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A two-step enrichment protocol for isolation of low count putative lactic acid bacteria from freshwater fish intestines

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Abstract
The present study reports a double enrichment cum selective isolation based protocol for successful isolation of lactic acid bacteria (LAB) from freshwater fish intestine where it exists in substantially lower population counts. The procedure involved enrichment of homogenized intestinal samples of freshwater fish in de Man, Rogosa and Sharpe (MRS) broth medium followed by further enrichment cum selection in *Lactobacillus* Anaerobic MRS with Vancomycin and Bromocresol green (LAMVAB) broth and final isolation in LAMVAB agar. Traditional isolation method using only MRS medium was also performed with the same samples. Individual colony morphology was recorded and biochemical identification was done. Comparison of the two isolation procedures showed that, total number of LAB along with total number of different individual species of LAB such as *Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus brevis, Pediococcus acidilactici, Weissella paramesenteroides* and *Enterococcus faecium* was more in the double enrichment cum selective isolation procedure using LAMVAB medium than that recovered from isolation procedure involving only MRS medium. Besides LAB, undesired spore forming rods and yeasts were found in the procedure involving only MRS medium thus indicating its low selectivity. Hence, the double enrichment cum selective isolation based protocol using LAMVAB medium was found to be reliable for successful recovery and isolation of LAB from intestines of fish.

Keywords: Freshwater fish intestine, isolation, lactic acid bacteria, MRS, LAMVAB

1. Introduction
A macroorganism and its microflora are in the state of a dynamic equilibrium which has settled and strengthened in the course of a long evolutionary development. The alimentary tract of fish represents an interface between the external environment and the body. Its complex, densely populated, highly diversified poly microbial ecology plays the main role in the immunobiological activity of the fish and has an important influence on its health and disease [1]. Of particular significance are the indigenous, residential microflora and its important constituent part, the group, Lactic acid bacteria (LAB) [2]. The ecological roles played by these microbes in the gut include among others, production of antimicrobial substance, modulation of immune systems, fermentation of non-digestible carbohydrates and increase availability of nutrients [3]. Knowledge on the presence of LAB as a natural flora in freshwater fish intestines may lead to its further applications in form of probiotic treatments to improve health of edible species [4]. However, such applications require exhaustive studies of digestive tract lactoflora.
Currently, LAB group comprises the following genera: *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella*. They are a group of Gram-positive rod and coccus-shaped organisms that have less than 55% mol G+C content in their DNA. They are non- spore forming, non-motile and produce lactic acid as their major end product during the fermentation of carbohydrates [5]. Various amino acids, vitamins and minerals are essential for their growth. Accordingly, they are commonly associated with nutritious environments like foods, fermented, decaying material and the mucosal surfaces of the gastrointestinal tract of animals [5]. Various media have been used to isolate and assay LAB. For most (fermented) food products, rich, slightly acidic media, such as MRS (de Man,

Rogosa and Sharpe) and Rogosa agar allow selective isolation of LAB, as they are generally the most abundant group of bacteria present [6]. But isolation of LAB from the intestine is generally difficult due to the presence of various other competing organisms in higher numbers. Moreover, most media used for the isolation of LAB also support the growth of these competing micro-organisms [7]. Though LAMVAB (*Lactobacillus* Anaerobic MRS with Vancomycin and Bromocresol green) has been used as a selective media for *Lactobacilli* [6, 8] but dilution-based LAB isolation is not always a sufficient manner for the recovery of these bacteria from freshwater fish intestine where it exists in substantially lower population counts than several other bacteria.

In freshwater fisheries, LAB probiotics is currently a frontier area of research. However, isolation of this important group of bacteria from fish intestine suffers due to lack of standard isolation protocols and procedures. In the present study therefore, the development of a double enrichment cum selective isolation based protocol for successful isolation of LAB from freshwater fish intestine was researched upon, formulated and validated.

