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ORIGINAL ARTICLE

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF *LACTOBACILLUS FERMENTUM* FROM FECAL SAMPLES OF *CASPIAN SHAD*

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ABSTRACT: The intestinal microflora of Caspian shad fish was studied to isolate and identify lactic acid bacteria as new probiotic. For this purpose, 50 samples from Aras River in East Azerbaijan province of Iran randomly selected and were tested from presence of Lactobacillus strains. Polymerase chain reaction was performed at volume of 25 microliters in specific primers of 16S rRNA genes. PCR was performed in a denaturation cycles. Then, biochemical properties of these genera such as acid producing potential, NACL tolerance, acid tolerance, protease and lipase activity and antibiotic resistance was detected. Results showed that of 50 samples, 3 samples were detected as Lactobacillus fermentum to wit 6% of total samples was positive. In conclusion can be declare that because lack of the documented articles in the microbiota content of the fecal samples from Caspian shad or other fishes thus authors suggest that there needs more studies must be fulfill to estimate an accurate amount of lactobacillus spp population in the feces of fishes.

KEYWORDS: Lactobacillus fermentum, PCR, Caspian Shad, fecal sample.

INTRODUCTION

The shads or river herrings comprise the genus Alosa, fish related to herring in the family Clupeidae. They are distinct from others in that family by having a deeper body and spawning in rivers. Several species can be found on both sides of the Atlantic Ocean and Mediterranean Sea. Alosa can also be found throughout the Caspian Sea. Many are found in freshwater during spawning and some are only found in landlocked freshwater. Shads are thought to be unique among the fishes in having evolved an ability to detect ultrasound (sound at frequencies above 20 kHz, which is the limit of human hearing; Mann *et al.,* 2001). This was first discovered by fisheries biologists studying a type of shad known as blueback herring, and was later verified in laboratory studies of hearing in American shad. This ability is thought to help them avoid dolphins that find prey using echolocation. Alosa are generally pelagic (Bobori *et al.,* 2001). They are mostly andromous or semiandromous with the exception of strictly freshwater landlocked species (Bobori *et al.,* 2001). Alosa are generally migratory and schooling fish (Bobori *et al.,* 2001). Males will usually mature about a year before females and spawn in the late spring to summer months (Travis, 2007; Bianco, 2002). Most individuals die shortly after spawning (Travis, 2007; Bianco, 2002). Alosa are seemingly very adaptive vertebrates and can change readily to adapt to their environment as species are found in a variety of temperatures and waters (Bianco, 2002). Lactobacilli are members of the normal microflora existing in the gastrointestinal (GI) tract of human and animals (Tannock *et al.,* 1990). In general, the lactobacilli level in intestine was suggested to be an index of animals' healthy status. Lactic acid bacteria (LAB) consist of a number of bacterial genera within the phylum Firmicutes. The genera Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus and Weissella are recognized as LAB (Ercolini *et al.,* 2001; Jay, 2000; Holzapfel *et al.,* 2001; Stiles and Holzapfel, 1997). Lactic acid-producing Gram-positive bacteria but belonging to the phylum Actinobacteria are genera such as Aerococcus, Microbacterium, and Propionibacterium (Sneath and Holt, 2001) as well as Bifidobacterium (Gibson and Fuller, 2000; Holzapfel *et al.,* 2001). Members of LAB share the property of being Gram-positive bacteria (Fooks *et al.,* 1999) that ferment carbohydrates into energy and lactic acid (Jay, 2000). Depending on the organism, metabolic pathways differ when glucose is the main carbon source: homofermentative bacteria such as Lactococcus and Streptococcus yield two lactates from one glucose molecule, whereas the heterofermentative (ie. Leuconostoc and Weissella) transform a glucose molecule into

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lactate, ethanol and carbon dioxide (Caplice and Fitzgerald, 1999; Jay, 2000; Kuipers *et al.*, 2000). In addition, LAB produces small organic compounds that give the aroma and flavor to the fermented product (Caplice and Fitzgerald, 1999). The Aras is a river located in and along the countries of Turkey, Armenia, Azerbaijan, and Iran. Its total length is 1,072 kilometers (666 mi). Given its length and a basin that covers an area of 102,000 km^2, it is one of the largest rivers of the Caucasus. The objective of this study was to identification and isolation of lactobacillus fermentum from the fecal samples of Caspian Shad by PCR method.

