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Gut Associated Lactic Acid Bacteria Isolated from the Estuarine Fish *Mugil cephalus*: Molecular Diversity and Antibacterial Activities against Pathogens

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Abstract In the present study we address the issue on gut associated lactic acid bacteria (LAB) isolated from the intestine of estuarine fish *Mugil cephalus* using de Man Rogossa and Sharpe (MRS) agar. LAB isolates were identified biochemically and screened for their ability to inhibit *in vitro* growth of various fish, shrimp and human pathogens. Most of the LAB isolates displayed an improved antagonism against fish pathogens compared to shrimp and human pathogens. Selected representative strains displaying high antibacterial activity were identified using 16S rRNA gene sequence analysis. Of the selected strains *Lactobacillus brevis* was the most predominant. Four other species of *Lactobacillus*, *Enterobacter hormaechei* and *Enterobacter ludwigii* were also identified. It was also observed that even among same species, considerable diversity with respect to substrate utilization persisted. Considering the euryhaline nature of grey mullet (*Mugil cephalus)*, the LAB isolated from the gut possessed good tolerance to varying salt concentrations. This finding merits further investigation to evaluate whether the isolated LAB could be used as probiotics in various fresh and sea water aquaculture.

Keywords Probiotics; Antibiotic resistance; Lactobacilli; Molecular diversity

Introduction

During the last decade commercial aquaculture has extensively increased and the usage of antibiotics has also increased manifold (Saleh, 2006). At high bacterial population densities, as is reported in aquaculture ponds – genetic exchange leading to the rise of resistant organism can occur through plasmids (Molina-Aja et al., 2002) or by viral transduction or even by direct transformation through DNA absorbed onto sediment particles (Moriarty, 1997).

Lactobacilli and other bacterial genera have been suggested to be used as probiotics as they have shown potential to inhibit pathogen colonization in the gut through a number of ways such as competitive exclusion (Gomez-Gill et al., 2000; RingØ et al., 2005; Balcazar et al., 2006; Gomez & Balcázar, 2008), production of organic acids (Atrih et al., 2001) and low molecular weight compounds such as reuterin (Vandenbergh, 1993). These organisms are environment friendly and are thus sustainable in the long run. The use of probiotics in aquaculture started in the 90's and numerous studies have been reported since then (Austin et al., 1995; Gildberg & Mikkelsen, 1998; RingØ & Vadstein, 1998; Gatesoupe, 1994; Nikoskelainen et al., 2001; Panigrahi et al., 2004; RingØ et al., 2005). It is well known that lactic acid bacteria (LAB) produce acids and di acetyl (Messens & De Vuyst, 2002), antifungal compounds (Corsetti et al., 1998), phenyl lactic acid (Lavermicocca et al., 2000) and bacteriocins to inhibit the *in vitro* colonization of pathogens (De Vugst & Vandamme, 1994; Gatesoupe, 1999; RingØ et al., 2005; Gatesoupe, 2007; Wang et al., 2008). Several studies on the gut microbiota of fish from different habitats (Cahill, 1990; RingØ et al., 1995; Hansen & Olafsen, 1999; RingØ & Birkbeck, 1999; Saha et al., 2006; RingØ et al., 2010) have revealed establishment of LAB in the gut though they are not dominant forms among the gut microflora (RingØ & Gatesoupe, 1998; RingØ et al., 2004; Lauzon & RingØ, 2011).

To our knowledge there is limited available information about the presence of LAB in the

digestive tract of flathead grey mullet (*Mugil cephalus*) (Lin et al., 2013), and therefore the present study addressed to evaluate the diversity of LAB in the gut of this fish and further addressed the antimicrobial activity of LAB against 29 fish, shrimp and human pathogens. The flathead grey mullet is a typical estuarine fish found in coastal waters in the tropics and subtropics (Saleh, 2006). The adult fish normally feed on algae and attains a size of 30 to 75 cm. Considering the nature of flat head grey mullet, it is presumed that its gut microbiota also can tolerate wide fluctuations in salinity. The ability of gut microbiota to tolerate a wide range in salinity might be a useful feature to be included in future evaluations of LAB strains with probiotic potential to combat important aquaculture pathogens under varied culture conditions; from fresh to sea water.

