antimicrobial activity of bacteria, CFSs were tested for their sensitivity to the proteolytic enzymes. Antimicrobial activities exhibited by *L. casei* AP8 and *L. plantarum* H5 were sensitive to proteolytic enzymes since proteolytic, but not lipolytic or glycolytic enzymes, completely inactivated the antimicrobial effect of both cell-free supernatants, confirming the proteinaceous nature of the inhibitors (Table 3). The effect of several chemicals on the antimicrobial activity was also evaluated. Interestingly, the cell-free extracts remained active after treatment with chemicals such as catalase, SDS, Triton X-100, Tween 20, Tween 80 and EDTA after 5 h of exposure (Table 2). Enhancing the antimicrobial activity in case of *L. casei* AP8 bacteriocin was observed after treating by EDTA and SDS against *L. monocytogenes* ATCC 19115. The stability study of

Indicator organism	Medium*	Temp. [°C]	Bac AP8	Bac H5
Gram Negative Group				
Aeromonas hydrophilus MJ 1120	**BHI**	**37**	**++**	**0**
Aeromonas hydrophilus MJ 1240	**BHI**	**37**	**+++**	**+**
Aeromonas salmonicida CC 1546	**BHI**	**37**	**+**	**+**
Aeromonas salmonicida RT 7895	**BHI**	**37**	**++**	**+**
Brochothrix thermosphacta RF 35	**BHI**	**37**	**++**	**+**
Escherichia coli ATCC 25922	**BHI**	**37**	**++**	**0**
Escherichia coli PTCC 1325	**BHI**	**37**	**++**	**++**
Photobacterium damselae ssp. Piscida	**BHI**	**37**	**0**	**0**
Pseudomonas aeruginosa PTCC 1310	**BHI**	**37**	**++**	**+**
Pseudomonas fluorescens HFC 1236	**BHI**	**37**	**++**	**0**
Salmonella enteritidis ATCC 13076	**BHI**	**37**	**++**	**++**
Salmonella spp SM 162	**BHI**	**37**	**+++**	**++**
Vibrio anguillarum MI12	**BHI**	**37**	**++**	**+**
Vibrio parahaemolyticus MI 23	**BHI**	**37**	**+++**	**0**
Vibrio parahaemolyticus MI 56	**BHI**	**37**	**+++**	**+**
Gram Positive Group				
Bacillus cereus ATCC 9634	**BHI**	**37**	**+++**	**+++**
Bacillus coagulans	**BHI**	**37**	**+++**	**++**
Bacillus licheniformis PTCC 1331	**BHI**	**37**	**++**	**0**
Bacillus subtilis ATCC 9372	**BHI**	**37**	**+++**	**+**
Clostridium perfringens ATCC 3624	**RCM**	**37**	**++**	**+**
Clostridium sporogenes PTCC 1265	**RCM**	**37**	**++**	**+**
Lactobacillus acidophilus ATCC 4356	**MRS**	**30**	**++**	**+**
Lactobacillus alimentarius AP 10	**MRS**	**30**	**+**	**++**
Lactobacillus brevis H56	**MRS**	**30**	**++**	**++**
Lactobacillus brevis AP 83	**MRS**	**30**	**++**	**++**
Lactobacillus casei PTCC 1608	**MRS**	**30**	**0**	**++**
Lactobacillus casei RN 78	**MRS**	**30**	**0**	**0**
Lactobacillus casei LB 10	**MRS**	**30**	**0**	**+**
Lactobacillus casei LB 46	**MRS**	**30**	**0**	**+**
Lactobacillus plantarum PTCC 1050	**MRS**	**30**	**0**	**0**
Lactobacillus plantarum AP 76	**MRS**	**30**	**+**	**0**
Lactobacillus plantarum H12	**MRS**	**30**	**+**	**0**
Lactobacillus sakei AP 43	**MRS**	**30**	**0**	**+**
Lactobacillus sakei	**MRS**	**30**	**0**	**0**
Lactococcous sp	**MRS**	**30**	**+**	**0**
Lactobacillus curvatus	**MRS**	**30**	**0**	**+**
Listeria innocua AN 15	**BHI**	**37**	**++**	**++**
Listeria monocytogenes ATCC 7644	**BHI**	**37**	**+++**	**+++**
Listeria monocytogenes PTCC 1163	**BHI**	**37**	**++**	**++**
Listeria monocytogenes PTCC1297	**BHI**	**37**	**++**	**++**
Staphylococcus aureus ATCC 25923	**BHI**	**37**	**+++**	**+**
Staphylococcus aureus PTCC 1112	**BHI**	**37**	**+++**	**+**