2. Materials and Methods

2.1. Isolation on different media

Live freshwater fish rohu (*Labeo rohita* Hamilton, 1822), catla (*Catla catla* Hamilton, 1822) and mrigal (*Cirrhinus mrigala* Hamilton, 1822) (n=2 of each specimen) of average weight 1.3±0.7 kg, were used in the study. Samples were collected at regular interval from different locations within 60 km radius of Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar, India. Fish were sacrificed and the whole intestine from each sample was removed under aseptic conditions. After extracting and discarding fecal material, the intestine was flushed thoroughly with sterile saline (0.85% w/v) to remove non-adherent bacteria and homogenized in a mortar and pestle by using sterile normal saline solution (1:10; wt: vol). 0.1 mL of each homogenate was inoculated in 10 mL of MRS broth (HIMEDIA, India) and incubated at 37 ºC for 48 h. The enriched broth culture was used in two different medium (methods) as follows;

(a) MRS agar (Primary medium) - Dilution series from enriched broth culture of each sample was prepared and from appropriate dilution, 1 mL was pour plated on MRS agar (HIMEDIA, India) plates and incubated at 37 ºC for 48 h. Individual colonies were studied on the basis of colony morphology and Gram staining.

(b) LAMVAB medium (Selective medium) - LAMVAB broth was prepared as described by Hartemink *et al*. [6]. Briefly, cysteine-HCL (0.5 g/L) and bromocresol green (0.05 g/L) was added to MRS broth (104.4 g/L). The pH of the solution was adjusted to 5.0±0.1 using 4M HCL and was sterilized at 121 ºC for 15 min. The solution was cooled and to 500 mL of this solution 10 mL of filter sterilized vancomycin hydrochloride solution (2 mg/mL in water) was added aseptically. For preparation of LAMVAB agar plates, agar, 40 g/L was sterilized and cooled to 50 ºC in a water bath and added to the above MRS-vancomycin mixture. However, in the present study, LAMVAB agar plates without bromocresol green were used.

0.1 mL of enriched broth cultures mentioned above were inoculated in 10 mL of LAMVAB broth and incubated at 37 ºC for 48 h under anaerobic conditions. Finally, after 48 h, dilution series from the enriched yellowish green LAMVAB broth culture of each sample was prepared and from appropriate dilution, 1 mL was pour plated on LAMVAB agar plates (without bromocresol green) and incubated anaerobically at 37 ºC for 48 h. Individual colonies were studied on the basis of colony morphology and Gram staining.

2.2. Biochemical characterization and identification

Each Gram positive, non spore forming and catalase negative isolates obtained from both the protocols were transferred into MRS broth and incubated at 37 ºC for 48 h. These presumptive LAB were kept in MRS broth containing 20% (v/v) glycerol at -80 °C. Further analysis was carried out from the stored cultures. A set of 26 tests (including morphology, Gram staining characteristic and catalase test) (Table 1) as described by Ricciardi *et al*. [9] was used to identify and classify the isolates. Strains were tested in duplicate to determine the test reproducibility. Identification of the strains were carried out according to Bergey's manual, Kandler and Weiss [10]; Hammes *et al*. [11] integrated with supplementary information for strains isolated from natural populations obtained from Boukhemis *et al*. [12]; Khedid *et al*. [13]; Huidrom *et al*. [14] and Yu *et al*. [15].

3. Results

Several colonies with different morphologies were observed in both the procedures. Besides LAB, spore forming organisms and yeasts were observed in the MRS medium. All the Gram positive, non spore forming, catalase negative isolates on biochemical characterization were identified as different strains of *Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus brevis, Pediococcus acidilactici, Weissella paramesenteroides* and *Enterococcus faecium* (Table 1). Total number of LAB along with total number of different species of LAB isolated from the two different isolation protocols is shown in Table 2.