1 Materials and methods

1.1 Isolation and screening for LAB from fish gut

Flat head grey mullet were procured from the landing centre at Fort Kochi, Kerala and 85 individuals were collected. The weight of the fish ranged from 34.2 – 140.0g, with average weight of 60g. The fishes were packed in ice boxes and transported to the laboratory within 2 hours for isolation of gut bacteria. The fish surfaces were washed in running tap water, weighed and aseptically eviscerated. Gut samples were surface washed with sterile physiological saline to remove extraneous matter. The weight of the gut ranged from 1.34 to 7.08g. Depending on the weight, the gut samples (with digesta) were mixed with 80-110 ml of sterile saline solution and were homogenized in a Masticator (IUL Instruments, Barcelona, Spain) for about 3-5 minutes, until the gut tissues appeared visibly macerated.

Homogenized gut tissues were transferred into 1% peptone broth containing 0.5% NaCl and were kept for enrichment for 24 h (Lantz et al., 1998). The enriched broth media were serially diluted to 10^{-1} and 10^{-2} dilutions and were plated onto de Man Rogosa and Sharpe (MRS) agar (MV 641, Hi Media, Mumbai) and incubated at 31-32℃ for 2-3 days. White colored, well separated colonies of 2-3 mm diameter with round margin were obtained suspended within the agar mass. Colonies were picked and transferred into MRS broth (MV 369, Hi Media, Mumbai) incubated at 31-32℃ for 2-3 days and streaked on MRS agar slants until purity for storage at room temperature of 31-32℃. Further analysis was carried out from the stored cultures.

1.2 Physical and biochemical examination of the gut isolates

Two hundred and forty three gut isolates were isolated and these were tested for colony and cell morphologies, cell grouping - cluster or chain formation, Gram-staining, spore, catalase production and pigmentation. Gut isolates suspected of belonging to lactic acid bacteria (LAB) were inoculated into MRS broth and incubated at different temperature points over varied incubation period. Growth was enumerated by measuring optical density at 600nm using a spectrophotometer (Systronics, India) after incubation at different temperatures (4 and 10℃ for 12 days and at 15, 37 and 45℃ for 5 days). Growth at 4, 6, 8 and 10% NaCl was observed after incubation at 30℃ for 5 days. Growth was measured at 600 nm. Any detectable turbidity more than 0.5 by the spectrophotometric analysis was considered as positive growth. The O.D values ranging between 0.5 and 1.0 was categorized as "++" whereas O.D values more than 1.0 were categorized as "+++". All isolates were checked for their ability to produce acid in purple carbohydrate fermentation broth base (Peptone - 10 gL^{-1}, NaCl - 5 gL^{-1} and Bromo Cresol Purple- 0.02 gL^{-1}) containing the various sugars (1% w/v) such as L-arabinose, sucrose, mannose, melibiose, lactose, aesculin, cellobiose, dextrose, rhamnose, maltose, galactose, xylose, sorbitol, salicin, fructose, mannitol, raffinose, trehalose, amygdalin, inositol, glycerol and ribose as described by Abegaz (2007).

1.3 Antibiogram of the gut isolates

Twenty nine LAB isolates were screened for the presence of antibacterial activity by using the disk diffusion method of Kirby-Bauer (1966) and the agar well diffusion method described by Perez et al. (1990). Sterile culture broth of LAB grown in MRS broth was used to test the antagonism against the target bacteria. The test organisms were inoculated into MRS broth and incubated at 37℃ for 3 days.

Before performing the assay, the broth was shaken well to get a uniform solution of the broth culture. Antibacterial activities of 29 LAB isolated were checked against several pathogens isolated from fish (4), shrimp (5) and human (20). The tested pathogens consisted of: *Aeromonas hydrophila*, *Vibrio harveyi*, *Pseudomonas aeruginosa*, *Vibrio metschnikovii*, *Vibrio coralliilyticus*, *Vibrio fluvialis*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio mimicus*, *Vibrio hollisae*, *Bacillus cereus*, *Campylobacter jejuni*, *Escherichia coli* and of *Salmonella*. The species of *Aeromonas* and *Pseudomonas* are part of the culture collections in our lab. The members of genus *Vibrio* were isolated from water samples from a shrimp farm at Edavanakkad of Ernakulam district (Kerala, India) and were identified in our laboratory following the protocol of Noguerola & Blanch (2008). The *Salmonella* serotypes used in the present study were provided by the WHO as part of a memorandum under the Global *Salmonella* Surveillance Programme. Inhibition zones around each LAB isolate was noted after overnight incubation at 37℃. Sensitivity of a pathogen to the LAB isolates was defined by the absence of visible growth of the test organism around the disc or the well containing the sterile culture broth. Inhibition zone less than 10 mm was considered as negative (-), 10-14 mm as moderate (+) and larger than 15 mm as good (++).

