



***In vitro* probiotic properties of *Bacillus* sp isolated from biofloc reared genetically improved farmed tilapia (*Oreochromis niloticus*)**

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ABSTRACT

A study was conducted to investigate the probiotic potential of the bacterial species from biofloc reared genetically improved farmed tilapia (GIFT) using *in vitro* quantitative assays. Based on the morphological, biochemical and 16S rRNA sequencing analysis, isolated bacterial species from GIFT gut were identified as *Bacillus infantis*, *Exiguobacterium profundum*, *Bacillus subtilis* and *Bacillus megaterium*. The *in vitro* probiotic properties such as bile salt hydrolase activity, bile tolerance, growth at different bile concentrations, antibiotic susceptibility test, antimicrobial activity, auto aggregation test, bacterial adhesion to hydrocarbons and resistance to gastric acidity were performed. All the isolates showed higher resistance to bile tolerance test and growth of cultures was observed from 0.5 to 8% bile salt concentrations. The distinct zone of hydrolysis was marked in the tested isolates in bile salt hydrolysis activity. Except *B. infantis*, all the other three isolates were predominantly resistant to the tested antibiotics. Antimicrobial activity against three pathogens, viz. *Vibrio parahaemolyticus*, *Vibrio harveyi* and *Aeromonas hydrophila* was observed in all the isolates. *E. profundum* and *B. subtilis* showed improved auto aggregation. Enhanced resistance to bile salt adhesion to hydrocarbon and *in vitro* gastric acidity (pH 3) was seen in *B. megaterium*. It is one of such unique studies confirming the probiotic effect of *Bacillus* sp. isolated mainly from GIFT biofloc culture. *B. subtilis* and *B. megaterium* exhibited remarkable *in vitro* probiotic properties and thus can be recommended as a successful probiotic strain for fish farming.

Key words: Antimicrobial activity, Biofloc, GIFT, *In vitro* properties, Probiotics

Aquaculture is one of the major food producing sector to meet the nutritional demand of overgrowing population with its global fish supply. The estimated production in 2050 is 82 million tonnes from the present level of production (FAO 2016). Tilapia is one of the widely cultured fishes in the world due to its positive culture traits as it can adapt to a wide array of different aquaculture systems (FAO 2016). The intrinsic feature of tilapia includes their disease resistance, adaptability to production systems, capacity to support large aquatic conditions and omnivorous feeding behaviour in the lower trophic level (Watanabe *et al.* 2002). However, the main challenge behind the development of a sustainable aquaculture industry lies with the availability of constrained natural resources and also with its negative impacts to the environment due to poor management practices (Costa-Pierce *et al.* 2012, Verdegem 2013). A healthy way to improve production and productivity of aquaculture can be achieved through intensification of

aquaculture systems and practices with limited discharge. Though tilapia possess potential cultivable traits for aquaculture, poorest growth performance was found in the intensification of the production and increased disease outbreaks (Telli *et al.* 2014). Among the advanced culture technologies for intensification, biofloc technology promises to solve the above mentioned problems for enhanced fish production with limited water exchange (Hargreaves 2013). Felix *et al.* (2015) reported that GIFT reared in biofloc driven systems had significantly higher weight gain and better FCR.

Multiple benefits of biofloc in sustainable disease management practices for protecting the culture animal with proper biosecurity measures was compared with the traditional aquaculture practices such as use of probiotics, prebiotics and antibiotics (Sinha *et al.* 2008).

The heterotrophic bacterial dominance established by maintaining C/N ratio in the water with the interventions in using different external carbohydrate sources or by using low protein feeds helps the bacteria to assimilate the ammonia for converting to microbial biomass (Avnimelech 1999). The heterotrophic bacteria in biofloc (Halet *et al.* 2007) produce some natural substances (Dinh *et al.* 2010, Iyapparaj *et al.* 2013) and suppress the growth of other

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pathogenic species like *Vibrio harveyi* (Defoirdt *et al.* 2007). The main objective of the present study was to isolate, identify and characterize the various probiotic properties of isolates derived from the biofloc driven GIFT fish gut.

