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Characterization of Lactic Acid Bacteria from the Gut of *Penaeus vannamei* as Potential Probiotic

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ABSTRACT

Background: The present objective of the study was to isolate and characterize the gut associated culturable lactic acid bacteria (beneficial bacteria) from the gut of *Penaeus vannamei* for their potential application as probiotic.

Methods: *Penaeus vannamei* (host) gut associated bacterial isolates were obtained from ten commercial brackishwater shrimp ponds (n=10) located in Kanchipuram, Chengalpattu, Tiruvallur and Villupuram districts of Tamil Nadu, during 2021-22 to test their efficiency as indigenous gut probiotic. Twenty-five shrimps from each pond, with salinity ranging from 5 to 25 ppt, were collected for isolation of beneficial bacterial isolates.

Result: Thirty lactic acid bacteria were isolated and identified from the gut of 250 *Penaeus vannamei,* using a 16S ribosomal DNA sequence. Six isolates *viz*., *Pediococcus pentosaceus* (ON495586), *Lactiplantibacillus plantarum* (ON491817), *Lactococcus lactis* (ON479264), *Enterococcus faecium* (ON478992), *Enterococcus hirae* (ON478991) and *Enterococcus durans* (ON564885) having better enzyme activity were taken and further subjected to *in vitro* analysis. It was found that these isolates had antibacterial activity against shrimp pathogens *V. campbelli, V. harveyi* and *V. parahaemolyticus* with zone of inhibition ranging between 12.33 to 21.00 mm; showed better growth at pH 7.0; tolerated the bile salts up to 1% concentration and endured salt concentrations up to 6.0%. In addition, above isolates demonstrated excellent auto-aggregative activity (74.45 to 91.14%) and hydrophobicity (77 to 99.93%). No antagonist activities were detected among the strains, suggesting its use as the multiple cocktail probiotic. Hence, the investigated isolates could serve as potential probiotics in shrimp aquaculture production systems.

Key words: Aquaculture, Lactic acid bacteria, *Penaeus vannamei*, Probiotic.

INTRODUCTION

In India, *Penaeus vannamei*, productivity was 7.52 MT/ha/year, whereas, Tamil Nadu and Pondicherry had productivity of 5.20 MT/ha/year (https://mpeda.gov.in) during 2020-21. Epidemics restrict shrimp production and antibiotic use by few farmers causes its build-up in meat, rendering them unsuitable for export. Probiotics impart a health benefit on the host (Hill *et al.,* 2014) in augmenting shrimp growth, disease prevention and improvement of the water quality in ponds. Probiotic imparts benefits via competitive exclusion of harmful bacteria, nutritional and enzymatic addition to shrimp digestion, augmentation of the shrimp immune system and antiviral activities (Ringo *et al.,* 2019). Probiotic strains obtained from individual fish host are likely to outperform those acquired from terrestrial hosts in their native habitat (Van Doan *et al.,* 2018). Hence, isolation, identification and *in vitro* screening to identify bacterial strains with probiotic characteristics could provide fresh insights on the autochthonous bacteria. Accordingly, the present study was designed to isolate probiotic bacteria from *Penaeus vannamei*, reared in brackishwater systems of Tamil Nadu.

MATERIALS AND METHODS

Penaeus vannamei gut associated beneficial bacteria were isolated from brackishwater shrimp ponds with salinity ranging from 5 to 25 ppt in Kanchipuram, Chengalpattu, Tiruvallur and Villupuram districts of Tamil Nadu, during 2021-22. The pond and farming details such as location, pond size, stocking density and shrimp size at the time of sampling are given in Table 1. The ponds were provided with commercial branded feeds such as Avanti, CP and Growel with the feed management practices suggested by the respective company. Farmers used commercial environmental probiotics as and when required during the culture period for the improvement of water and soil quality. The gut of 25 shrimp from each pond (n=10) were pooled, homogenized and serially diluted and inoculated in

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Lactobacillus MRS Agar (Himedia-M641I) supplemented with 1% $CaCO_3$ and incubated anaerobically 30°C for 48 hrs. The bacterial isolates were stored in glycerol suspensions at -80°C for further screening and characterisation (Wang *et al*., 2020).