Table 1 Biochemical identification of the LAB isolated from freshwater fish intestines

Identified species	***L. plantarum***	***L. fermentum***	***L. brevis***	***W. paramesenteroides***	***P. acidilactici***	***E. faecium***
Colony characteristics	**White, creamy, round, entire margin**	**Gray white, slightly convex, concentric circle at centre**	**Off white, flat, matt finish, rhizoid edge**	**Off white, dry, flat, edge not neat**	**Milky white, mucoid, circular, concave, entire**	**Pin point, off white, round, entire**
Morphology	Cocco bacillary	Long rods	Rods highly variable in length	Lenticular/ Spherical shape	Coccus, tetrad	Coccus
CO_2 from glucose	+	+	+	+	-	-
Growth at 15 ºC	+	-	+	+	-	+
Growth at 45 ºC	-	-	-	+	+	-
Arginine dihydrolysis	-*	+	+	-	+	+
Glucose	+	+	+	+	+	+
L-arabinose	+	+	-	+*	+*	+
Galactose	+*	+*	-*	+	+	+
Sucrose	+	+	-	+	-	+
Lactose	w*	w	-	-	-*	+
Maltose	+	+	+	+	-	+
Mannitol	+	w	-	-	-	+
Mannose	+	w	-	+	+	+
Melezitose	+	-	-	-	-	w*
Melebiose	+	+	+	+	-	+
Raffinose	w*	+	-	-	-	w*
Ribose	+	+	+*	-	+	+
Trehalose	+	+	-	+	+*	+
Dextrose	+	w*	+*	+	+	+
Inositol	w*	+*	-	w	-	-
Sorbitol	+	-	-	-	-*	w
Salicin	+	-	-	-	+*	+
Bile esculin	+	+*	+	-*	+	+
Growth in 2% NaCl	+	+	+	+	+	+
Growth in 4% NaCl	+	+	+	+	+	+
Growth in 6% NaCl	+	+	+	+	+	+

Colony characteristics indicates the morphology obtained in LAMVAB agar plates; - = negative; + = positive; w = weakly positive; * = properties differing among strains of same type

Table 2 Comparison of recovery of LAB from various samples using two isolation protocols

Samples	**Medium**	***L. plantarum***	***L. fermentum***	***L. brevis***	***W. paramesenteroides***	***P. acidilactici***	***E. faecium***	**Total LAB**	**Spore forming rods**	**Yeast**
Rohu 1	MRS	8	6	4	0	4	0	22	8	6
	LAMVAB	14	10	5	0	8	0	37	0	0
Rohu 2	MRS	11	7	5	0	7	3	33	10	5
	LAMVAB	17	10	8	0	10	5	47	0	0
Catla 1	MRS	6	6	5	0	5	0	22	5	3
	LAMVAB	10	8	8	0	10	0	36	0	0
Catla 2	MRS	8	5	4	0	5	0	22	6	4
	LAMVAB	12	8	12	0	10	0	40	0	0
Mrigal 1	MRS	6	4	4	5	3	0	22	4	3
	LAMVAB	9	7	8	9	6	0	39	0	0
Mrigal 2	MRS	5	4	5	4	6	0	24	7	4
	LAMVAB	11	8	8	5	9	0	41	0	0

4. Discussion

The intestine of fish is a very complex microbial ecosystem, in which several hundred bacterial species are present [1]. Detection of a single group of bacteria will always involve elimination of most of the other bacteria. It is thus obvious that contamination (plate in numbers counts) is a serious problem, especially in the detection of groups or species that are not among the numerically dominant groups. Unfortunately, information on suitability of selective media for isolation of a particular group of bacteria from intestinal flora is often not available [8]. In samples such as fermented food products, LAB are usually the abundant and dominant group of bacteria present. Hence, their recovery and selective isolation from these samples using rich and slightly acidic media such as MRS and Rogosa agar is quite easy. However, isolation of LAB from the intestine is generally difficult due to the

presence of various other higher numbers of competing organisms whose growth are also supported in most of the media mentioned above [6]. Hence, dilution-based LAB isolation is not a sufficient manner for the recovery of these bacteria from freshwater fish intestine. Plating the samples directly on MRS agar or selective media often result in scanty colony development or no colony at all [16]. It is thus advisable to enrich the cultures in the nutrient rich media and then go for selective isolation. Hence, a double enrichment cum selective isolation based protocol was followed which was found to be quite reliable.