* BHI: brain hearth infusion, MRS: de Man-Rogosa-Sharpe agar and RCM: reinforced clostridial medium. 0 no zone of inhibition; +, 1 mm<zone<5 mm; ++, 5 mm<zone<8 mm; +++, zone>8 mm.; PTCC: Persian Type Culture Collection; ATCC: American Type Culture Collection.

Table 4. Antimicrobial activity of potential bacteriocin producing strain *L. casei* AP8 *and L. plantarum* H5 as examined with selected bacterial indicator strains.

inhibitory compounds of *L. casei* AP8 and *L. plantarum* H5 in different conditions indicated the high resistance of these agents. The antimicrobial compounds were able to resist most of these factors to which it was exposed even during prolong incubation period (Table 6). Cell free extracts prepared from both the isolates are found to be thermo-stable. When *L. casei* AP8 bacteriocin was heated at 40-100° C for 30 min, it retained inhibitory activity against *L. monocytogenes* ATCC 19115. However, a loss in activity in the ranges of 35% was observed when heated at 120°C for 15 min (Table 6). The Antilisterial activity of *L. plantarum* H5 bacteriocin was resistant to heat treatments of 40-100°C for 30 min and remained constant after heating at 121ºC for 15 min. Both investigated bacteriocins were most stable at 4°C and - 20°C and able to retain their antilisterial activity for 30 days without any decrease. *L. casei* AP8 bacteriocin was active in a wide range of pH, as full activity was retained at pH values between 3 and 10. *L. plantarum* H5 bacteriocin remained stable after incubation for 2 h at pH values between 2.0 - 12.0.

Treatment	Concentration	%Residual antimicrobial activity	
		L. casei AP8	*L. plantarum* H5
Enzymes			
Trypsin	1 mg/ml^{-1}	0	0
Papain	1 mg/ml^{-1}	100	100
Proteinase K	1 mg/ml^{-1}	0	0
Pronase E	1 mg/ml^{-1}	0	0
α- amylase	1 mg/ml^{-1}	100	100
Catalase	1mg/ml^{-1}	100	100
Organic solvents			
Butanol	10% [v/v]	100	100
Ethanol	10% [v/v]	100	100
Methanol	10% [v/v]	92	100
Ethyl ether	10% [v/v]	100	100
EDTA	5 mmol l^{-1}	100	83
Sodium deoxycholate	1mg ml^{-1}	100	100
Sulphobetaine 14	1mg ml^{-1}	92	100
SDS	1% [w/v]	100	100
Tween 20	1% [v/v]	100	100
Tween 80	1% [v/v]	92	100

Table 5. Effect of enzymes and chemicals on the antimicrobial activity of two selected strains *L. casei* AP8 and *L. plantarum* H5. For details see text

Treatment (Storage, Temperature and pH stability)	Residual antimicrobial activity	
	L. casei AP8	*L. plantarum* H5
4 ºC, -20ºC/ 30 d	+	+
40-100 ºC/30 min	+	+
121 ºC/10 min	+ [-35%]	+
121 ºC/15 min	-	+
pH= 2	-	+
pH= range 3-10	+	+
pH= 11	-	+
pH= 12	-	+

No inhibition= -; inhibition= +

Table 6. Effect of cold storage, different temperatures and pH on inhibitory activity against *Listeria monocytogenes* ATCC 19115. For details see text.