1.4 Molecular identification and construction of the phylogenic tree

The 14 LAB isolates displaying highest antibacterial activity against the tested pathogens was screened by the 16S rRNA genes as described by (RingØ et al., 2006). All sequences were analyzed and edited in BioEdit and blasted against the sequences available in GenBank. Searches for sequence similarity were performed in GenBank data library with the help of BLAST programme. The sequences were then imported into BioEdit Sequence Alignment Editor, where in CLUSTAL W Multiple Alignment (Hitachi Software Engineering Co.) was used to assemble and align them. Phylogenetic tree was constructed by using the Neighbor- Joining method, the Bootstrap value being 1000.

1.5 Nucleotide sequence accession numbers

The nucleotide sequences for the 16S rRNA analyses described in this report have been submitted to the GenBank under the accession no. KJ156315 (LAB 155), KJ156316 (LAB 156), KJ156317 (LAB 158), KJ156318 (LAB 159), KJ156319 (LAB 160), KJ156320 (LAB 161), KJ156321 (LAB 168), KJ156322 (LAB 176), KJ156323 (LAB 177), KJ156324 (LAB 179), KJ156325 (LAB 180), KJ156326 (LAB 229), KJ156327 (LAB 240) and KJ156328 (LAB 243). Isolate number are given in brackets.

2 Results and Discussion

2.1 Antibacterial activity

Antibacterial activity of LAB isolates from the gut of *M. cephalus* against various fish, shrimp and human pathogens is given in Tables 1 and 2. LAB is known to exert their antimicrobial property by the individual or joint production of organic acids, H_2O_2 and/or by the production of different classes of bacteriocins (Klaenhammer, 1993; Vandenbergh, 1993; RingØ et al., 2005; Tiwari et al., 2008). As untreated culture broths were used for the antibiogram in the present study, the antibacterial property cannot be attributed to any particular component. LAB are known to be more effective against the Gram-positive pathogens rather than the Gram-negatives (Stoffels et al., 1992; Abee et al., 1995; Rodriguez et al., 2005; RingØ, 2008). However, in the current study antibacterial activities were noted against the Gram-negative pathogens encountered in aquaculture such as: *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Aeromonas hydrophila* and *Pseudomonas aeruginosa*. LAB isolates tested in the present study also showed good antibacterial activity against different serovars of *Salmonella* (Table 2). In the present study it was observed that gut associated LAB displayed antibacterial activity against one or a number of Gram-negatives tested. Isolates belonging to the *Lactobacillus* genus were noticed to possess antimicrobial activity against both fish and human pathogens. The most promising gut bacteria with respect to inhibition; *Lactobacillus casei* (LAB 156) inhibited growth of 25 out of the 29 pathogenic bacteria strains tested.

Table 1 Antibiogram profile of gut LAB isolates from *M. cephalus* against selected fish and shrimp pathogens

Sl. No.	Isolate No.	Fish Pathogens				Shrimp pathogens				
		A. hydrophila	*P. aeruginosa*	*V. harveyi*	*V. parahaemolyticus*	*V. coralliilyticus*	*V. fluvialis*	*V. hollisae*	*V. mimicus*	*V. metschnikovii*
1	155	+++	+++	++	+++	++	++	-	-	-
2	156	+++	+++	++	+++	++	++	+++	-	++
3	158	+++	+++	+++	++	++	++	-	-	++
4	159	+++	++	+++	+++	++	++	-	-	++
5	160	+++	+++	+++	+++	-	-	-	-	-
6	161	+++	+++	+++	+++	++	+	-	-	++
7	168	+++	+++	+++	+++	-	-	-	-	++
8	176	++	++	+	+++	-	-	-	-	++
9	177	+++	++	++	+++	++	++	-	-	++
10	179	+++	+++	++	+++	++	++	-	-	-
11	180	+++	+++	+++	+++	++	++	-	-	+++
12	229	++	-	-	ND	++	++	++	-	++
13	240	-	++	-	ND	++	++	++	-	++
14	243	ND	ND	ND	ND	ND	ND	ND	ND	ND