MATERIALS AND METHODS

For this purpose, 50 samples from Aras River in East Azerbaijan province of Iran randomly selected and were tested from presence of Lactobacillus strains. For this mean, Amount of 25 g of homogenized fecal sample was diluted in 25 ml of sodium citrate 2%. Then, 10 ml of the resulting dilution transferred to 90 ml MRS broth medium and then incubated at 37 °C for 24 hours.

Then, the amount of one-tenth ml of the resulted medium were cultured in the MRS agar media as surface method and then incubated as anaerobic at 37 °C for 48-72 hours.

The colonies formed were evaluated by the catalase test and Gram staining and samples of rod-shaped bacteria, Gram-positive and catalase negative was incubated as anaerobic in MRS broth culture medium at 37 °C for 24 hours for DNA extraction. For extraction of DNA, we used of prepared fluid from MRS media in the 1.5 ml tubes; then these tubes centrifuged at 12000rpm for 5 minute.

The supernatant fluid was poured out and the resulting precipitate was used for DNA extraction. Then, we used of liquid nitrogen for breaking the cell wall and exposure of DNA. Finally, extracted fluid was transferred to the microtubes by 1 ml of buffer solution (table 1) then incubated at 60 °C for 1 hour.

After 1 hour, they centrifuged at 12000rpm for 5-10 minute and supernatant fluid was transferred to the new 1.5 ml tubes and isoamyl alcohol was added its same volume and shanked 4-3 times slowly over the bottom then centrifuged at 12000 rpm for 10 minute.

The superficial transparent liquid was obtained by sampler and transferred to the new tubes and cold isopropyl alcohol was added at same volume and kept at -20°C for 30 minute. Then were centrifuged at 12000rpm for 10 minute and after disposing of superficial fluid, remnants were used for PCR.

Table 1: constituent of buffered used for DNA extraction

Tris-base	12.1 gr/L (ph:8)
EDTA-Na_2	7.44 gr/L
PVP (2%)	1 gr/L
NaCl	81.82 gr/L
C-TAB	20 gr/L
Distilled water	Until 1000 ml

Solutions required for electrophoresis on agarose gel:

TAE buffer (50X)

Tris-base	242 gr/L
EDTA (0.5 molar)	100 ml/L (ph:8)
Glacial Acetic Acid	57.1 ml/L

TAE buffer (1X)

TAE (50X)	20 ml/L
Distilled water	Until 1000 ml

Loading buffer (6X)

Bromo PHenol Blue	0.03 (w/v%)
Xylene Cyanol FF	0.03 (w/v%)
Glycerol EDTA	60 ml

PCR protocol:

Polymerase chain reaction was performed at volume of 25 microliters in specific primers of 16S rRNA genes as shown below:

LACF: 5´- AGAGTTTGATCMTGGCTCAG -3´
LACR: 5´- TACCTTGTTAGGACTTCACC- 3´

PCR solution was prepared as below constituent:

DNA Template	50 ng (1 µl)
Primer Forward	0.5 µl
Primer Reverse	0.5 µl
Master Mix	12.5 µl
Dionized H2O	10.5 µl
Total volume	25 µl

PCR was performed in a denaturation cycles as follow:

Initial denaturation cycle was performed at 94 °C for 5 minute, 32 cycles were performed at 94 °C for 1 minute, primer binding step at 57 ° C for 1 min, expanding at 72 ° C for 1.5 min and finally, one final expanding cycle at 72 °C for 10 min. after staining of the gel by Sibere green solution and assessment under UV light, primary information on DNA template was achieved and result of PCR was obtained by appearance of bands.

Then, biochemical properties of these genera such as acid producing potential, NACL tolerance, acid tolerance, protease and lipase activity and antibiotic resistance was detected.

RESULTS

DNA extracted from Lactobacillus samples are shown in figure 1. Due to the size of the 16S rRNA gene, which is about 1,500 pair's nucleotide, resulted amplified with above mentioned primers by PCR confirmed a 1500 bp fragment the nucleotide. Of 50 samples, 3 samples were detected as Lactobacillus fermentum to wit 6% of total samples was positive.

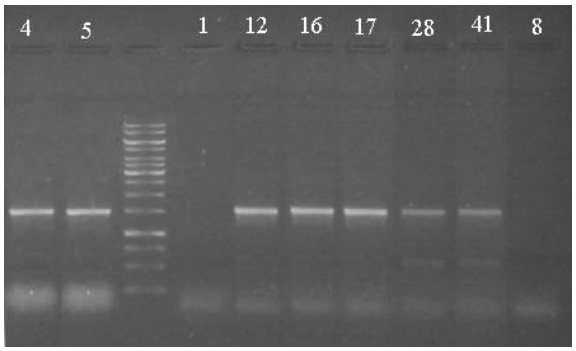

Figure 1: DNA extracted from Lactobacillus fermentum.

