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Original Article

Effects of autochthonous bacteria and prebiotic supplementation on the growth and survival of *Clarias batrachus*

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A B S T R A C T

Studies were conducted to identify the autochthonous bacteria from the intestines of walking catfish, *Clarias batrachus*, and to evaluate the efficacy of the gut microbes supplementation on the growth and survival of the catfish under laboratory condition. Autochthonous bacteria were isolated from the intestines of experimental fish using Nutrient agar (NA) and de Man, Rogosa, and Sharpe (MRS) agar in duplicate. Primarily, 16 gut bacteria were isolated and three isolates were selected *viz.*, *Lactobacillus* sp., *Bacillus* sp., and *Lactococcus* sp. after the phenotypic study. For biosafety evaluation of the selected bacteria, healthy *C. batrachus* fingerlings (average weight: 10.4 ± 0.8 g) were challenged with these bacteria at a dose of 10^7 CFU/mL by immersion technique for 7 days. Gut microbiota supplemented diets were prepared and analyzed for their proximate composition. A 3-week long feeding trial was conducted with ten 35 L capacity rectangular glass aquaria to evaluate the growth performance and survival of 96 *C. batrachus* fingerlings (average weight: 9.28 ± 1.5 g) fed with the above three gut microbiota supplemented diet and gut microbiota + prebiotic (molasses, yeast, and rice bran) supplemented diet. Morphometric measurements of the experimental fish and water quality parameters were determined weekly. The bio-safety evaluation revealed that the isolates were safe for *C. batrachus* with no clinical signs or mortalities during the challenge test. The study revealed better growth and survival of *C. batrachus* with gut bacteria supplemented diets but showed much better performance in the cases of all growth parameters while prebiotic was additionally supplemented. Thus, the above mentioned autochthonous gut probiotics and prebiotics could be recommended as an effective eco-friendly health management approach in *C. batrachus* culture. Further detail study is necessary to establish the fact.

Introduction

Aquaculture is currently the fastest-growing food production sector in the world. Bangladesh is one of the world's leading fish producing countries with a total production of 4.28 million MT in 2017-18, while aquaculture production contributes 56.24% of total production, which is 2.41 million MT (DoF, 2018). Presently, Bangladesh ranked 3rd in capture fisheries and 5th in aquaculture production (excluding aquatic plants) in the world (FAO, 2018). According to GAA's report, the largest producers of catfish species, China, Vietnam, Indonesia, Bangladesh, and India produced about 5 million MT in 2018 (GAA, 2019). Walking catfish (*Clarias batrachus*) and stinging catfish (*Heteropneustes fossilis*) are two well-known indigenous farmed catfish species, which have already contributed 2.33% of total inland production in 2017-18 (DoF, 2018). *C. batrachus* (locally called Magur) is one of the most familiar aquaculture species in Bangladesh, which is well known for disease resistance capability, fast growth rate, higher adaptability in adverse environmental conditions (Argungu *et al.*, 2013, Li *et al.*, 2018). High stocking density with greater production rates also make these species as an ideal cultivar for increasing aquaculture production. However, recent studies demonstrated that during natural disease outbreaks in many aqua farms, especially in winter, many diseased *C. batrachus* showed severe clinical signs including hemorrhagic and ulcerative body lesions (Patwary *et al.*, 2008). Microorganisms have been implicated in this problem and its control in aquaculture

is a challenge (Ringo, 1999), especially bacterial infections remain primary constraints to its continued expansion (Abd El-rhman *et al*., 2009; Pieters *et al*., 2008; El-Haroun *et al*., 2006). Several studies suggest that certain bacteria like *Aeromonas* spp., *Pseudomonas* spp., fungi like *Ahanomyces invadans, Saprolegnia spp*., some parasites and other factors such as environmental stress, nutritional deficiency, etc. are mainly responsible for the disease outbreak (Mishra *et al*., 2017). An unwise application of conventional antibacterial agents or the use of different toxic chemicals like malachite green, sumithion, malathion, etc. against various fish pathogens may have harmful effects on aquatic organisms or the aquatic environment as well as it can lead to severe problems such as bacterial resistance and unacceptable residual effects in aquaculture. Moreover, the residual effects and the pathogen's resistance against antibiotics can be a catastrophic threat for human health (Alam *et al*., 2011; CDC, 2013; Prestinaci *et al*., 2015).

