Isolation and characterization of *Lactobacillus* species from intestinal contents of beluga (*Huso huso*) and Persian sturgeon (*Acipenser persicus*)

Ghanbari, M.[1*]; Rezaei, M.[2]; Jami, M.[1] and Nazari, R. M.[3]

[1]Department of Fishery, Hamun International Wetland Research Institute, Zabol University, Zabol, Iran; [2]Department of Fishery, Faculty of Natural Resources and Marine Sciences, Tarbiat Modares University, Noor, Mazandaran, Iran; [3]Fisheries Organization, Shahid Rajaei Aquaculture Center, Sari, Iran

[*]**Correspondence:** M. Ghanbari, Department of Fishery, Hamun International Wetland Research Institute, Zabol University, Zabol, Iran. E-mail: Ghanbari.msc@gmail.com

(Received 1 Jan 2008; revised version 18 Jan 2009; accepted 21 Jan 2009)

Summary

Lactic acid bacteria are characterized as gram-positive, usually non-motile, non-sporulating bacteria that produce lactic acid as a major or sole product of their fermentative metabolism. In this study, the presence of lactobacilli were investigated in the intestines of beluga (*Huso huso*) and Persian sturgeon (*Acipenser persicus*), inhabiting Caspian sea. The obtained data showed that various species of lactobacilli populations were found at high levels in the intestines of fishes. Total number of lactobacilli was about $10^{5.3}$ and $10^{6.4}$ cfu/g of intestinal content for beluga and Persian sturgeon, respectively. Physiological and biochemical characteristics of 84 strains isolated from intestines of beluga and Persian sturgeon revealed that these strains can be categorized into 2 metabolic groups; facultative and obligate heterofermentatives. The most common presumptive lactobacilli species were *Lactobacillus sakei* and *Lactobacillus plantarum*. The current study is the first report on the isolation of lactobacilli from the intestine of beluga and Persian sturgeon.

Key words: Lactobacilli, Beluga, Persian sturgeon, Caspian Sea

Introduction

Lactic acid bacteria are gram-positive, non-sporulating and catalase negative rods or cocci that ferment various carbohydrates mainly to lactate and acetate. Various amino acids, vitamins and minerals are essential for their growth (Kandler and Weiss, 1986). Accordingly, they are commonly associated with nutritious environments like foods, decaying material and the mucosal surfaces of the gastrointestinal and urogenital tract (Kandler and Weiss, 1986; Havenaar *et al*., 1992; Walstra *et al*., 1999), where they enhance the host protection against pathogens (Havenaar *et al*., 1992). Various authors have shown that lactic acid bacteria are also part of the normal intestinal flora of fish (Ringø and Gatesoupe, 1998). Most of the evidences come from salmonid species like Arctic charr (*Salvelinus alpinus*), Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Ringø *et al*., 2000; Ringø and Gatesoupe, 1998; Ringø and Olsen, 1999; Spanggaard *et al*., 2000; Gonzalez *et al*., 2000). Few studies have described lactic acid bacteria in other fishes (Kvasnikov *et al*., 1977; Cai *et al*., 1999). Kvasnikov *et al*. (1977) 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*. (1999) 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*. Recently, Bucio Galindo *et al*. (2006) 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 (data not shown).

The aim of the present study was to make a survey on the presence of lactobacilli in the intestinal content of beluga (*Huso huso*) and Persian sturgeon (*Acipenser persicus*), two high marketing values species of Caspian sea sturgeon fish, as well as, to make a bank collection of strains for further screening research.

Materials and Methods

Fish intestine samples

Two species of Persian sturgeon and beluga were collected from the south coast of Caspian sea in Iran in the spring 2006. 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 (Bucio Galindo *et al*., 2006).

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 (Hartemink and Rombouts, 1999). 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 (Hartemink and Rombouts, 1999). 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. (Hartemink *et al*., 1997). 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*. (2006). 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.

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 (1986) and Hammes and Vogel (1995).

Statistical analysis

Statistical analysis using Student's t-test 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.

Results

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 (Kandler and Weiss, 1986) (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).

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

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

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).

Discussion

In this study, we isolated, quantified and characterized *Lactobacillus* from two species of sturgeon fish inhabiting Caspian sea to make a bank collection of strain for further research (Table 3). These fishes are highly valuable species for fisheries and aquaculture in Iran. Presumptive lactobacilli

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

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 did not 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 3: *Lactobacillus* species isolated from the intestines of sturgeon fish

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

species found in this study (Table 2) were relatively similar to the species described by Bucio Galindo *et al.* (2006). 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 (Kandler, 1986). 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* (Garvie, 1984). 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 (Hicks *et al*., 2000). The carbohydrate portion constitutes above 40% of the weight of the mucus (Stephen, 1985) or higher values (Hicks *et al*., 2000).

Fish at all life stages may expose to the bacteria from the environment. Some of them are detrimental and others are beneficial. Current methods for control of pathogens in the fish farms should be improved by studying the beneficial bacteria. As *Lactobacillus* has many documented health effects (Ouwehand *et al*., 1999), and are naturally present in the gastrointestinal tract of man and animals (Fuller, 1989), we started studies aimed to investigate the intestinal *Lactobacillus* in fish; with the goal of selecting a strain to be used as a feed supplement for warm freshwater fish. Knowledge on the presence of *Lactobacillus* as a natural flora in fish may lead to further applications to improve fish health. Consequensly, the discovered lactobacilli in this study can be candidates as probiotic bacteria. They should resist processing and storage conditions and be alive and active even after gastrointestinal passage. It's notable that selection of probiotic strains is achieved by screening procedures for several characteristics *in vitro*, such as inhibitory activities against several pathogens, resistance to gastric secretions, bile tolerance and growth in faecal material. They should be safe and impart benefits to the host (Fuller, 1989; Havenaar *et al*., 1992). Further studies should be aimed at revealing the factors that determine the lactobacilli occurance in the digestive tract of sturgeon fish, in order to study the more properties of their isolated strains for using them as probiotics for normalization of intestine microflora in these commercial fish.