DISCUSSION AND CONCLUSION

This study was carried out on fecal samples of Caspian shad fishes while there was no documented articles in this field so, this study is unique because it is a first report of Lactobacillus fermentum isolated from fecal sample of above mentioned fish. In this study revealed that of 50 fecal samples, 3 samples were positive for existence of Lactobacillus fermentum. Wang *et al.,* (2009) showed after intake of L. plantarum L2 for 28 days, a significant increase in live L. plantarum was found in the rats' feces, small intestine and colon. The bacterial levels remained high even after the L. plantarum L2 administration had been stopped for two weeks. Strain-specific PCR and FISH provided clear and direct evidence of colonization of the rat gastrointestinal tract by L. plantarum L2. Additionally, a significant increase in CD19-positive cells in the ileum was observed after intake of L. plantarum L2. In conclusion, dietary supplementation with L. plantarum L2 induced significant colonization of the gastrointestinal tract of rats, and this was associated with significant alteration of the immune response in the gastrointestinal mucosa.

Daily consumption of FMD enabled a probiotic Lact. casei strain maintained in the gastrointestinal tract of volunteers at a stable relatively high population level during the probiotic feeding period (Tuohy *et al.,* 2007).

The results of Brunser *et al.,* (2006) confirmed the presence of higher counts of bifidobacteria and lactobacilli in the microbiota of BF infants compared to formula-fed infants before dietary diversification, and that La1 survives in the infant digestive tract.

Garrido *et al.,* (2005) demonstrated that at baseline, 37.7% of the total fluorescent bacteria were Eubacterium rectale, 18.3% Fusobacterium prausnitzii, 13.2% Bacteroides, 8.6% Atopobium, 2.30%, Clostridium histolyticum, 2.05% Bifidobacterium and 0.95% Lactobacillus. Fecal excretion of La1 increased during the intake period and decreased during the post-ingestion period, so that no La1 was observed in the stools of the volunteers seven weeks after the intake product has been finished. La1 intake increased the populations of C. histolyticum ($p=0.049$), Lactobacillus ($p=0.056$) and Bifidobacterium ($p=0.067$), and decreased those of F. prausnitzii ($p=0.005$) while it did not affect Bacteroides, E. rectale and Atopobium populations. These bacterial populations returned to their baseline levels during the post-ingestion period. The regular intake of a La1-containing product beneficially affects the homeostasis of the human fecal microbiota, probably contributing to the health-promoting effects of this probiotic.

On study was carried out by Urlings *et al.,* (1993) on fecal microbiota. It is suggested that the measured increase of amino acid breakdown, and (or) the acidic pH of the fermented diet, caused these unfavorable results. To examine the effect of the fermented diet on the gut flora, fecal samples were analyzed. The fermented diet changed the composition of the gut flora significantly. In the group that received the fermented diet the number of lactobacilli and the mesophilic aerobic count increased and the number of Enterobacteriaceae and enterococci decreased compared with the control group.

On study by Alexopoulos *et al.,* (2011) on microbial ecology of fish species ongrowing in Greek sea farms and their watery environment was done and he state that in most of the watery ecosystems coming from the different sampling areas, total and fecal coliforms as well as total and fecal streptococci were abundant in all water samples. Enterococcus, Escherichia coli and Pseudomonas were present at a level of 3 logs cfu/100 ml. The anaerobic Clostridium perfringens was found in vegetative (21.3%) and spore forms (13.3%). It is of interest to note that pathogens as Pasteurella piscicida and Vibrio anguillarum were isolated only in a small number of samples. Staphylococcus aureus was detected in 4% of the samples, other Staphylococcus sp. in 29.3%, E. coli in 30.7%, Salmonella sp. in 1.3%, Pseudomonas sp. in

13.3%, Clostridia lec(-) in 49.3%, Bacillus sp. in 38.7%, Vibrio sp. in 18.7%, Lactobacillus and Lactococcus sp. in 36% και 29.3% respectively. Vegetative forms of C. perfringens were detected in 22.7%. Although, our results showed no significant correlations between the sea water and fish microflora, more focus on this bipolar interacting system should be necessary in order to avoid any possible disturbance in the balance of the healthy farming ecosystem with the host organisms. In conclusion can be declare that because lack of the documented articles in the microbiota content of the fecal samples from Caspian shad or other fishes thus authors suggest that there needs more studies must be fulfill to estimate an accurate amount of lactobacillus spp population in the feces of fishes.