Genomic DNA was extracted from bacterial isolates by cetyltrimethylammonium bromide (CTAB) method (Minas *et al.,* 2011) and stored -80°C. Bacterial 16S rRNA genes was amplified using universal primers 27F (5′-AGAGTTT GATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACC TTGTTACGACTT-3′) (Lane, 1991) sequenced and blasted against nucleotide database using NCBI-BLASTn program and the bacterial strains were identified. Construction of phylogenetic tree of the lactic acid bacterial isolates from shrimp gut was done based on 16S rRNA gene sequences. The Maximum Likelihood approach and the Tamura-Nei model were used to infer the evolutionary history (Tamura *et al*., 1993). MEGA11 was used to perform evolutionary analysis (Tamura *et al.,* 2021).

The gut bacterial isolates were evaluated for digestive enzyme activity *viz*., the protease (Bhowmik *et al.,* 2015), amylase, lipase and cellulase (Das *et al.,* 2014) using plate screening method. Antibacterial activity of the isolates was studied against the *Vibrio campbelli, V. harveyi* and *V. parahaemolyticus* obtained from Aquatic Animal Health and Environment Division, ICAR-CIBA, Chennai, by agar well diffusion assay (Wanna *et al.,* 2021; Kaewchomphunuch *et al.,* 2022; Rajyalakshmi *et al.,* 2021). The plates were examined for clearing zones around the wells after 24 hours of incubation at 30°C, with sterile MRS broth as negative control and tetracycline disc (TE: SD037- Hi media) as positive control. Six isolates of lactic acid bacteria (LAB) spread across four genera, which exhibited highest antibacterial and enzymatic activity were evaluated *in vitro* against varied NaCl concentrations (1,2,3,4,5 and 6%); pH (3.0,7.0 and 10.0); bile salt concentrations (0.4, 0.6, 0.8 and 1%) (Li *et al.,* 2020) and kinetics were measured using a Multimode reader (Spark 10M, TECAN) at 600 nm.

The auto aggregation test was used to detect specific cell-cell interactions. The isolates were grown in MRS broth at 30°C for 20 hours and the bacterial pellet obtained by centrifugation at 5000 G for 10 minutes, washed twice and resuspended to a final count of 10^8 CFU/ml with PBS. The auto-aggregation test was done and auto-aggregation percentage calculated (Wanna *et al.,* 2021). Hydrophobicity of the isolates was performed by growing the isolates in MRS broth at 30°C for 24 hours, centrifuged for 15 minutes at 3000 G. The resultant pellet was used for measurement cell-surface hydrophobicity (Liu *et al.,* 2020). Cross streaking assay was done by streaking **s**ingle colony of the isolates ON495586, ON491817, ON479264, ON478992, ON478991 and ON564885, incubating them anaerobically at 30°C for 24 h on the MRS agar plates and examining for inhibition zone near the contact point of the streaking lines (Kaewchomphunuch *et al.,* 2022). The statistical analysis was performed with SPSS Version 17.0 using one way ANOVA and mean comparison employing Duncan's multiple range test.

RESULTS AND DISCUSSION

Molecular characterization of the LAB from shrimp gut

Thirty LAB strains were isolated, identified, their sequences submitted to the NCBI and accession numbers were obtained. A phylogenetic tree (Fig 1) was constructed using the partial 16S rRNA sequences. In the present study, thirty isolates of LAB spread across four genera were identified from the shrimp gut. Research has shown that in shrimp, *P. pentosaceus* can improve shrimp innate immunity, physiological stability and pathogen resistance (Truc *et al.,* 2021); *L. plantarum* was used effectively as a potential probiotic in shrimp farming to improve *P. vannamei* production efficiency, immunity strength and disease resistance (Wei *et al.,* 2022). Supplementation of the bacteria *viz*., *Lactococcus lactis*, *Pediococcus pentosaceus* and *Bacillus subtilis* in white leg shrimp augmented growth, enhanced digestive enzyme function, resistance to disease,

Table 1: Pond and farming details in the study area.

Pond no.	District	Location		Pond size (ha)	Stocking density (Nos./m²)	*Water salinity (ppt)	*Size of the shrimp (Mean±SD
		Latitude	Longitude				
1	Tiruvallur	13°27′2.26″N	80°7′21.42″E	0.7	38	20	14.80±1.13
2	Tiruvallur	13°28′47.79″N	80°6′37.56″E	0.8	42	22	14.20±1.25
3	Tiruvallur	13°21′35.58″N	80°17′5.20″E	0.65	30	6	15.83±1.55
4	Tiruvallur	13°21′44.91″N	80°17′11.00″E	0.5	35	5	16.23±1.21
5	Chengalpattu	12°39′28.79″N	80°11′48.92″E	0.8	50	19	12.80±1.13
6	Chengalpattu	12°39′16.50″N	80°11′44.82″E	0.7	48	20	12.30±1.08
7	Kanchipuram	12°33′29.70″N	80°9′45.12″E	0.8	34	12	16.20±0.75
8	Kanchipuram	12°32′42.85″N	80°9′44.97″E	1.0	45	13	15.93±1.07
9	Villupuram	12°11′55.03″N	79°55′33.66″E	0.8	40	24	17.10±1.40
10	Villupuram	12°11′16.86″N	79°55′3.89″E	0.75	37	25	17.53±1.25

*Parameters at the time of sampling.