3.5. Growth and bacteriocin production

Figure 1 shows the growth and bacteriocin production curves of *L. casei* AP8 and *L. plantarum H5* cultured at 30°C. For *Lactobacillus* casei AP 8 cell growth reached the stationary phase at 12 h of cultivation. Kinetics of bacteriocin production showed that its synthesis and/or secretion started at 4 h growth in the exponential phase of growth and maximum activity was observed at the early stationary phase of growth (1800 AU ml^{-1}) and had stabled for 6 h before the bacteriocin activity decreased (Figure 1). The pH values decreased from 6.5 to 3.7 at the end of incubation. For *L. plantarum* H5, bacteriocin activity was detectable in the culture supernatant after 5hr when an absorbance of 0.55 at 600 nm of the culture broth. Production of bacteriocin increased throughout logarithmic growth. In the stationary phase, *L. plantarum* H5 showed maximum bacteriocin activity (3400 AU/mL) and stabilized for 2 hr. But since then, bacteriocin activity declined gradually and stabilized at 1600 AU/ml during the following 4 h. In the stationary phase, extracellular pH was maintained, however, bactericidal activity decreased, excluding a possibility of lactic acid as a bactericidal mechanism.

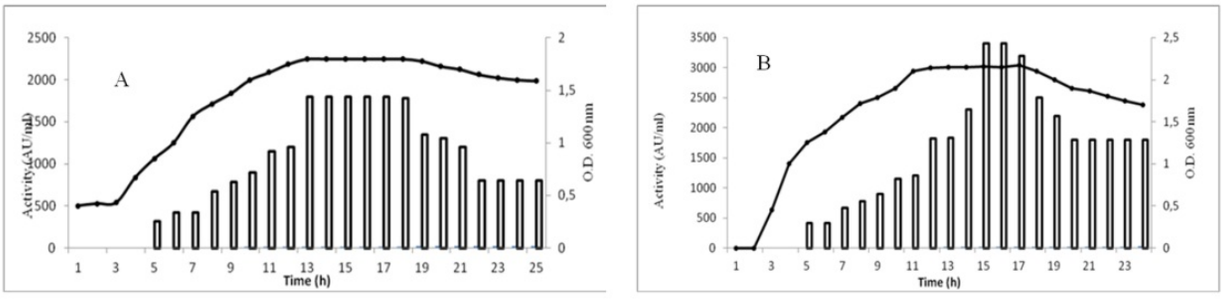

Figure 1. Antimicrobial activity [bars] against *L. monocytogenes* ATCC19115 of *L. casei* AP8 [A] and *L. plantarum* H5 [B] observed during growth in MRS medium [●] and expressed in AU/ml. Results are represent the mean of three independent experiments.

To investigate the reduction of viable cells of target organism in presence of inhibitory substances, twenty mL of each filter-sterilized bacteriocin-containing cell-free supernatant were added to 100 mL of *L. monocytogenes* ATCC 19115 (4 h old at 30°C). The optical density at 600 nm and viable cell count were determined every hour during 24 h. In the control samples inoculated with indicator strain the viable cell count reached to 10^{11}CFU/ml after 24 h incubation at 37°C. The inhibition kinetics using the bacteriocin AP8 (Figure 2) indicated a bactericidal mode of action against *L. monocytogenes*. Addition of the bacteriocin *L. casei* AP8 to early logarithmic-phase cells of indicator strain resulted in grows inhibition after 1h, followed by complete growth inhibition (slow decline) for the remaining time (20 h). In the case of *L. plantarum* H5 bacteriocin the inhibition kinetics showed a bacteriostatic mode of inhibition against indicator strain. Addition of bacteriocin H5 to culture of *L. monocytogenes* showed a growth inhibition after 1 h followed by slow growth. Experiment with stationary-phase cells did not showed any inhibition. No increase in the activity of bacteriocin AP8 and H5 were observed after treatment of the producer cells with 100 mmol/l NaCl at low pH, suggesting that these bacteriocins do not adhere to the surfaces of the producer cells.