Note: (-): no zone of inhibition, (+): 5mm<zone<10mm, (++): 10mm<zone<15mm, (+++): zone > 15mm, (ND): not performed

Good antibacterial activity was also noticed against various type strains of *Escherichia coli* and against the Gram-positive bacterium; *Bacillus cereus*. The antibacterial activity against several strains of *Salmonella* ranged mostly between 5-15 mm but high antibacterial activities; was noticed against *Salmonella oranienberg*, *Salmonella meleagridis* and *Campylobacter jejuni* 395; 5 out of 18, 2 out of 18 and 4 out of 18 respectively. On the other hand, moderate to good activity was observed against *V. cholerae* strains V4 (by LAB 156, 159 and 240) and V129 by strain no. LAB 156, 158 and 160. During the last two decades there have been published several studies showing that LAB inhibit growth of pathogenic *Vibrio* (Strøm, 1988; Olsson, 1995; Jöborn et al., 1997; Harzevili et al., 1998; Ringø, 2008). StrØm (1988) reported *in vitro* growth inhibition of *Vibrio* spp., *Vibrio anguillarum* (the causative agent of classical vibriosis), *Vibrio salmonicida* (the causative agent of cold water vibriosis) and *Proteus vulgaris*. In two later studies by Olsson (1995) and Jöborn et al. (1997) it was demonstrated that an uncharacterized inhibitory substances produced by *Carnobacterium inhibens* inhibited the growth of *V. anguillarum* and *Aeromonas salmonicida*; the causative agents of furunculosis. In a more recent study, RingØ (2008) tested the antibacterial activity of several LAB strains against several fish pathogens; *A. salmonicida*, *V. anguillarum*, *V. salmonicida*, *V. splendidus*, *Moritella viscosa* (winter ulcer) and *Carnobacterium maltaromalticum*. Of the shrimp pathogens tested in the present study; *Vibrio metschnikovii* and *Vibrio hollisae* displayed high degree of resistance against the LAB isolates. Generally LAB strains isolated from *M. cephalus* displayed distinctly higher antagonistic activity against the fish pathogens tested compared to the human and shrimp pathogens. This finding may be due to the fact that, LAB strains were isolated from fish gut, and we put forward the hypothesis that they are better adapted at countering fish pathogens rather than pathogens from shrimp and human sources. However, this controversial hypothesis merits further investigation.

2.2 Molecular diversity and biochemical properties

Isolates showing promising antimicrobial activity (14 No.s) were selected for the 16S rRNA analysis. *Lactobacillus brevis* (35.7%) was the most predominant (Table 3, Figure 1). It was noticed that *L. brevis* strains (LAB 158, 159, 160, 169 and 180) differed from each other in their ability to utilize carbohydrate tested. LAB 158 and 160 matched in its ability to utilize carbohydrate except esculin and LAB 169 showed positive to ribose and amygdalin. Isolate LAB 156 identified to genus *Lactobacillus*, displayed 90% similarity to *Lactobacillus casei* ATCC 334 strain.