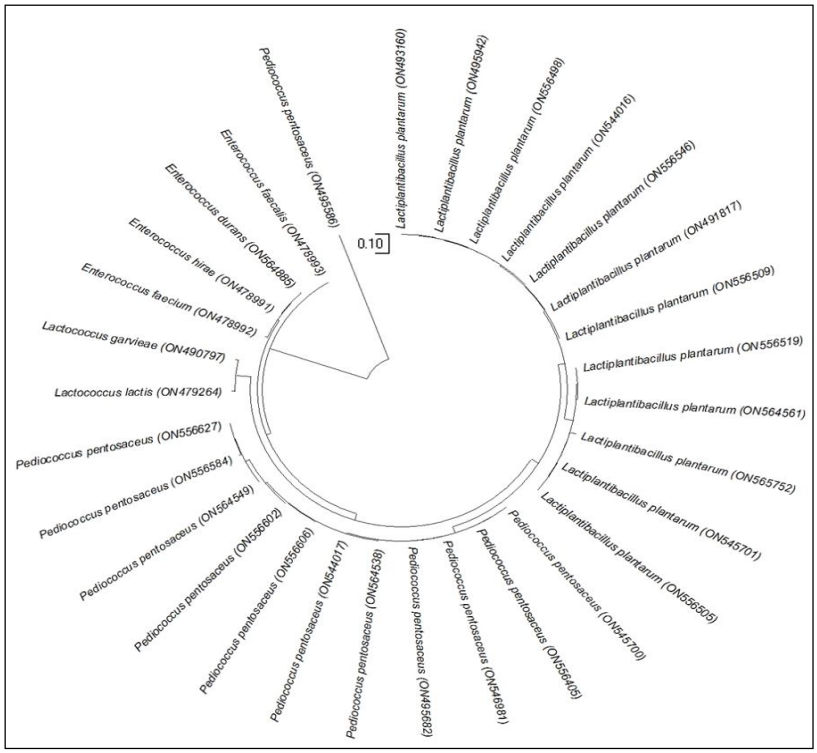


Fig 1: Construction of phylogenetic tree of the LAB from the gut of *Penaeus vannamei.*

enhanced immunity and gene expression (Won *et al.,* 2020). *Enterococcus faecium* and *Enterococcus durans* involved the production of various antimicrobial compounds against both gram positive and negative bacteria (Hanchi *et al.,* 2018). *Enterococcus hirae* isolated from the seabass intestine has the antibacterial activity against *Vibrio harveyi* (Masduki *et al.,* 2020).

Enzyme activity

Thirty isolates of LAB were screened for the enzyme activity *viz*., amylase, lipase, cellulase and protease. The growth of isolates on tributyrin agar plates revealed clearing around colony depicting better lipase activity (Fig 2-Panel A); growth of isolates on starch agar plates presented transparent halo zone surrounding the colony when flooded with Gram's iodine solution after 24 h, showing better amylase activity (Fig 2-Panel B); growth of isolates on carboxy methyl cellulose agar exhibited halo surrounding a colony and after addition of 1% Congo red, representing better cellulase activity (Fig 2-Panel C) and growth of isolates on gelatin peptone agar showed halo after flooding with 15% Mercuric chloride followed by washing with 1M NaCl, indicating better protease activity (Fig 2-Panel D). Feed supplemented with *Lactococcus lactis* Sub sp. *Lactis* isolated from shrimp gut showed enhanced cellulose, lipase, amylase and protease levels substantially (Adel *et al.,* 2017) contributed by *Lactobacillus* enzyme secretions as well as secretion from probiotic stimulated cells (Zuo *et al*., 2019). The isolates which had the better enzyme activity from each genus were further subjected to antibacterial activity, pH resistance, bile salt tolerance, NaCl tolerance, aggregation and hydrophobicity studies for further selection as candidate probiotic.