With the growing claim for environmentally pleasant aquaculture, the application of non-antibiotic eco-friendly agents such as probiotic is being considered as one of the most significant tools for health management in the field of aquaculture. The term "probiotic" comes from Greek *pro* and *bios* which refers to "prolife" (Schrezenmeir and Vrese, 2001) and defined as living bacteria, when administrated in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001). Several studies reported that probiotics can increase appetite, improve the digestibility of nutrients and feed utilization, enhance survivability and adaptability to stress, and improve reproduction rate (Martínez Cruz *et al.*, 2012; Opiyo *et al*., 2019), and are recognized as an alternative therapy for health management instead of vaccinations and chemotherapy (Panigrahi *et al*., 2010). Although, the application of probiotics in the livestock sector is quite familiar, however, the concept of probiotics in aquaculture is a little bit newer (Tukmechi *et al*., 2007) but research on it's application is increasing due to the demand for environment-friendly aquaculture (Abdelhamid *et al*., 2009) in terms of the use of eco-friendly alternatives to the therapeutic use of antimicrobials (Merrifield *et al*., 2010). Many commercial probiotic products prepared from various bacterial species such as *Bacillus* sp., *Lactobacillus* sp., *Enterococcus* sp., *Carynebacterium* sp., and the yeast *Saccharomyces cerevisiae* among others. Besides, a consensus has already been developed that probiotic treatment using autochthonous gut microbiota might lead to better protection of fish against multiple diseases and helpful for growth improvement. Antimicrobial substances produced by bacilli isolated from the intestines of Japanese coastal fish (Sugita *et al*., 1998), and an Indian Major Carp, *Labeo rohita* (Giri *et al*., 2011) have been documented as bio-control agents. Moreover, antagonistic activities of *Pseudomonas* sp. against *Aeromonas* (Das *et al*., 2006; Giri *et al*., 2011) and *Vibrio* sp. (Vijayan *et al*., 2006) have been reported. In addition, non-digestible feed ingredients, known as prebiotics are supplemented with probiotics to boost the activities of beneficial gut probiotics (Gibson and Roberfroid, 1995). Prebiotics are provided to gut microbes to utilize it not only as the feed of gut microbes but also they modify the gastrointestinal environment, gut microflora profile, enhance feed efficiency, provide nutrients, energy to boost the immunity of host and prevent the pathogenic bacterial growth and their colonization within the gut region (Passos *et al*., 2018; Amenyogbe *et al*., 2020). The combined effect of probiotics and prebiotics may increase the non-specific immune response of fish or directly modulate the immune system that may give beneficial effects in the cases of disease resistance, health promotion, and survival of fish (Akter *et al.*, 2015). The research aims to isolate probiotics from *C. batrachus* intestine and to determine the growth performance and survival of this fish using the gut microbiota supplemented diets along with or without prebiotics.

Materials and Methods

Study Area and Duration

The experiment was conducted at Fish Disease Laboratory and Fish Nutrition Laboratory, Faculty of Fisheries, Bangladesh Agricultural University (BAU), Mymensingh-2202 from April, 2018 to July, 2018.

Isolation of Gut Microbiota

A total of 10 healthy walking catfish, *C. batrachus* (average weight 47.86 ± 3.7 g) were randomly collected from different fish markets and catfish farms located in Mymensingh region and brought to the Fish Disease Laboratory of the Department of Aquaculture, Bangladesh Agricultural University and kept them for acclimation for 24 hours. The bacterial culture media *i.e.*, Nutrient agar (NA) and de Man, Rogosa, and Sharpe (MRS) agar (Hi-media, India) were prepared according to the manufacturer's instructions. After 24 h of starvation, fish were sacrificed and the digestive tracts were collected by sterile forceps, homogenized in sterile physiological saline (0.85%) at 4ºC (Figure 1), and gut microbiota were isolated using NA and MRS agar in duplicate and incubated at 37ºC for 48 h. The well-separated mostly available colonies with distinct morphology were selected for pure culture and further analysis.

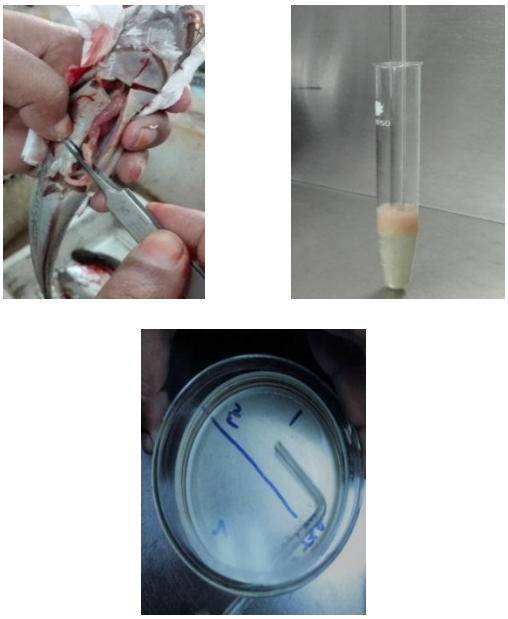

Figure 1. Collection and isolation of gut microbiota. a) Collection of Gut; b) Homogenized with physiological saline; c) Isolation in agar media.

Phenotypic Identification

The Morphological and colony characteristics were studied using MRS agar plates. The physiological characteristics of all the obtained isolates were studied. The biochemical characteristics (Indole, Catalase, Voges-Proskauer, Methyl Red, Citrate, Hydrogen sulfide production, Nitrate reduction, Gelatin hydrolysis test, Bile-Esculin) and sugar fermentation

tests were also carried out using standard reference Bergey's Manual of Systematic Bacteriology (Bergey *et al*., 2009).

