Research Article
AMERICAN JOURNAL OF PHARMACY AND HEALTH RESEARCH
www.ajphr.com
2016, Volume 4, Issue 5
ISSN: 2321–3647(online)

Probiotic profiling of *Lactobacillus spp.* isolated from the intestine of *Sperata seenghala* and *Labeo bata*

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ABSTRACT

A very common probiotic bacterium is *Lactobacillus*. In the current study for the first time the isolation has been done from *Sperata seenghala* and *Labeo bata*. The objective of the current study was to isolate and identify *Lactobacillus* bacteria from the intestines of these two fishes. The total bacterial counts were $2.1x10^6$ and $1.8x10^5$ CFU/g. Isolation was done by dissection, homogenization and filtration. The isolates were identified as *Lactobacillus* by gram staining, microscopic observation and biochemical tests. The isolates showed growth within pH 3 to 8 with maximum growth at pH 7. They stood firm at bile salt concentrations of 0.0, 0.1, 0.2 and 0.3%. Antibiotic sensitivity test was done by disc diffusion method. The *Lactobacillus* isolated from *S. seenghala* was resistant to Ampicillin, Ceftazidime, Cefuroxime, Co-trimoxazole and Nalidixic Acid. Isolate from *L. bata* was resistant to Ampicillin, Azithromycin, Ceftazidime, and Cloxacillin. Both these isolates can be denoted as potential probiotics and they will serve as very useful tools for future research.

Keywords: Probiotic, *Lactobacillus*, *Sperata seenghala*, *Labeo bata*, pH and bile salt tolerance, antibiotic sensitivity

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Received 01 May 2016, Accepted 09 May 2016

Please cite this article as: Karim R *et al.*, Probiotic profiling of Lactobacillus spp. isolated from the intestine of Sperata seenghala and Labeo bata. American Journal of Pharmacy & Health Research 2016.

INTRODUCTION

Fish is one of the most important foods for human beings. Intestine of fish is a great source of *Lactobacillus* bacteria [1]. Worldwide aquaculture is a popular and fastest food producing sector. To minimize the loss of this sector the use of probiotics has become a common topic now. The term probiotics refers to bacteria that are helpful [2]. The genus *Lactobacillus* are rod shaped, gram positive, non spore forming, nonpigmented, catalase negative and microaerophillic to strictly anaerobic organisms. *Lactobacilli* possess two major advantages- GRAS (Generally Recognized As Safe) and probiotics [3]. Probiotics are useful bacteria that help in intestinal microflora balance, promote good digestion, inhibit the growth of harmful and pathogenic bacteria, boost immune function and increase resistance to infection. Beside these, they also contribute in removal of carcinogens, lowering of cholesterol, and allergy lowering effect, synthesizing and enhancing the bioavailability of nutrients [4]. *Lactobacillus* bacteria also possess some inhibitory properties because of their capacity to produce different antimicrobial compounds such as lactic acid, acetic acid, ethanol, formic acid, acetone hydrogen peroxide, diacetyle and bacteriocin. These contain preservative ability which protects fish from other natural competitive [5,6,7]. Most probiotics contain single or multiple strains of lactic acid bacteria (LAB) and they are the part of natural microflora [8]. The genus *Lactobacillus* is one of the largest groups of LAB which is used in food fermentation and so it has much economic importance [9]. It also has a great probability to contribute in finding remedy against potential fish pathogens [10].

To locate probiotic one has to evaluate few criteria such as acid and bile salts tolerance and also antibiotic susceptibility since the bacterium has to survive in the diversity offered by adverse intestinal environment to stressful condition and also to prevail when traditional antibiotics are in action simultaneously [11,12]. The aim of this study was the isolation, identification and probiotic characterization of *Lactobacillus spp.* from the intestine of *Sperata seenghala* (Local name: Ayr) and *Labeo bata* (Local name: Bata), two members from two very important pisces families, i.e. Bagridae and Cyprinidae. To very best of our knowledge, this is the first report of isolating intestinal microbiota from *S. seenghala.* This is also the pioneer approach of isolating *Lactobacillus spp.* from *L. bata*. It is quite understandable that occurrence of *Lactobacillus* in the sources belong to this region has to be well reported. The claimed probiotics could aid in future research of bio-therapeutics for sure.