Antibacterial activity

The isolates from the gut of the *Penaeus vannamei* ON491817, ON495586, ON478992, ON479264, ON478991 and ON564885 exhibited antibacterial activity against shrimp bacterial pathogens *V. campbelli, V. harveyi* and *V. parahaemolyticus* with zone of inhibition ranging between 12.33 to 21.00 mm. Among the isolates, ON479264 had no effect on growth of *V. campbelli* and *V. harveyi*. However, the isolate, ON491817 showed excellent antibacterial activity against *V. campbelli* and *V. harveyi,* whereas the isolates ON478991 and ON564885 exhibited better antibacterial activity versus *V. parahaemolyticus* (Fig 3). Prior investigations on antibacterial activities expressed results with bacterial strains *Lactobacillus paracasei, Pediococcus acidilactici* and *Lactobacillus rhamnosus* inhibited *Vibrio alginolyticus, Vibrio harveyi, Vibrio parahaemolyticus* and *Vibrio cholera* most effectively with exhibition of zone of inhibition of 23-24 mm (Rajyalakshmi *et al.,* 2021), whereas *P. pentosaceus* against shrimp pathogens *V. harveyi* and *V. parahaemolyticus* (Wanna *et al.,* 2021). *Enterococcus hirae* isolated from intestine of Seabass found to inhibit the growth of *V. harveyi* with a zone of inhibition of 11±6 mm (Masduki *et al.,* 2020). Organic acids, bacteriocins and hydrogen peroxide are the antimicrobial metabolites produced by probiotics (Ispirli *et al.,* 2015). The cell free culture supernatant (CFCS) of *L. acidophilus* and *P. pentosaceus* found to inhibit the growth of *E. coli* strains, that may be due

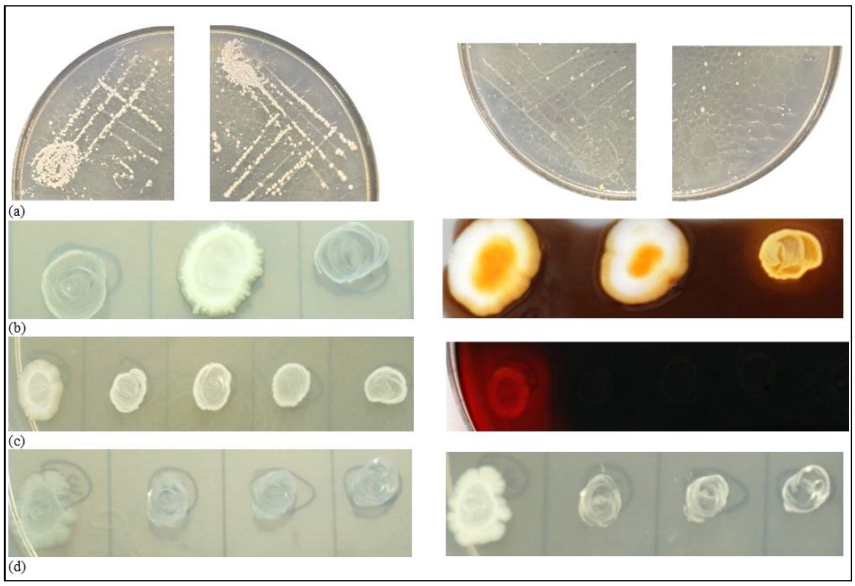


Fig 2: Enzyme activity of the LAB isolates.

Panel (a): Tributyrin agar plates revealed clearing around colony for lipase positive (Left) and negative (Right) isolates.

Panel (b): Starch agar plates inoculated with bacteria (Left). Amylase enzyme positive (Right).

Panel (c): Carboxy methyl cellulose agar inoculated with bacteria (Left) and cellulase positive colony (Right)

Panel (d): Gelatinpeptone agar inoculated with bacteria (Left). Protease positive isolates (Right).