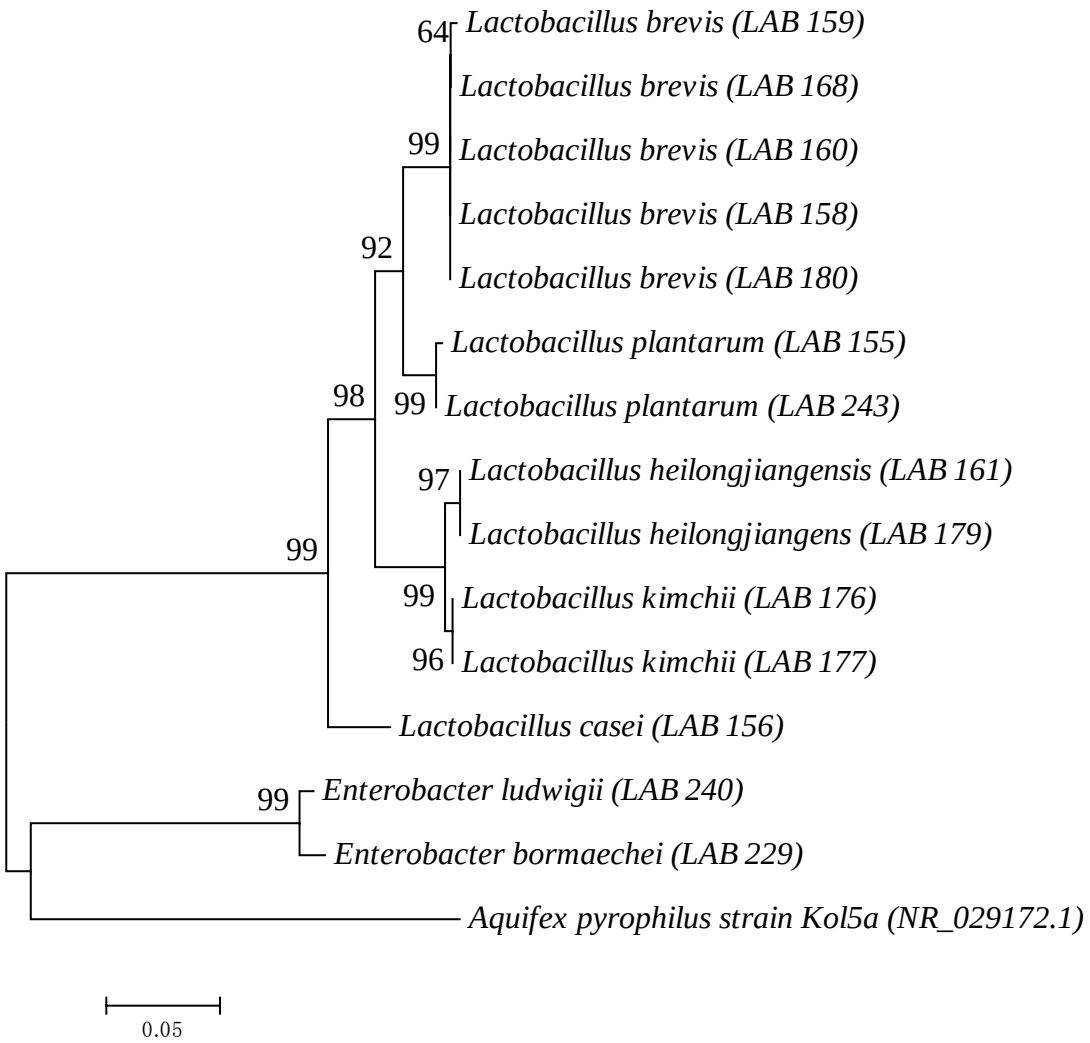

Figure 1 Phylogenetic tree of the isolates from *M. cephalus* with an out-group constructed by Neighbour Joining Method

Isolate LAB 155 and 243 were identified as *L. plantarum* and were monophyletic. These two isolates displayed a similar pattern only in utilizing aesculin, glycerol, ribose, lactose and fructose. Isolates LAB 161 and 179 was identified as *Lactobacillus heilongjiangensis* and showed 98% similarity to *Lactobacillus heilongjiangensis* strain S4-3. These isolates were monophyletic but differed with respect to utilize; lactose, trehalose, dextrose, sorbitol, salicin, aesculin, rhamnose, sucrose, inositol and glycerol (Table 3). Isolates LAB 176 and 177 were most closely related to *Lactobacillus kimchii* strain MT-1077 displayed 99% similarity. Isolate LAB 229 and 240 which were identified as *Enterobacter hormaechei* and *Enterobacter ludwigii* was monophyletic.

There are numerous examples of LAB being isolated from the gut of fresh water and marine fish (RingØ & Gatesoupe, 1998; RingØ et al., 2004; RingØ et al., 2005; Askarian et al., 2011; Lauzon & RingØ, 2011). Flat head grey mullet used in the present study is common in the Indian waters and is a widely consumed fish in India (Radhakrishnan, 2010). It survives and grows very well in estuarine waters, since these areas produce substantial quantities of organic matter thus enhancing secondary production (Nagvenkar et al., 2006). Since these estuarine waters are also a zone of active mixing of fresh and saline waters with wide variations in salinity it is assumed that the fishes thriving in these estuaries are able to tolerate wide fluctuations in salinity. The results of the present study on salt tolerance did indicate that, the gut isolates were able to tolerate variations in salt concentration (Table 4).