MATERIALS AND METHOD

Sample Collection

20 fish samples of each species were collected from Freshwater Fish Breeding Center, Chachra, Jessore, Bangladesh (23.14°N, 89.2°E) with taxonomical confirmation by the responsible scientist on duty there and kept in lab aquarium in the Department of Fisheries and Marine Bioscience, Jessore University of Science and Technology (JUST). All the fishes were weighted between 150 to 300gm and 12 to 18 cm in length. Fish sample for experiment was randomly selected from these. At first the body of the fish was washed with 70% alcohol. The fish was then dissected, intestine was collected and washed with 0.85% saline water and distilled water for 3 to 4 times. Then the intestine was cut into small parts which and 1gm was taken. It was then homogenized using mortar pastel [13]. All the steps were done under sterile condition.

Isolation of *Lactobacillus* Bacteria

Applying serial dilution technique, 0.1 ml of the homogenized intestine sample was spread over plate count agar (PCA) (Merck) and incubated at 37°C for 72 hours to count the colony forming unit (CFU). Rest of the homogenate was poured into MRS broth medium through filtration. It was incubated for 24 to 48 hours at 30^0C. After that serial dilution was done with 0.1ml of culture. Sample culture was then spread on MRS agar and incubated at 30^0C for 72 hours [14]. White colony was observed and from this single colony subculture was done on new MRS agar for 3 or 4 times to find axenic culture. Isolated pure bacterial single colonies were stored for further study on agar slants [15].

Identification of *Lactobacillus* Bacteria

The gram staining technique with microscopic observation of the morphological characteristics and classical biochemical test sets were applied for the identification of bacterium. The slide was examined under light microscope with a magnification of 1000X [16].

Probiotic Profiling

pH Tolerance

Acid tolerance of the isolate was investigated in MRS broths with different pH including 2, 3, 4, 5, 6, 7 and 8 were prepared using 1% HCl and 1(N) NaOH (Sigma) [17]. The broths media along with control bottles were autoclaved at 121°C for 20 min and then inoculated with overnight culture of the selected strain in MRS broth followed by incubation at 30°C. Optical density (OD) as growth rate of bacteria was measured by spectrophotometer at 600 nm after 2 hrs incubation. The viability of the isolates was also controlled by duplicate inoculation on MRS agar [11, 18, 19].

Bile Salt Tolerance

Testing bile salt tolerance of the isolate was on the carts. The test was executed in MRS broth which included 0.0, 0.1, 0.2 and 0.3% (w/v) Oxgall bile salt (Sigma, USA). Duplicate bottles of MRS broth containing filtered different concentrations of bile salt were inoculated by 30 µl of cultured strain and incubated at 30°C. Growth rate was assessed by measuring the optical density by spectrophotometer at 600 nm after 0, 2, 4, 6, 8 and 12 hrs incubation [11, 18, 20].

Antibiotic Sensitivity Test

In this study antibiotic sensitivity test was done according to disk diffusion method [21,22]. Here 15 antibiotic discs were used, i.e. Azithromycine (AZM), Cloxacillin (COX), Erythromycin (E), Vancomycin (VA), Chloramphenicol (C), Gentamicin (GEN), Ciprofloxacin (CIP), Cefotaxime (CTX), Gatifloxacin (GAT), Tetracycline (TE), Ceftazidime (CAZ), Cefuroxime (CXM), Ampicillin (AMP), Co-trimoxazole (COT) and Nalidixic acid (NA). MRS agar plates were inoculated with 100µl 24hrs broth culture prior to placing antibiotic discs on them. Then it was incubated overnight at 30^0C [20]. Resistance and susceptibility were estimated by measurement of zone of inhibition [23].

Statistical analysis

Each experiment was run in triplicate and mean values were calculated with SD. SPSS version 11.0 was used for the data analysis.

RESULTS AND DISCUSSION

Total Bacterial Count in Intestine

The total bacterial colony was counted by CFU counting technique on PCA. The counted colony that has been estimated was $2.1x10^6$ and $1.8x10^5$ Cfu/g from the intestines of *S. seenghala* and *L. bata* respectively.

Identification of *Lactobacillus*

Isolated bacteria from both the fishes showed white to yellowish colony on MRS agar and were subjected to gram staining, microscopic observation and biochemical tests. Both the fish intestines contain bacteria having gram positive, non-sporulating, catalase negative rods which were confirmed to be *Lactobacillus* [9]. Thus, LAB isolated from *S. seenghala* was denoted as LAB_1 and isolate from *L. bata* was LAB_2 (Figure. 1).