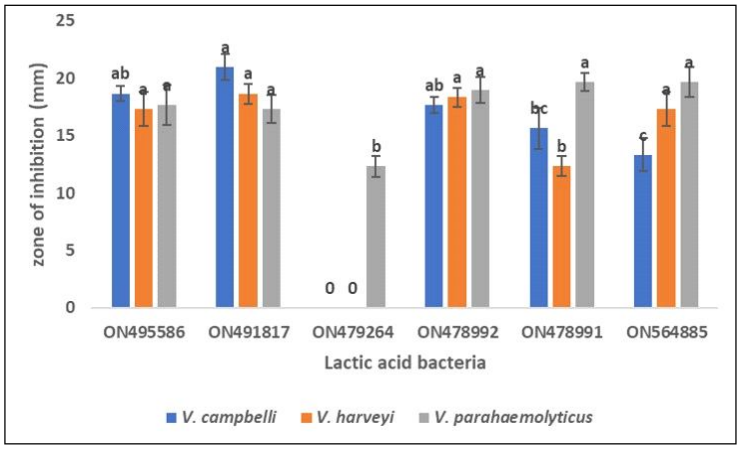


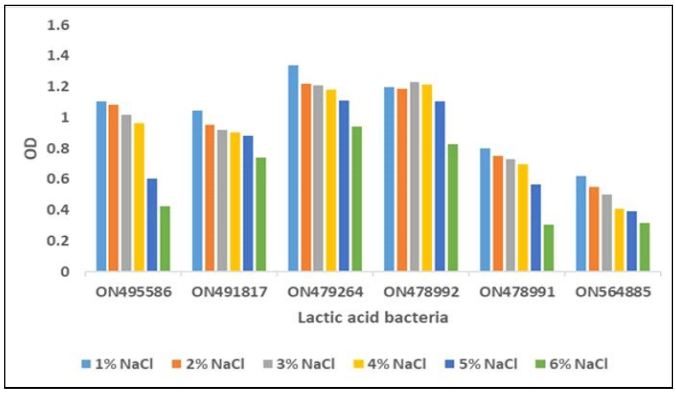
Fig 3: Antibacterial activity of LAB from the gut of *Penaeus vannamei.*

to the fact that several active constituents function together in CFCS (Kaewchomphunuch *et al.,* 2022). Furthermore, the CFCS in our investigation requires additional research to identify active compounds in order to substantiate the inhibitory activity shown against the *Vibrio* spp.

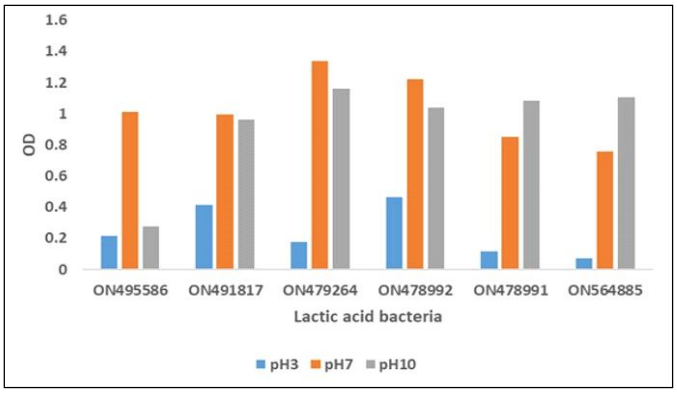
NaCl, pH and bile salt tolerance

The strains ON478991, ON564885 showed better growth at 1-2% of NaCl concentration compared to higher concentrations at 12 hours of incubation (Fig 4a). All the strains of LAB showed better growth at pH 7.0 at 12 hours of incubation. The viability was not there for all the strains at pH 3. However, except the strain ON495586, all other strains showed better tolerance to show viability even at pH of 10, showing that these bacterial strains can be used as probiotic even when there is a change in the pH to 10 (Fig 4b). It was found that the all six strains (ON478992, ON79264, ON491817, ON495586, ON478991 and ON564885) tolerated the bile salts up to 1% concentration (Fig 4c). Probiotics are exposed to a variety of environmental variables upon intake by the host and throughout passage through the gastrointestinal tract.

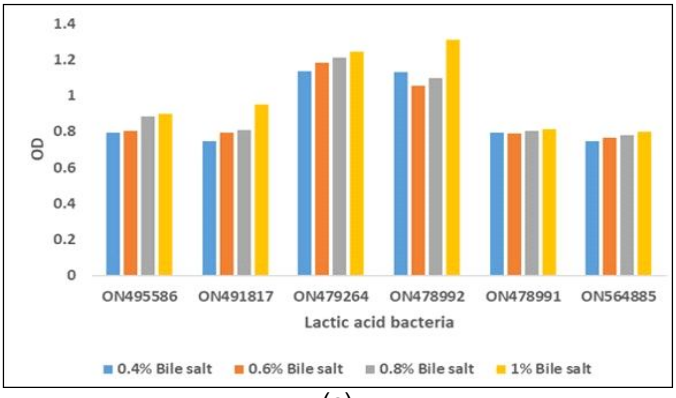
P. pentosaceus grow in a broad range of salt between 1 to 6% as well as bile salt concentrations 0.6 to 1%, further can be acclimated to acidic conditions of pH 3 (Wanna *et al*., 2021). LAB strains were particularly resistant to acid and bile salt (Li *et al.,* 2020). *Enterococcus faecium* was shown to grow at pH levels ranging from 2.0 to 4.0 for 8 hours, bile