Table 4 Effect of temperature and different concentrations of NaCl on gut associated LAB from *M. cephalus*

Sl. No	LAB isolate	Growth at Different temperature (℃)					Growth at NaCl concentration (%)			
		4	10	15	37	45	4	6	8	10
1	155	+	+	+	+++	+	++	++	+	-
2	156	+	+	++	+	+	++	++	+	-
3	158	+	+	+++	+++	+	++	+	+	-
4	159	+	+	+	++	++	+	+	+	-
5	160	+	+	+++	++	+	++	+	+	-
6	161	+	+	+++	+++	+	++	+++	+	-
7	168	+	+	+++	+++	+	++	+	+	-
8	176	+	+	++	+++	+	++	+	+	-
9	177	+	+	+	+++	+	+++	++	+	-
10	179	+	+	+++	+++	+	+	+	+	-
11	180	+	+	++	++	++	+	+++	+	-
12	229	*	*	*	*	*	*	*	*	*
13	240	*	*	*	*	*	*	*	*	*
14	243	*	*	*	*	*	*	*	*	*

Note: (+++): O.D value more than 1.0, (++): O.D value 0.5 to 1.0, (+): O.D value less than 0.5, (-): No Growth, (*): Unviable

Isolate LAB 155, 156, 158,159, 160 and 161 were catalase-positive even though they have been identified to belong to genus *Lactobacillus*, which are generally catalase negative. However, according to the classification by Kandler & Weiss (1986) some strains are weak catalase positive. Twelve isolates out of the 14 for which 16S rRNA analysis was performed, belonged to the genus *Lactobacillus*. In a previous study Nagvenkar et al. (2006) studied the gut microbiota of *M. cephalus* and reported the presence of *Pseudomonas* sp., *Vibrio* sp., *Streptococcus* sp., *Enterobacter* sp., *Vibrio parahaemolyticus*, *Bacillus subtilis*, *Serratia marcescens* and *Proteus vulgaris*. However no LAB was isolated. In the present study, growth studies of LAB were conducted at different temperatures and at different concentrations of NaCl and displayed that the LAB isolates grew well between 15-37℃ and at 0 to 6% NaCl (Table 4). These results are in accordance with the results reported by Abegaz (2007) where all LAB grew at 10, 15 and 37℃ and in 4 and 6% NaCl.

LAB peptides like bacteriocin offer us a plausible option vis-à-vis antibiotics: they are effective in nanomolar range, are non-toxic and easily broken down by proteolytic enzymes (Ogunbanwo et al., 2003). The isolates used in the present study displayed wide antibacterial activity, smallest spectrum of inhibition being 6 out of 29. It is proposed that isolate LAB 156; *Lactobacillus casei* (GenBank accession no: KJ156316) which displayed antagonistic activity against 25 out of 29 test strains of the test pathogens might be used as a probiotics in the future; but further studies are needed. It can be concluded that, LAB isolated from the gut of *M. cephalus* provide an opportunity to develop a sustainable and organic means of combating the aquaculture pathogens. It has been demonstrated by Balcázar (2003) that a mixture of bacterial strains (*Bacillus* and *Vibrio* sp.) had a beneficial effect on the growth and survival of juveniles of white shrimp besides improving their immunity against *Vibrio harveyi* and white spot syndrome virus. It was also demonstrated that nonspecific immune system could be activated by *Clostridium butyricum* in rainbow trout fishes against vibriosis (Sakai et al., 1995). (Nikoskelainen et al., 2003) stimulated respiratory burst in rainbow trout by the administration of lactic acid bacteria *Lactobacillus rhamnosus* (strain ATCC 53103). It may not be possible to replace the usage of antibiotics altogether, but probiotic can definitely reduce the consumption of antibiotics as they can provide disease resistance to the host.