The initial isolation procedure, in both the methods, involved enrichment of the samples in MRS broth medium. Earlier findings have shown MRS medium has been successfully applied as an enrichment medium for isolation of LAB from various samples [17, 18, 19]. However, in further isolation steps using only MRS medium, besides LAB, a huge number of spore forming rods and yeasts were found indicating its low selectivity. This low selectivity of MRS medium has also been observed previously [6, 8]. Moreover, total number of LAB recovered from each sample in MRS medium was found to be less in comparison to that isolated from double enrichment cum selective isolation procedure using LAMVAB medium. The protocol involving LAMVAB broth and agar was found to be highly selective for isolation of LAB as almost all the colonies characterized and identified were confirmed to be LAB. No spore forming rods and yeasts were recovered. Earlier findings [4, 8] have also reported high selectivity of LAMVAB medium. Total number of different species of LAB such as *Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus brevis, Pediococcus acidilactici, Weissella paramesenteroides* and *Enterococcus faecium* was also found to be more in the protocol using LAMVAB medium in comparison to those recovered from MRS medium.

LAB are highly acid-resistant, with growth being possible at an initial pH of 5.0 [11]. Most other intestinal bacteria are not able to grow at this acidic pH. Contrary to other Gram-positive bacteria, most *Lactobacilli, Pediococci, Weissella, Leuconostoc* and *Enterococci* are resistant to the antibiotic vancomycin [6, 20]. Hence, LAMVAB is selective due to the low pH, which inhibits the most common Gram-negative bacteria and the presence of vancomycin, which inhibits the competing Gram-positive flora in the intestine. As anaerobic conditions favor the isolation and growth of LAB, cysteine in the LAMVAB acts as a reducing agent thus improving the isolation of these micro-aerophiles [6]. The yellow discoloration of the LAMVAB broth medium is due to acid production by LAB thus indicating its presence. However, final isolation was done on LAMVAB agar plates without bromocresol green in order to get clear colony morphology on the plate. Color of the colonies might not always be considered to be characteristics as not all LAB would show green or blue color in LAMVAB agar plates [6].

5. Conclusion

Lactic acid bacteria being fastidious in nature require specific isolation procedures. Presence of such bacteria in freshwater environment is normally less. Further, presence of these bacteria in fish intestine is reportedly small and often masked by the presence of other bacteria during the process of isolation. Hence, specific modifications over traditional methods are required to enhance its probability of isolation from such complex environmental samples. The double enrichment cum selective isolation based protocol using LAMVAB media was found to be quite reliable for successful recovery and isolation of LAB from intestines of freshwater fish. It allows growth and isolation of all intestinal LAB and suppresses all other bacteria and yeasts encountered in the intestinal samples.