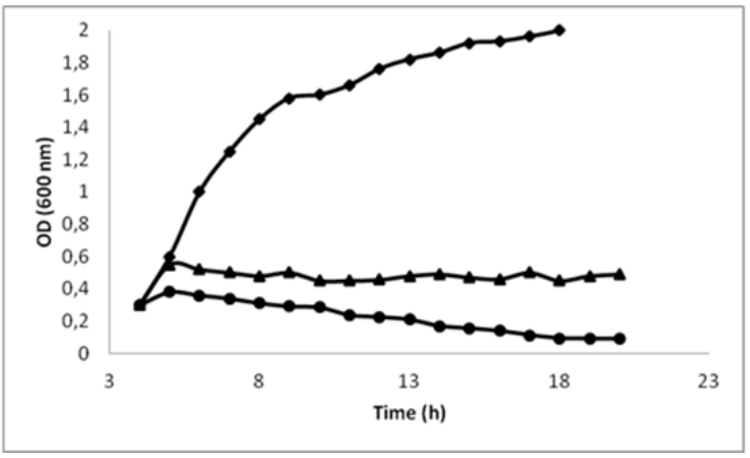

Figure 2. Antimicrobial effect of the CFS of *L. casei* AP8 [▲] and *L. plantarum* H5 [●] on the growth of *L. monocytogenes* ATCC 19115 at 30°C. Growth of *L. monocytogenes* ATCC 19115 without added bacteriocins [control, ♦].

3.6. Partial purification and molecular size of bacteriocins AP8 and H5

Ammonium sulfate precipitation method with 60% saturated ammonium sulphate is used for partial purification of both bacteriocins. Results showed an increase (10-15%) in the inhibitory activity of both bacteriocins against *L. monocytogenes* ATCC 19115 after precipitation. The SDS-PAGE analysis of the partially purified samples showed peptide bands for bacteriocins AP8 and H5 in size of approximately 5 and 3 kDa respectively (Figure 3).

4. Discussion

In this study, we isolated, quantified and characterized *Lactobacillus* species from two species of sturgeon fish inhabiting Caspian Sea to make a bank collection of strain for further research. These fishes are highly valuable species for fisheries and aquaculture in

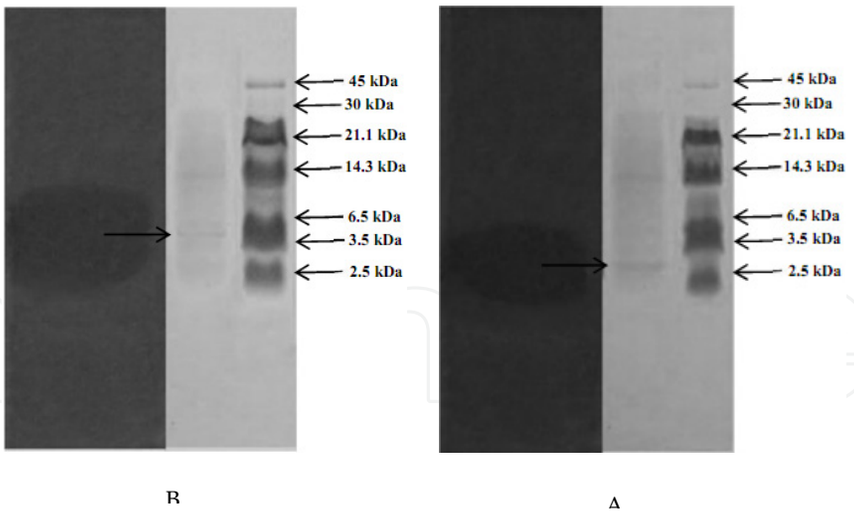

Figure 3. Tricine-SDS-PAGE gel of partially purified bacteriocins [precipitated by 60% saturated ammonium sulphate] *L. casei* AP8 [A] and *L. plantarum* H5 [B] along with the standard MW markers. The gel was overlaid with *L. monocytogenes* ATCC 19115 [approx. 10^6 CFU/ml], embedded in BHI agar, after incubation at 30 °C for 24.