REFERENCES

Alexopoulos A, Plessas S, Voidarou C, Noussias H, Stavropoulou E, Mantzourani I, Tzora A, Skoufos I, Bezirtzoglou E. Microbial ecology of fish species ongrowing in Greek sea farms and their watery environment. Anaerobe 2011;17(6):264-6.

Bianco PG. The Status of the Twaite Shad, Alosa agone, in Italy and the Western Balkans. Marine Ecology 2002;23:51–64.

Bobori DC, Koutrakis ET, Economidis PS. Shad Species in Greek Waters – An Historical Overview and Present Status. Bulletin Français De La Pêche Et De La Pisciculture 2001;362-363:1101-108.

Brunser O, Figueroa G, Gotteland M, Haschke-Becher E, Magliola C, Rochat F, Cruchet S, Palframan R, Gibson G, Chauffard F, Haschke F. Effects of probiotic or prebiotic supplemented milk formulas on fecal microbiota composition of infants. Asia Pac J Clin Nutr 2006;15(3):368-76.

Caplice E, Fitzgerald GF. Food fermentation: role of microorganisms in food production and preservation. Int J Food Microbiol 1999;50:131-149.

Ercolini D, Moschetti G, Blaiotta G, Coppola S. Behavior of variable V3 region from 16S rDNA of lactic acid bacteria in denaturing gradient gel electrophoresis. Curr Microbiol 2001;42:199-202.

Fooks LJ, Fuller R, Gibson GR. Prebiotics, probiotics and human gut microbiology. Int Dairy J 1999;9:53-61.

Garrido D, Suau A, Pochart P, Cruchet S, Gotteland M. Modulation of the fecal microbiota by the intake of a Lactobacillus johnsonii La1-containing product in human volunteers. FEMS Microbiol Lett 2005;248(2):249-56.

Gibson GR, Fuller R. Aspects of in vitro and in vivo research approaches directed toward identifying probiotics and probiotics for human use. J Nutr 2000;130:391S-395S.

Holzapfel WH, Haberer P, Geisen R, Björkroth J, Schillinger U. Taxonomy and important features of probiotic microorganisms in food nutrition. Am J Clin Nutr 2001;73:365S-373S.

Jay JM. Fermentation and fermented dairy products, pp. 113-130. In: Modern Food Microbiology, 6th edition. An Aspen Publication, Aspen Publishers, Inc. Gaithersburg, USA 2000.

Kuipers OP, Buist G, Kok J. Current strategies for improving food bacteria. Res Microbiol 2000;151:815-822.

Mann DA, Higgs DM, Tavolga WN, Souza MJ, Popper AN. Ultrasound detection by clupeiform fishes. J Acoust Soc Am 2001;109(6):3048-54.

Sneath PHA, Holt JG. Bergey's Manual of Systematic Bbacteriology, 2nd edition. A Waverly Company, Williams & Wilkins, Springer-Verlag, NY, USA 2001;1:64.

Stiles ME, Holzapfel WH. Review article: Lactic acid bacteria of foods and their current taxonomy. Int J Food Microbiol 1997;36:1-29.

Tannock GW, Fuller R, Pedersen K. Lactobacillus succession in the piglet digestive tract demonstrated by plasmid profiling. Appl Environ Microbiol 1990;56(5):1310-1316.

Travis R. Age, growth and fecundity of Alabama shad (Alosa alabamae) in the Apalachicola River, Florida Clemson University 2007.

Tuohy KM, Pinart-Gilberga M, Jones M, Hoyles L, McCartney AL, Gibson GR. Survivability of a probiotic Lactobacillus casei in the gastrointestinal tract of healthy human volunteers and its impact on the faecal microflora. J Appl Microbiol 2007;102(4): 1026-32.

Urlings HA, de Jonge G, Bijker PG, van Logtestijn JG. The feeding of raw, fermented poultry byproducts: using mink as a model. J Anim Sci 1993;71(9):2427-31.

Wang B, Li J, Li Q, Zhang H, Li N. Isolation of adhesive strains and evaluation of the colonization and immune response by Lactobacillus plantarum L2 in the rat gastrointestinal tract. Int J Food Microbiol 2009;132(1):59-66.