(a)



(b)



(c)

Fig 4: a) NaCl tolerance b) pH tolerance and c) Bile salt tolerance of LAB from the gut of *Penaeus vannamei.*

contents ranging from 0.2 to 1.2% (Mao *et al.,* 2020). *Enterococcus hirae* grow at pH levels between 2 to 10, with the optimum growth occurring at pH 7, propagated up to 4% NaCl with excellent growth at 1.5% NaCl (Masduki *et al.,* 2020). Exposure of *L. plantarum* tolerated NaCl concentrations up to 6% (Wang *et al.,* 2018). In our study, the LAB strains grow showed better growth at pH 7.0; tolerated the bile salts up to 1% concentration and salt concentrations ranging up to 6.0% and hence its selection as candidate probiotic.

Aggregation, hydrophobicity and cross streaking assay

The probiotic bacteria must have the auto-aggregation property to build a barrier and inhibit unwanted microorganisms from attaching (Saito *et al.,* 2019). All of the isolates (ON491817, ON495586, ON478992, ON479264, ON478991 and ON564885) examined in this investigation showed auto-aggregation percentage of 74.45 to 91.14% at 24 hrs. Thus, the bacterial isolates could serve as the promising gut probiotic for usage in shellfish culture (Fig 5). Similar auto aggregation activity of bacterial strains was seen in; *Pediococcus pentosaceus,* 40.40 to 75.00% (Wanna *et al.,* 2021); *Lactobacillus*, 39.58 to 56.37% (Liu *et al.,* 2020).

In the current investigation, LAB isolates from the gut of shrimp demonstrated excellent hydrophobicity ranging between 77 to 99.93%, showing better ability to adhere onto

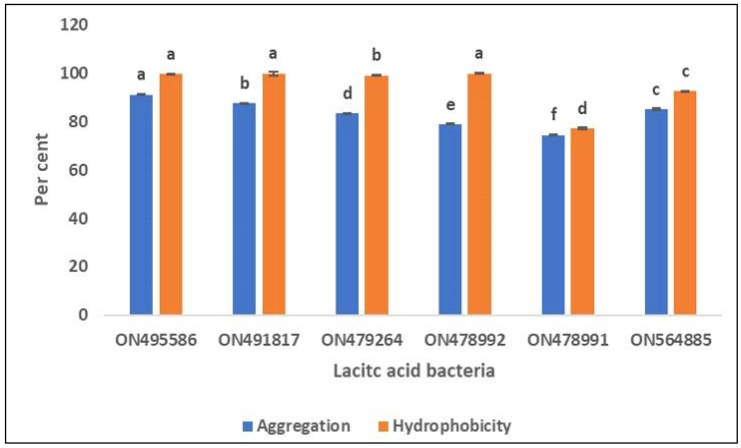


Fig 5: Aggregation (24 hrs) and Hydrophobicity of the LAB from the gut of *Penaeus vannamei.*

intestinal epithelial cells (Ortiz *et al.,* 2015) and hence as candidate gut probiotic (Fig 5). The isolates ON495586, ON491817, ON479264, ON478992, ON478991 and ON564885 showed no inhibition zone near the contact point of the streaking lines, showing no antagonist activities among all LAB strains, similar to observations of Kaewchomphunuch *et al.,* 2022. Hence, these strains could be used in the multiple strain probiotic preparation, (Puvaneswari *et al.,* 2021).

CONCLUSION

The screening, characterization and probiotic evaluation of host endogenous probiotic strains from *P. vannamei*'s gut provided six candidate probiotic strains showing better enzyme activities, exhibited anti-bacterial activity, withstanding pH change, better growth in presence of bile salt and tolerant to varying NaCl concentrations. Further, these isolates possessed aggregation and hydrophobicity properties with no antagonism between isolates. Thus, these isolates have the required probiotic properties for use as single as well as multiple strain probiotic for brackishwater shrimp farming. Further *in vivo* studies on evaluation of these candidate probiotic strains in *P. vannamei* is being carried out for its development as the probiotic with functional feed in shrimp aquaculture.

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Conflict of interest: None.

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