Iran. Presumptive lactobacilli species found in this study were relatively similar to the species described by Bucio Galindo et al. [28]. These authors reported *L. alimentarius, L. coryneformis, L. casei, L. sakei, L. pentosus, L. plantarum, L. brevis and L. oris,* as lactobacilli presented in the intestinal content of studied fish. However, the fish species analysed in that study were different from the two species in this study which were collected from a lake environment. The biochemical characteristics used for identification of *Lactobacillus* may suggest some ideas in relation to the occurrence of the strains in nature. Most of *Lactobacillus* examined in this study (80%) had the capacity to ferment lactose and galactose. Generally, most lactobacilli are able to ferment lactose, by uptake of this disaccharide by a specific permease and splitting it by S-galactosidase for further phosphorylation of galactose and glucose [12]. Because, lactose is only present in milk and milk derivates, it is possible that these strains have evolved from environments related with mammals, as was suggested for other lactose positive *Lactobacillus* [40]. Lactose may be present or was present in the environment as a waste; resulting from livestock production, and disposal effluents from dairy factories. Another component, often fermented by the strains was the amino-sugar N-acetyl-glucosamine, a compound present in peptidoglycans, in blood, chitin and as one of the main constituents of mucus in the gastrointestinal tract [41]. The carbohydrate portion constitutes above 40% of the weight of the mucus [42] or higher values [41].

It could be shown that two strains, *Lactobacillus casei* AP8 and *Lactobacillus plantarum* H5 isolated from intestinal bacterial flora of beluga (*Huso huso*) and Persian sturgeon (*Acipenser persicus*) were able to produce antibacterial substances. According to the findings it was likely that the antibacterial effect was due to the formation of bacteriocin. Results from enzyme inactivation studies demonstrated that antimicrobial activity of isolates AP 8 and H5 was lost or unstable after treatment with all the proteolytic enzymes, confirming the protein status of metabolites and indicating the presence of bacteriocins. Furthermore,

treatment with lipolytic or glycolytic enzymes did not affect the activity of antimicrobial compound produced by strain, suggesting that produced bacteriocins do not belong to the controversial group IV of the bacteriocins, which contain carbohydrates or lipids in the active molecule structure [45-47]. It is important to note that, their activities were not due to hydrogen peroxide or acidity, as antimicrobial activity was not lost after treatment with catalase. Both of the presumptive were considered to be heat stable. Although heat stability of antibacterial substances produced by *Lactobacillus* spp. has been well established [39,48,49,50-53] heat stability of *L. casei* AP8 121ºC for 10 min is novel. The result of pH stability were not coherent with previous report that had indicated the tolerance of bacteriocins to acidic pH rather than alkaline [36,54]. The loss of antimicrobial activity of AP8 bacteriocin at pH > 10 might be ascribed to proteolytic degradation, protein aggregation or instability of proteins at this extreme pH [39,48,55]. *L. casei* AP8 bacteriocin showed an increase in the inhibitory activity after treatments with SDS and EDTA, may be due to the ability of these compounds to break down the proteinaceous complex from its large form into smaller more active unite [21]. Similar to Lactocin RN78 and Plantaricin LC74, both bacteriocin *L. casei* AP8 and *L. plantarum* H5 were found to be stable after treatment with organic solvents like butanol, ethanol and methanol confirming their proteinaceous and soluble nature [18,21,56]. Pronounced inhibitory potential against various species of Gram-positive bacteria were shown, including pathogenic and spoilage microorganisms such as *A. hydrophila, A. salmonicida, C. perfringens*, *B. cereus* and *L. monocytogenes*. Observed effects were consistent with reports about bacteriocins produced by other strains of LAB [1,3,17,19,20,25,41,49,55,57 59,60]. Although bacteriocins from LAB usually are ineffective against Gram-negative bacteria and rather relate to a narrow antimicrobial spectrum [9,51,53], both presumptive bacteriocins AP8 and H5 showed broad antimicrobial activity against several genera of Gram-positive and Gram-negative bacteria. Even representatives of *Pseudomonas, Salmonella, E. coli, A. hydrophila*, *A. salmonicida and V. anguillarum* could be inhibited. Moreover a high level of inhibitory activity against *Listeria monocytogenes* was observed. Earlier studied have shown that several marine bacteria may produce inhibitory substances against bacterial pathogens in aquaculture systems [1,16,19]. Hence the use of such bacteria releasing antimicrobial substances in now gaining importance in fish farming as a natural alternative to administration of antibiotics [1,61-63]. In kinetic studies, both crude bacteriocins were continuously produced during logarithmic phase followed by optimal production during stationary growth phase, suggesting that these peptides may be secondary metabolites. Similar results were reported for some bacteriocins produced by some LAB isolates [5,64,65] and is contrary for other *Lactobacillus* species bacteriocins [1,16,25,55,60,66]. Bacteriocin H5 showed a decrease in activity towards the end of stationary growth may be due to proteolytic degration, protein aggregation, and feedback regulation as has been observed for Lactacin ST13BR, Lactacin B, Helveticin J and Enterocin1146 [53,55,67]. *L. casei* AP8 crude bacteriocin demonstrated a bactericidal mode of action, as the immediate decrease in the optical density of *L. monocytogenes* was observed in mix culture. In the case of H5 bacteriocin a bacteriostatic mode of action was observed. Crud H5 bacteriocin showed a growth inhibition, followed by decrease activity for remained time,