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7. References

1 Denev S, Staykov Y, Moutafchieva R, Beev G. Microbial ecology of the gastrointestinal tract of fish and the potential application of probiotics and prebiotics in finfish aquaculture. International Aquatic Research 2009; 1:1-29.
2 Jankauskiene R. Defence mechanisms in fish: frequency of the genus Lactobacillus bacteria in the intestinal tract microflora of carps. Biologija, 2002; 2:3-17.
3 Talpur AD, Memon AJ, Khan MI, Ikhwanuddin M, Daniel Danish MM, Abol-Munafi AB. Inhibition of pathogens by lactic acid bacteria and application as water additive multi isolates in early stages larviculture of P. pelagicus (Linnaeus, 1758). The Journal of Animal and Plant Sciences. 2012; 22(1):54-64.
4 Bucio A, Hartemink R, Schrama JW, Verreth J, Rombouts FM. Presence of lactobacilli in the intestinal content of freshwater fish from a river and from a farm with a recirculation system. Food Microbiology, 2006; 23(5):476-482.
5 Salminen S, Wright A. Lactic Acid Bacteria: Microbiological and Functional Aspects. Third Edition Boca Raton FL: CRC Press, 2004.
6 Hartemink R, Domenech VR, Rombouts FM. LAMVAB-a new selective medium for the isolation of lactobacilli from faeces. Journal of Microbiological Methods. 1997; 29(2):77-84.
7 Nelson GM, George SE. Comparison of media for selection and enumeration of mouse fecal flora populations. Journal of Microbiological Methods. 1995; 22(3):293-300.
8 Hartemink R, Rombouts FM. Comparison of media for the detection of bifidobacteria, lactobacilli and total anaerobes from faecal samples. Journal of Microbiological Methods. 1999; 36(3):181-192.
9 Ricciardi A, Parente E, Piraino P, Paraggio M, Romano P. Phenotypic characterization of lactic acid bacteria from sourdoughs for Altamura bread produced in Apulia (Southern Italy). International Journal of Food Microbiology. 2005; 98(1):63-72.
10 Kandler O, Weiss N. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG. (Ed.), Bergey's Manual of Systematic Bacteriology. 1986, 2, Bathimore: Williams and Wilkins, 1994, 1209-1234.
11 Hammes WP, Weiss N, Holzapfel WH. The genera Lactobacillus and Carnobacterium. In: Balows A, Truper HG, Dworkn M, Harder W, Schleifer KH. (Ed.), Prokaryotes, a Handbook for the biology of bacteria: Ecophysiology, Isolation, Identification, Application, Springer-Verlag, New York, 1992; 2:1508-1534.
12 Boukhemis M, Djeghri Hocine B, Tahar A, Amrane A. Phenotypic characterization of Lactobacillus strains

isolated from different biotopes. African Journal of Biotechnology. 2009; 8(19):5011-5020.
13 Khedid K, Faid M, Mokhtari A, Soulaymani A, Zinedine A. Characterization of lactic acid bacteria isolated from the one humped camel milk produced in Morocco. Microbiology Research, 2009; 164(1):81-91.
14 Huidrom S, Deka M, Deblakshmi R. Identification of lactic acid bacteria from the faeces of infant. Recent Research in Science and Technology, 2012; 4(6):54-56.
15 Yu J, Gao W, Qing M, Sun Z, Wang W, Liu W *et al*. Identification and characterization of lactic acid bacteria isolated from traditional pickles in Sichuan, China. Journal of General and Applied Microbiology. 2012; 58(3):163-172.
16 Audisio MC, Torresa MJ, Sabate DC, Ibargurena C, Apella MC. Properties of different lactic acid bacteria isolated from Apis mellifera L. bee-gut. Microbiological Research, 2011; 166(1):1-13.
17 Rodriguez Palacios R, Staempfli HR, Duffield T, Weese JS. Isolation of bovine intestinal *Lactobacillus plantarum* and Pediococcus acidilactici with inhibitory activity against Escherichia coli O157 and F52009. Journal of Applied Microbiology. 2008; 106(2):393-401.
18 Niazi Amraii H, Abtahi H, Jafari P, Mohajerani HR, Fakhroleslam MR, Akbari N. *In Vitro* study of potentially probiotic lactic Acid Bacteria strains isolated from traditional dairy products. Jundishapur Journal of Microbiology. 2014; 7(6):e10168.
19 Mahasneh AM, Hamdan S, Mahasneh SA. Probiotic properties of Lactobacillus species isolated from local traditional fermented products. Jordan Journal of Biological Sciences. 2015; 8(2):81-87.
20 Swenson JM, Facklam RR, Thornsberry C. Antimicrobial susceptibility of vancomycin-resistant Leuconostoc, Pediococcus, and Lactobacillus species. Antimicrobial Agents and Chemotherapy, 1990; 34(4):543-549.