Probiotic Profiling

pH Tolerance

Erratic changes have been observed through statistical analysis in the growth rate of both the

isolated LABs. Their growth varied within pH 2 to 8. They both showed zero growth at pH 2, while maximum at the neutral pH (Figure 2).

Bile Salt Tolerance

Both the LABs showed significant proliferation at 0.0, 0.1, 0.2 and 0.3% bile salt concentration after 2 hrs of growth, however, their growth descended with the increase of bile salt concentration. The trending pattern showed resemblance even after 4, 6, 8 and 12 hrs of incubation with enhanced proliferation (Figure 3).

Antibiotic Sensitivity Test

Antibiotic sensitivity was done by disc diffusion method. Here in this study 15 antibiotic discs were used. Both the bacterial strains were resistant to numerous antibiotics. LAB1 was resistant to Ampicillin, Ceftazidime, Cefuroxime, Co-trimoxazole and Nalidixic Acid; while moderately resistant to Azithromycin, Cloxacillin, Erythromycin and Vancomycin; intermediate resistant to Cefotaxime, Chloramphenicol and Gentamicin; and susceptible to Tetracycline. LAB2, moreover, showed resistance to Ampicillin, Azithromycin, Ceftazidime and Cloxacillin. It was moderately resistant to Cefuroxime, Chloramphenicol, Erythromycin, Nalidixic Acid, Vancomycin, Gatifloxacin and Gentamicin; intermediate resistant to Cefotaxime and Co-trimoxazole and susceptible to Ciprofloxacin and Tetracycline. The details of the results have been given in table 1.

Table 1:Antibiotic sensitivity test results on two isolated LABs

Antibiotics	Concentration (µg/disc)	Zone Inhibition (in mm)		Sensitivity	
		LAB_1	LAB_2	LAB_1	LAB_2
Ampicillin (AMP)	25	0	1	R	R
Azithromycin (AZM)	30	5	0	MR	R
Cefotaxime (CTX)	30	12	11	IR	IR
Ceftazidime (CAZ)	30	0	0	R	R
Cefuroxime (CXM)	30	0	6	R	MR
Chloramphenicol (C)	30	10	6	IR	MR
Ciprofloxacin (CIP)	05	15	15	S	S
Cloxacillin (COX)	30	5	4	MR	R
Co-trimoxazole (COT)	25	0	10	R	IR
Erythromycin (E)	30	5	5	MR	MR
Gatifloxacin (GAT)	30	15	6	S	MR
Gentamicin (GEN)	10	10	8.5	IR	MR
Nalidixic Acid (NA)	30	0	6	R	MR
Tetracycline (TE)	30	15	16	S	S
Vancomycin (VA)	30	5	7	MR	MR

N.B. LAB_1 = Isolate from *S. seenghala*, LAB_2 = Isolate from *L. bata* R = Resistant, MR = Moderately Resistant, IR = Intermediate Resistant, S = Susceptible