suggesting that indicator organism became resistant to the bacteriocin or bacteriocin was destroyed by proteolytic enzymes [55]. Treating of bacteriocins AP 8 and H5 with NaCl at low pH did not result in increased levels of antilisterial activity, suggesting no adsorption of bacteriocins to their producer cells in agreement with result reported before for *Lactobacillus* strains bacteriocins [55,64,66].

More accurate techniques could be used to determine the molecular mass of molecules, yet the SDS-PAGE technique provides valuable information about the presence of the peptides [3]. In recent years, a large number of new bacteriocins produced by *L. plantarum* have been identified and characterized and the molecular masses of all the bacteriocins produced have been reported in the range of 3-10 kDa [5,39,55]. However, to our knowledge, there is no bacteriocin produced by any *L. casei* strain with a molecular mass of 5 kDa with similar characteristics to strain investigated in this study. Thus, it is possible that this bacteriocin may be a novel bacteriocin produced by *L. casei*. The physiochemical properties of bacteriocins from *L. casei* AP8 and *L. plantarum* H5 were similar to those of other bacteriocins of lactobacilli belonging to the group IIa lactic acid bacteria with respect to molecular weight, heat and pH stability and also sensivity to proteolytic enzymes [9,45,51]. Characteristics unifying all members of class IIa bacteriocins are 1) below 10kDa [1] their potent activity against *Listeria* spp., 2) their resistance to elevated temperatures and extreme pHs, and 3) their cystibiotic feature attributed to the presence of at least one disulfide bridge, which is crucial for antibacterial activity [15,45,51,55]. Class IIa bacteriocins were formerly considered as "narrow"-spectrum antibiotics, with antimicrobial activity directed against related strains. Recently, some class IIa bacteriocins, such as bacteriocin OR-7, enterocin E50-52, and enterocin E760, have been shown to be active against both Gram-negative and Gram-positive bacteria, including *Campylobacter jejuni*, *Yersinia* spp., *Salmonella* spp., *Escherichia coli* O157:H7, *Shigella dysenteriae*, *Staphylococcus aureus*, and *Listeria* spp. [15, 54, 45, 51, 3].

5. Conclusion

Bacteriocins AP8 and H5 showed a wide spectrum of antibacterial activity against seafood borne pathogens like *Listeria*, *Clostridium*, *Bacillus* spp, *S. aureus* and even Gram-negative pathogens like *Pseudomonas, Salmonella* and *E. coli*. Some of these foodborne pathogens can produce toxins resulting in human illness. In addition to the broad inhibition spectrum, theirs technological properties and especially cold, heat and storage stability, indicate that bacteriocins AP8 and H5 have potential for application not only as biopreservative agents to control pathogens in food products that are pasteurized and cook-chilled but also as bioprotect compounds at aquaculture. Accordingly *L. casei* may be of great interest as probiotics strains because of their ability to adhere to intestinal epithelial cells and being of human origin. Several authors have reported the production of bacteriocins by *L. casei* and *L. plantarum* strains from plant, dairy or meat origin. However, very few bacteriocins from *L. plantarum* have been reported to be isolated from fish and also based on our knowledge this is the first report of a *L. casei* bacteriocin isolated from fish.

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