MATERIALS AND METHODS

Isolation and characterisation of dominant bacteria: GIFT fishes reared in biofloc ponds (220±4.8 g) were collected and examined for infection using external morphological analysis (Johansen *et al.* 2006). Sampled fishes were killed and dissected under sterile environment. Homogenates of intestinal tracts (oesophagus to rectum) were prepared and preserved in physiological solution (0.85% NaCl). Three pathogenic bacteria, viz. *Vibrio parahaemolyticus* ATCC 17803, *Vibrio harveyi* ATCC-BAA-2752 and *Aeromonas hydrophila* ATCC 35654 were obtained from American Type Culture Collections (ATCC, Chromachemie Laboratory Pvt Ltd, Karnataka, India) and used as target pathogen. All strains were preserved at -20°C in growth medium containing 20% glycerol to maintain a viable inoculum throughout the study. The intestinal homogenate was serially diluted and 10⁻², 10⁻⁴, 10⁻⁶ and 10⁻⁸ were plated on de Man-Rogosa-Sharpe (MRS) agar as per standard microbiological methods. The plates were incubated for 24 h at 37°C, morphologically different colonies were picked and further streaked to isolate pure colonies. The four different isolates obtained were characterised morphologically and biochemically as per the method of Bergey's Manual of Determinative Bacteriology.

DNA isolation, PCR amplification and phylogenetic tree construction: The DNA was isolated from 16 h old culture using phenol-chloroform method. The isolated DNA was amplified for its 16s rRNA region using the Forward primer (27F-5'AGA GTT TGA TCM TGG CTC AG 3') and Reverse primer (1492R-5' CGG TTA CCT TGT TAC GAC TT 3'). The amplified DNA was purified using QIA quick PCR purification kit (Qiagen), and sequenced using Sanger's method. The aligned sequences were submitted to the GenBank database using the BLAST program of NCBI (<http://www.ncbi.nlm.nih.gov>) and accession numbers were obtained. The phylogenetic tree was constructed to find the closely related isolates using Mega 6.0 software.

Bile tolerance: MRS supplemented with 0.3% (w/v) bile as well as without bile (control) was inoculated with four bacterial isolates (incubated for 16±2 h at 37°C). The growth of four isolates in bile was measured at 600 nm using spectrophotometer. Time to attain the exponential phase at 0.3% bile concentration was measured to find bile tolerance and the sensitivity (Chateau *et al.* 1994).

Growth at different bile concentrations: Different concentration of Ox-bile [0.5, 1.0, 2.0, 4.0 and 8.0% (w/v)] were added to the MRS broth and inoculated with freshly grown (incubated for 16±2 h at 37°C) bacterial cultures. The growth of the test cultures was examined by reading the optical density (OD) at 650 nm after incubating for 24 h at 37°C. MRS broth without Ox-bile was used as

negative control (Nithya and Halami 2013).

Bile salt hydrolase activity: Four isolates were grown overnight in MRS broth and spotted using sterile cotton buds on to MRS agar plates containing 0.5% (w/v) ox-bile and 0.37 g/l CaCl₂. Plates were incubated for 72 h at 37°C and observed for the appearance of precipitation zones as per Sieladie *et al.* (2011).

Antibiotic susceptibility test: Antibiotic susceptibility of isolated bacteria was performed by disc diffusion method (CLSI 2016). Ampicillin (10 mcg), amoxicillin (10 mcg), cefixime (10 mcg), cephalixin (10 mcg), chloramphenicol (25 mcg), ciprofloxacin (10 mcg), erythromycin (15 mcg), tetracycline (25 mcg), levofloxacin (10 mcg), norfloxacin (10 mcg) discs were used. Freshly grown culture (16±2 h) were suspended to attain a density according to McFarland 0.5 turbidity standard and diluted to 1:100. The diluted suspension were streaked on Muller Hinton agar plates and incubated at 37°C for 24 h. The diameters of inhibition zones were measured and results were recorded as sensitive (S) and resistance (R).

Antimicrobial activity: Three pathogenic strains, namely *Vibrio harveyi*, *Vibrio parahaemolyticus* and *Aeromonas hydrophila* were grown in 5 ml sterile nutrient broth for 3 h. The freshly grown bacterial isolates were spread on to nutrient agar plates using sterile cotton buds. Wells were created using gel puncture and 30 µl of the selected pathogens were seeded in the respective wells. For the diffusion of culture, the seeded plates were kept in a refrigerator for 20 min and incubated at 37°C for 48 h. After incubation, plates were observed for presence or absence of inhibition zones as per Tambekar *et al.* (2010).