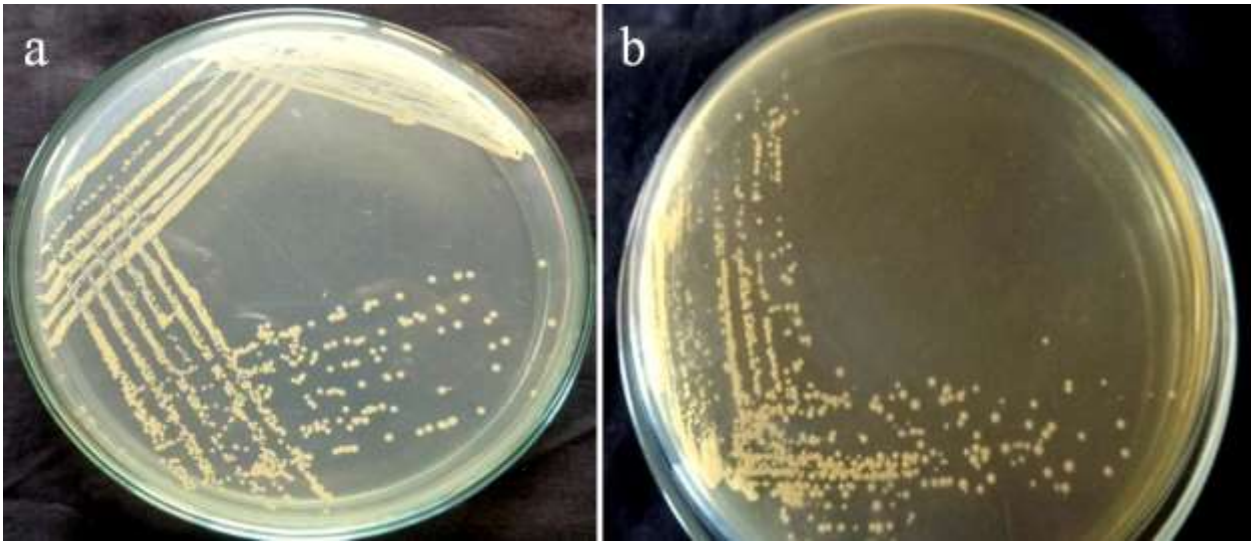

Figure 1: Two isolated LABs on MRS agar plate (a) Isolate from *S. seenghala* (LAB_1) (b) Isolate from *L. bata* (LAB_2)