Acknowledgements

The authors thank the personnel of the Shahid Rajaei Aquaculture Center for their assistance. We are also gratefull to Dr. Soltani from Faculty of Veterinary Medicine, University of Tehran, Mr. Shahreki from Medicine Science University and Dr. Adolfo Bucio Galindo from Wageningen University, The Netherlands, for their helpful opinions. This research was funded by Tarbiat Modares University.

References

Bucio Galindo, A; Hartemink, R; Schrama, JW; Verreth, JAJ and Rombouts, FM (2006). Presence of lactobacilli in the intestinal content of freshwater fish from a river and from a farm with a recirculation system. Food Microbiol., 23: 476-482.

Cai, YM; Suyanandana, P; Saman, P and Benno, Y (1999). Classification and characterization of lactic acid bacteria isolated from the intestines of common carp and freshwater prawns. J. Gen. Appl. Microbiol., 45: 177-184.

Fuller, R (1989). Probiotics in man and animals. J. Appl. Bacteriol., 66: 365-378.

Garvie, EI (1984). Taxonomy and identification of dairy bacteria. In: Davies, FL and Law, BA (Eds.), *Advances in the microbiology and biochemistry of cheese and fermented milk*. London: Elsevier Applied Science Publishers. PP: 35-65.

Gonzalez, CJ; Encinas, JP; Garcia-Lopez, ML and Otero, A (2000). Characterization and identification of lactic acid bacteria from freshwater fishes. Food Microbiol., 17: 383-391.

Hammes, WP and Vogel, RF (1995). The genus *Lactobacillus*. In: Wood, BJB and Holzapfel, WH (Eds.), *The lactic acid bacteria, the genera of lactic acid bacteria*. Vol. 2, London, Blackie Academic and Professional. PP: 19-54.

Hartemink, R; Domenech, VR and Rombouts, FM (1997). LAMVAB - a new selective

medium for the isolation of lactobacilli from faeces. J. Microbiol. Methods. 29: 77-84.

Hartemink, R and Rombouts, FM (1999). Comparison of media for the detection of bifidobacteria, lactobacilli and total anaerobes from faecal samples. J. Microbiol. Methods. 36: 181-192.

Havenaar, R; Ten Brink, B and Huis in't Veld, JHJ (1992). Selection of strains for probiotic use. In: Fuller, R (Ed.), *Probiotics: the scientific basis*. (1st Edn.), London, Chapman and Hall. PP: 209-224.

Hicks, SJ; Theodoropoulos, G; Carrington, SD and Corfield, AP (2000). The role of mucins in host-parasite interactions. Part I– Protozoan parasites. Parasitol. Today. 16: 476-481.

Kandler, O and Weiss, N (1986). Genus Lactobacillus beijerinck 1901, 212^{AL}. In: Sneath, PHA; Mair, NS; Sharpe, ME and Holt, JG (Eds.), *Bergey's manual of systematic bacteriology*. Vol. 2, Baltimore: Williams and Wilkins. PP: 1209-1234.

Kvasnikov, EI; Kovalenko, NK and Materinskaya, LG (1977). Lactic acid bacteria of freshwater fish. Microbiology. 46: 619-624 (In English).

Ouwehand, AC; Kirjavainen, PV; Grönlund, MM; Isolauri, E and Salminen, S (1999). Adhesion of probiotic micro-organisms to intestinal mucus. Int. Dairy J., 9: 623-630.

Ringø, E; Bendiksen, HR; Wesmajervi, MS; Olsen, RE; Jansen, PA and Mikkelsen, H (2000). Lactic acid bacteria associated with the digestive tract of Atlantic salmon (*Salmo salar* L.). J. Appl. Microbiol., 89: 317-322.

Ringø, E and Gatesoupe, FJ (1998). Lactic acid bacteria in fish: a review. Aquaculture. 160: 177-203.

Ringø, E and Olsen, RE (1999). The effect of diet on aerobic bacterial flora associated with intestine of Arctic charr (*Salvelinus alpinus* L.). J. Appl. Microbiol., 86: 22-28.

Ringø, E; Strøm, E and Tabachek, JA (1995). Intestinal microflora of salmonids: a review. Aquacult. Res., 26: 773-789.

Spanggaard, B; Huber, I; Nielsen, J; Nielsen, T and Gram, L (2000). The microflora of rainbow trout intestine: a comparison of traditional and molecular identification. Aquaculture. 182: 1-16.

Stephen, AM (1985). Effect of food on the intestinal microflora. In: Hunter, JO and Alun Jones, V (Eds.), *Food and the gut*. Sussex, England, Baillière Tindall. PP: 57-77.

Thapa, N; Pal, J and Tamang, JP (2006). Phenotypic identification and technological properties of lactic acid bacteria isolated from traditionally processed fish products of the Eastern Himalayas. Int. J. Food Microbiol., 107: 33-38.

Walstra, P; Geurts, TJ; Noomen, A; Jellema, A and van Boekel, MAJS (1999). *Dairy technology, principles of milk properties and processes*. New York, Marcel Dekker, Inc., P: 727.