Auto aggregation test: Auto aggregation ability of the bacterial isolates were determined as per the method described by Canzi *et al.* (2005). Pure bacterial culture (10 ml) was kept in static condition for incubation (3 h at 15°C). After incubation, 1 ml of the broth was transferred to another test tube from the upper suspension after incubation. The transferred suspension OD was measured at 600 nm. Auto aggregation was calculated by using the equation

$$\text{Auto aggregation in \%} = 1 - \left[\frac{\text{OD value of upper suspension}}{\text{OD value of total culture}} \right] \times 100$$

Bacterial adhesion to hydrocarbons (BATH): Adherence ability of the isolates to xylene as a hydrocarbon was performed as per Canzi *et al.* (2005). The freshly grown culture (16±2 h) was centrifuged to harvest the cell pellet for 15 min at 10,000 g at 4°C. The cell pellet was washed and resuspended in 0.1 M PBS to attain a OD value (600 nm) of 0.5. Equal amount of xylene was added to this suspension and the two phase system was vortexed for 3 min. After 1 h of incubation at 27±2°C, the aqueous phase was measured for its absorbance at 600 nm.

Adhesion percentage = [(A0-A)/A0] × 100 was used to estimate the BATH (A0 and A were absorbance at 600 nm before and after solvent extraction).

Resistance to gastric acidity: Four isolates were grown

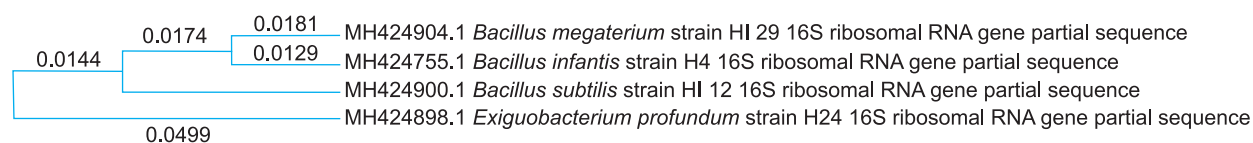


Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences

overnight in MRS broth at 37°C. An aliquot of 0.5 ml of the overnight culture was inoculated into 50 ml MRS broth adjusted to pH 3 and 7. Bacterial growth was measured for the absorbance at 620 nm after 6 and 24 h of incubation at 37°C. The surviving index of the isolates was calculated as the percent difference of optical density (OD) at pH 7.0 (ΔOD_{pH7}) and pH 3 (ΔOD_{pH3}) as per Shruthy *et al.* (2011). Following equation was used to estimate the resistance

$$\text{Surviving (\%)} = \Delta OD_{pH7} - \Delta OD_{pH3} / \Delta OD_{pH7} \times 100.$$

RESULTS AND DISCUSSION

Biochemical characterization of isolated colonies: The isolated bacterial species from biofloc reared GIFT gut were identified as gram positive rods (Table 1).

Table 1. Biochemical characterization of *Bacillus* sp.

Biochemical characteristic	<i>Bacillus infantis</i>	<i>Exiguobacterium profundum</i>	<i>Bacillus subtilis</i>	<i>Bacillus megaterium</i>
Indole	-ve	-ve	-ve	-ve
Methyl red	-ve	-ve	-ve	-ve
Voges Proskauer's	+ve	-ve	+ve	-ve
Citrate utilization	+ve	+ve	+ve	+ve
Glucose	+ve	+ve	+ve	+ve
Adonitol	-ve	+ve	+ve	-ve
Arabinose	-ve	+ve	+ve	-ve
Lactose	+ve	+ve	+ve	-ve
Sorbitol	-ve	+ve	+ve	-ve
Mannitol	+ve	+ve	+ve	-ve
Rhamnose	+ve	+ve	+ve	-ve
Sucrose	+ve	+ve	+ve	-ve

DNA isolation, PCR amplification and phylogenetic tree construction: Based on 16S rRNA gene sequence analysis the isolates, HI12 (568 bp), HI29 (577 bp), H24 (1001 bp) and H4 (577 bp) were found to be *Bacillus subtilis* MH424900, *Bacillus megaterium* MH424904, *Exiguobacterium profundum* MH424898 and *Bacillus infantis* MH424755. The phylogenetic tree is shown in Fig. 1.