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Table 2 Antibiogram profile of gut LAB isolates from *M. cephalus* against selected human pathogens

Human pathogens

Sl. No	Isolate No	Bc	Ccm	E1	E110	E131	E135	V5	V4	V129	8.1	8.2	8.4	8.5	9.1	9.2	9.3	9.4	9.5	9.6	9.7
1	155	+++	++	+++	+++	+++	+++	++	-	++	++	++	-	-	-	-	-	-	-	++	-
2	156	+++	+++	++	+++	+++	+++	++	+++	+++	+++	++	++	++	-	-	++	++	++	++	-
3	158	+++	-	++	++	+++	+++	+++	++	+++	+++	-	++	-	-	-	-	-	-	++	-
4	159	+++	++	++	+++	++	+++	+++	+++	++	+++	++	++	-	++	-	++	++	-	++	-
5	160	+++	++	+++	++	+++	+++	++	-	+++	++	-	-	-	-	-	++	-	-	++	-
6	161	+++	+++	+++	+++	+++	++	++	-	-	+++	++	++	++	-	-	-	++	++	++	-
7	168	+++	-	++	+++	+++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	176	+++	++	-	+++	++	+	++	-	++	++	+++	++	+++	++	-	++	++	++	++	++
9	177	+++	-	+++	++	++	++	++	++	++	++	++	-	++	++	-	++	-	++	-	++
10	179	+++	-	+++	++	+++	++	++	++	++	-	-	-	-	-	-	-	-	-	-	++
11	180	+++	-	+++	++	+++	++	++	++	++	-	-	-	-	-	-	+	-	-	-	-
12	229	ND	++	ND	ND	ND	ND	++	++	ND	+	-	++	-	++	-	++	++	-	-	++
13	240	ND	++	ND	ND	ND	ND	+++	+++	ND	-	-	+++	-	-	++	++	++	-	++	++
14	243	ND	++	ND	ND	ND	ND	ND	ND	ND	-	-	++	-	-	++	++	-	-	+++	++

Note: Bc: *B. cereus*, Ccm: *C. jejuni* 395, E 1, 110, 131, 135: WHO Type strains of *E. coli*, V5, 4, 129: Strains of *V. cholerae*, 8.1: *S. oranienberg*, 8.2: *S.. thompson*, 8.4: *S. javiana*, 8.5: *S. meleagridis* 9.1: *S. enteritidis*, 9.2: *S. brandenburg*, 9.3: *S. muenster*, 9.4: *S. bredeney*, 9.5: *S. sandiego*, 9.6: *S. worthington*, 9.7: *S. albany*, (-): no zone of inhibition, (+): 5mm<zone<10mm, (++): 10mm<zone<15mm, (+++): zone > 15mm, (ND): not performed

Table 3 Carbohydrate utilization pattern and identity of gut associated LAB from *M. cephalus*

Sl. No	Isolate No	Lc	Cl	Tr	Rf	Fr	Dx	Sr	Mn	Sa	Es	Xy	Ma	Ar	Rh	Me	Sc	Ml	Ga	Ri	Am	In	Gly	Identification
1	155	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*L. plantarum*
2	156	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	*L. casei*
3	158	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	*L. brevis*
4	159	+	+	+	-	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	*L. brevis*
5	160	-	+	+	-	+	+	-	-	+	-	+	-	+	-	+	+	+	+	+	+	-	-	*L. brevis*
6	161	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*L. heilongjiangensis*
7	168	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	*L. brevis*
8	176	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	*L. kimchii*
9	177	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	*L. kimchii*
10	179	-	+	-	-	+	-	-	-	-	-	+	+	+	-	+	-	+	+	+	+	-	-	*L. heilongjiangensis*
11	180	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	*L. brevis*
12	229	+	+	N	N	+	+	+	+	N	+	N	+	+	+	+	+	-	+	+	+	N	N	*E. hormaechei*
13	240	-	-	N	N	+	+	+	+	N	-	N	-	-	-	-	+	-	-	+	-	N	N	*E. ludwigii*
14	243	+	-	N	N	+	-	-	-	N	+	N	-	-	-	-	-	-	+	+	-	N	N	*L. plantarum*

Note: Lc: Lactose, Cl: Cellobiose, Tr: Trehalose, Rf: Raffinose, Fr: fructose, Dx: Dextrose, Sr: Sorbitol, Mn: Mannitol, Sa: Salicin, Es: Esculin, Xy: Xylose, Ma: Mannose, Ar: Arabinose, Rh: Rhamnose, Me: Melibiose, Sc: Sucrose, Ml: Maltose, Ga: Galactose, Ri: Ribose, Am: Amygdalin, In: Inositol, Gly: Glycerol, (+): positive, (-): Negative, (N): Not performed