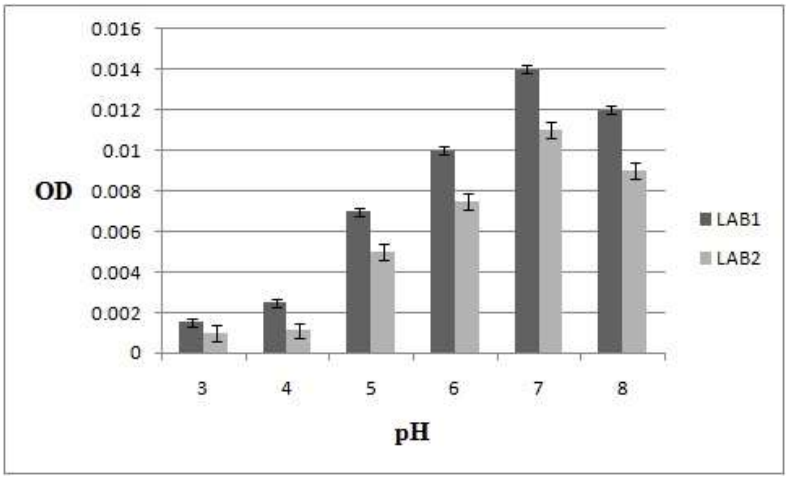

Figure 2: pH tolerance of both the isolates after 2hrs incubation

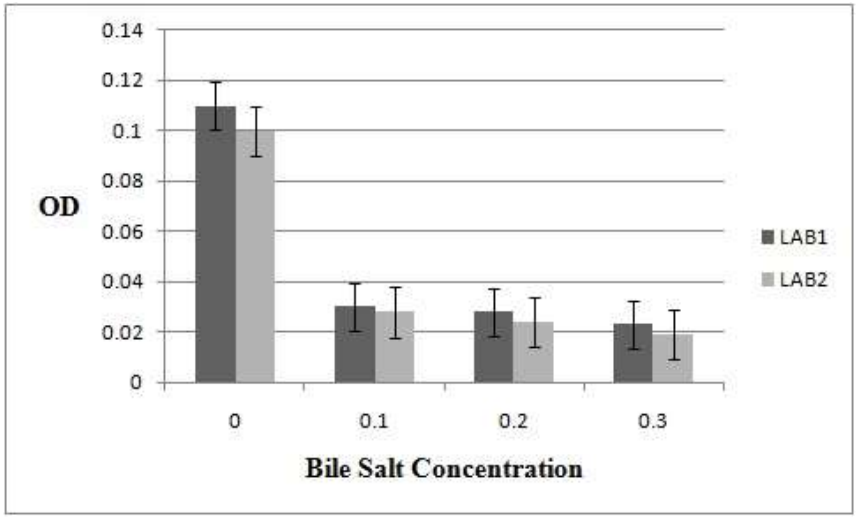

Figure 3: Bile salt tolerance of both the isolates after 2hrs incubation

DISCUSSION

This report provides worthy evidences of the prevalence of *Lactobacillus* in the intestine of *S. seenghala* and *L. bata* for the very first time. Intestinal bacterial load of these two fishes were $2.1x10^6$ and $1.8x10^5$ Cfu/g respectively. Allameh et al. (2012) reported bacterial load of $1.5x10^5$ cfu/g in the intestine of *Channa striatus* [10]. Al-Harbi and Uddin (2005) reported $1.6x10^8$ cfu/g population in the intestine of *Oreochromis niloticus* [24]. *Lactobacillus* was isolated from both the fish intestines. There are reports having similar results. *Lactobacillus* have been isolated from the intestines of *Hypophthalmichthys molitrix* [25], *Oreochromis mossambicus* [26], black tiger shrimp [27], *Labeo rohita* [2] etc. Bucio et al. (2006) reported the presence of *Lactobacilli* in the intestines of freshwater fishes from a river and from a farm with a recirculation system [28].

Another important aspect of the current research was the probiotic analysis of the isolated LABs. To serve the purpose, pH tolerance and bile salt tolerance test were done since the gut environment has a typical condition as far as acidity and bile salt concentration are concerned. Both the LABs showed their best growth at pH 7, however, they were able to grow moderately within pH 3 to 6. On the contrary, their growth was arrested at pH 2 and started to decline when pH crossed 7. According to previous researches, LAB has to survive low pH to be claimed as potential probiotic[29]. In this current work, two isolated LABs resisted low pH which is an evidence for their ability to transit through hostile intestinal environment. Kim and Austin (2008) reported resistance of probiotic carnobacterial strains within pH 5 to 10 isolated from rainbow trout intestine [20]. Nguyen et al. 2007 found a *Lactobacillus* strain surviving pH 6 to 10 [29].

Moreover, both the LABs were tested for their bile salt tolerance against four concentrations i.e. 0.0, 0.1, 0.2 and 0.3%. Although an increase in bile salt quantity decreased the proliferation rate of the two isolated LABs, it is quite substantial that they could survive different bile salt concentrations. This is another notable probiotic feature since the fish intestine possess variable bile salt concentration [30]. Survival of *Lactobacillus* at 0.1 to 0.4% bile salt concentration has been reported by many other workers as well[11, 18, 27]. Tolerance of these two probiotics to bile salt and acidic environment proves they are endurable under stress.

Two labs were also subjected to a very extended scale of antibiotic sensitivity test by using 15 distinguished antibiotics. Resistance to specific antibiotic is important since the probiotic can be given at the same time when antibiotic treatment is required. Secondly, the overlapping nature of these two lifeguards helps microflora of intestine to recover more quickly[11, 20]. LAB_1 was resistant to Ampicillin, Ceftazidime, Cefuroxime, Co-trimoxazole and Nalidixic Acid and LAB_2

exhibited resistance to Ampicillin, Azithromycin, Ceftazidime, and Cloxacillin. Similar results have been described by many scientists. Allameh et al. (2012) reported antibiotic susceptibility profiles showed that this strain was resistant to Streptomycin, intermediate to Amoxicillin and Kanamycin and sensitive to Gentamycin, Tetracycline, Chloramphenicol, and Ampicillin [10]. Kim and Austin (2008) determined the antibiotic susceptibility of *Carnobacterium* strains [20]. They reported resistance to Ampicillin, Gentamycin, Kanamycin, Streptomycin and Penicillin G but sensitivity to Chloramphenicol, Tetracycline and Cotrimaxazole. They also claimed that antibiotic-resistant probiotic is gainful in the case of antibiotics administration to fish and the establishment of the beneficial microorganisms in the intestine for prolonged periods.

CONCLUSION

In this study two LABs have been isolated from *S. seenghala* and *L. bata* and identified as *Lactobacillus* successfully. The total bacterial cell was counted as $2.1x10^6$ and $1.8x10^5$ CFU/g. They were able to grow within pH 3 to 8 and survived bile salt at 0.0, 0.1, 0.2 and 0.3%. The *Lactobacillus* isolated from *S. seenghala* was resistant to Ampicillin, Ceftazidime, Cefuroxime, Co-trimoxazole and Nalidixic Acid. Isolate from *L. bata* was resistant to Ampicillin, Azithromycin, Ceftazidime, and Cloxacillin. They are claimed as probiotics.

ACNOWLEDGEMENT

The research work is funded by the Ministry of Science and Technology, Government of Bangladesh (Grant No. 39.012.002.02.01.018.2015-40).

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