Bile tolerance: The bile tolerant concentration in the selection of probiotic species for humans and fishes is 0.3% (w/v) (Brashears *et al.* 2003). The three isolates, viz. *B. infantis*, *B. subtilis* and *B. megaterium* showed a delay in growth below 15 min and thus categorized as bile resistant. However, *Exiguobacterium profundum* recorded 60 min delay in growth and grouped under weakly tolerant. *Bacillus* species tested in the present study were categorized in both 'resistant' and 'weakly tolerant' groups (Table 2). All the four isolates showed no effect to the bile tolerance though *Exiguobacterium profundum* bacteria exhibited delayed

Table 2. Bile tolerance of *Bacillus* cultures

Isolate	Delay in growth (min)	Tolerance
<i>Bacillus infantis</i>	10	Resistant
<i>Bacillus subtilis</i>	15	Resistant
<i>Exiguobacterium profundum</i>	60	Weakly tolerant
<i>Bacillus megaterium</i>	15	Resistant

growth for 1 h which did not impact its probiotic potential. The findings of the current study confirmed that these isolates can be used as gut probiotics to thrive in fish intestine.

Growth at different bile concentrations: The cultures revealed difference in growth at tested concentrations of Ox-bile (0.5–8% w/v) (Fig. 2). The bacterial culture expressed growth from 0.5 to 8% of bile concentrations, however decrease in the growth of bacteria with increase in bile concentration was recorded. The findings of the present study were similar to that of Nithya and Halami (2013). The *Exiguobacterium profundum* exhibited more stable growth with high bile tolerant potency when compared to other three bacterial species. *Bacillus subtilis* was found to be the low bile tolerant and showed poor growth.

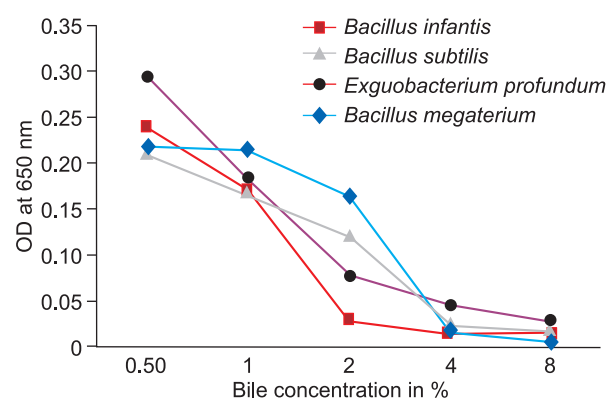


Fig. 2. Growth profile of *Bacillus* cultures in MRS broths supplemented with different concentrations of Ox-bile. The cultures were categorized according to the observed delay of growth (d) in presence of Ox-bile as resistant ($d \leq 15$ min), tolerant ($15 < d \leq 40$ min), weakly tolerant ($40 < d < 60$ min) and sensitive ($d \geq 60$ min) (Chateau *et al.* 1994).

Bile salt hydrolase activity: Out of the four isolates screened for the bile salt hydrolase activity, showed positive results with the formation of distinct zone of hydrolysis. The white precipitates around the colonies confirm enzymatic digestion ability of bile salts to primary bile salts thereby reducing the levels of serum cholesterol as suggested by Begley *et al.* (2006). Thus these bacterial

isolates can exert a probiotic effect on culture animals by reducing their serum cholesterol level.

Antibiotic susceptibility test: Out of four bacterial isolates, *Bacillus subtilis*, *Exiguobacterium profundum* and *Bacillus megaterium* showed a higher resistivity pattern. The lowest antibiotic susceptibility was found in *Bacillus infantis* with higher sensitivity to the tested antibiotics (Table 3). These results confirmed the absence of transferable antibiotic resistant genes and they can be categorized as safe probiotic strains as per the breakpoint levels of EFSA (2008). Among the four isolates, *Bacillus subtilis* showed intrinsic resistance to antibiotics and can be considered as putative probiotic strain. Apparently, *Bacillus subtilis* 2335 showed *in vitro* activity against *H. pylori* by producing antibiotic amicoumacin (Pinchuk *et al.* 2001). Thus this bacterial strain can replace antibiotic treatment effectively to fight against the pathogens (Sorokulova *et al.* 2008).

Table 3. Antibiotic susceptibility test

Antibiotic	<i>Bacillus infantis</i>	<i>Bacillus subtilis</i>	<i>Exiguobacterium profundum</i>	<i>Bacillus megaterium</i>
Ampicillin (10 mcg)	S ⁺	R	R	R
Amoxicillin (10 mcg)	S ⁺	R	R	R
Cefixime (10 mcg)	R	R	R	R
Cephalexin (10 mcg)	S ⁺	R	R	R
Chloramphenicol (25 mcg)	S ⁺	S ⁺	S ⁺	S ⁺
Ciprofloxacin (10 mcg)	S ⁺	R	S ⁺	S ⁺
Erythromycin (15 mcg)	R	R	R	R
Tetracycline (25 mcg)	S ⁺	R	S ⁺	S ⁺
Levofloxacin (10 mcg)	S ⁺	S ⁺	S ⁺	R
Norfloxacin (10 mcg)	S ⁺	R	R	R

Zone size: 0 to 5 mm, Resistant (R) and 6 to 15 mm, Sensitive (S⁺).

Antimicrobial activity: The antimicrobial activity of bacterial isolates against the infectious pathogens is one of the determining factors of probiotic potential. *B. subtilis* and *B. megaterium* showed higher antimicrobial activity against the selected pathogens (Table 4). This may be due to the spore forming ability of *Bacillus* species which in turn trigger the immune response thereby exerting a probiotic effect. The results were concurrent with the findings of Liu *et al.* (2014) and Irasema *et al.* (2011) against pathogenic vibrios and *Aeromonas hydrophila* (Aly *et al.* 2008). This might be due to the antimicrobial compound production by the *Bacillus* sp. (Morikawa *et al.* 1992, Perez *et al.* 1993, Drablos *et al.* 1999, Gullian *et al.* 2004,

Table 4. Zone of inhibition against the three pathogens

Pathogen	<i>B. infantis</i>	<i>B. subtilis</i>	<i>E. profundum</i>	<i>B. megaterium</i>
<i>Vibrio parahaemolyticus</i>	✓	✗	✓	✗
<i>Vibrio harveyi</i>	✓	✗	✗	✗
<i>Aeromonas hydrophila</i>	✓	✗	✗	✗

Chaurasia *et al.* 2005, Yilmaz *et al.* 2006).

Auto aggregation test, bacterial adhesion to hydrocarbons (BATH) and resistance to gastric acidity: Auto aggregation activity was found to be higher in *Exiguobacterium profundum* (98%) followed by *Bacillus subtilis* (96%), *B. infantis* (93%) and low aggregation was observed in *Bacillus megaterium* (43%). The highest aggregation property of three bacterial isolates (*Bacillus infantis*, *Exiguobacterium profundum* and *Bacillus subtilis*) confirmed their therapeutic properties in preventing the animals from disease outbreak (Nithya and Halami 2013).

The higher bacterial adhesion to hydrocarbon activity was 56.09% in *Bacillus infantis*, 29.89% in *Bacillus megaterium* and very low activity was expressed in *Exiguobacterium profundum* (10.41%) and *Bacillus subtilis* (15.25%) (Table 5). The culture *Bacillus infantis* exhibited better adhesion to hydrocarbons (>50%) supporting that this strain might have higher level of adherence and colonization ability. This was concurrent with the findings of Otero *et al.* (2004) suggesting that higher adhesion and colonization ability in *B. flexus* Hk1 and *B. licheniformis* Me1 strains.

Resistance to gastric acidity is one of the key indicators for the good probiotics. *Bacillus megaterium* showed good resistance against gastric acidity followed by *Exiguobacterium profundum* and *Bacillus subtilis*. Therefore, these bacteria can survive in intestine of the culture animal under acidic pH confirming its probiotic potential. The presence of similar heterogeneity within the *Bacillus* species in acidic environments has been reported by Hyronimus *et al.* (2000).

All the tested cultures survived in the high bile salt concentration and low pH which confirms its absorbance and presence in fish gut tissue. The experimented cultures showed resistance to bile salt hydrolase activity, bile

Table 5. Auto aggregation test, bacterial adhesion to hydrocarbons (BATH) and resistance to gastric acidity

Probiotic property	<i>Bacillus infantis</i>	<i>Exiguobacterium profundum</i>	<i>Bacillus subtilis</i>	<i>Bacillus megaterium</i>
Auto aggregation test (%)	93	98.86	96.6	43.46
Bacterial adhesion to hydrocarbons (%)	56.09	10.41	15.25	29.89
Resistance to gastric acidity (%)	41.66	86.59	85.7	90.39

tolerance and hydrophobicity towards hydrocarbons. The results of auto aggregation test, antibiotic susceptibility and antimicrobial activity of isolates revealed the bacterial performance in the order of *Bacillus megaterium* > *Bacillus subtilis* > *Exiguobacterium profundum* > *Bacillus infantis*. *Bacillus megaterium* and *Bacillus subtilis* showed the highest probiotic effect based on the *in vitro* properties.

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