Determination of Bio-safety Effects

The candidate gut bacterial flora were tested on *C. batrachus* to determine the possible harmful effects. Healthy fish weighing 10.4 ± 0.8 g were collected from a private fish farm for the bio-safety test. The fishes were acclimatized in aquaria with aeration for nine days and checked for the disease before using in the challenge test. Ten fish were immersed in each bacterial suspension separately and maintained in the glass aquarium for 6 h/ day for seven days. Employed immersion dose of *Lactobacillus* sp., *Lactococcus* sp., and *Bacillus* sp. were 10^7 CFU/fish (Table 2). Control fish did not receive any bacterial suspension and were kept in another aquarium. The average water temperature was 32.1 ± 0.5ºC.

Fingerling Collection and Acclimatization

Healthy *C. batrachus* fingerling having an average weight of 9.28 ± 1.5 g) were obtained from a private fish hatchery adjacent to the BAU, Mymensingh 2202 campus, and transported to the laboratory in oxygenated plastic bags. They were allowed to acclimatize in the laboratory conditions for a week with continuous oxygen supply and fed commercial pelleted feed (floating feed, Mega; 5% of fish body weight) twice daily at 9:30 am and 5:00 pm before use for the experiment.

Experimental Setup

The experimental set up was divided into two groups; basal diets supplemented with autochthonous gut bacteria + prebiotics and basal diets supplemented with autochthonous gut bacteria without prebiotics.

Stocking of fish fingerling

Experiments were conducted in ten glass aquaria (35 L capacity) filled with 30 L fresh and clean groundwater. One hundred and twenty *C. batrachus* fingerlings were divided into ten equal groups so that each aquarium contains 12 fishes. Stocking density was 1 fish per 2.5 L, and all aquaria were provided with continuous aeration. The fish were fed with a commercial diet (floating feed, Mega) supplemented with gut probiotics and gut probiotic + prebiotic supplementation. Control groups received the same commercial diet without any supplementation. Each aquarium was cleaned daily by 75% water exchange after siphoning out fish feces and uneaten feed. Water temperature was measured daily.

Preparation of probiotic supplemented feed

Three putative isolates from guts *viz.*, *Lactobacillus* sp., *Lactococcus* sp., and *Bacillus* sp., were used in this experiment. After 24 h of incubation (*Lactobacillus* sp. and *Lactococcus* sp. on MRS; *Bacillus* sp. on NA) at 37ºC, bacteria were washed using sterile physiological saline (0.85%) and suspended. A twenty-eight (28) g of rice starch (water from the boiling rice) was taken, cooled to room temperature, *Lactobacillus* sp., *Lactococcus* sp., *Bacillus* sp. suspensions and a miture of these three bacterial suspensions were mixed separately in it to mix with fifty-six (56) g of the basal diet (Mega feed) to prepare gut probiotic supplemented diets (D1, D2, D3, and D4, respectively) which contains the dosage of 10^8-10^9 CFU/g. The prepared feed was dried inside the room for 12 h using a fan, kept in an airtight plastic bag, stored in the refrigerator at 10°C, and used for feeding of the above mentioned *C. batrachus* fingerlings to determine growth and survival. The untreated basal diet was served as control (D5). No replication was used during the feeding experiment.

Preparation of prebiotic supplement

To prepare the prebiotic supplement, 250 g molasses, 100 g rice bran, and 4 g locally available yeast powder were mixed properly in a bucket and 250 ml water was added to the mixture. The mixture was then covered by a lid and kept in a cool, dry, and dark place for 72 h and 50 mL of water was added with for proper fermentation. From this mixture, 4 g was added daily into the selected group of aquaria.

Feeding experiment

Fish in the aquarium were fed with experimental diets twice daily at 9:00 am. and at 5 pm. at a rate of 10% of their body weight. Regular monitoring was done whether the feed was consumed or not.

Sampling of fish and water

Fish were sampled weekly, caught by the hand-held scoop net and the bodyweight of the individual fish was measured carefully using an electric balance. Water quality parameters *e.g.*, alkalinity (mg/L), total ammonia (mg/L), water temperature (˚C), and pH were monitored weekly throughout the study period. The alkalinity values were measured using the "AQUA BASE" alkalinity test kit denoted as milligram per liter (mg/L). Water temperature was measured by hand thermometer and denoted as ˚C. The water pH of the individual aquarium was recorded using a portable pH test kit (Manufactured by ADVANCED PHARMA CO., LTD.).

Analytical Methods

Proximate composition of feed samples

The probiotics supplemented diets were analyzed for protein, lipid, carbohydrate, ash, moisture, and crude fiber content. Analysis of the proximate composition of feed samples was done according to AOAC (1990) in the Fish Nutrition Laboratory, Department of Aquaculture, BAU, Mymensingh.

Estimation of protein

Crude protein of the samples was estimated by using Kjeltec Auto 1030 Analyzer. Calculations of crude protein of the samples were done using the following formula:

% Nitrogen = 0.014 × N × (T-B)/Weight of sample

% Crude protein = % Nitrogen × 6.25 (for animal) = Nitrogen × 5.58 (for plant)

Where,

T = Reading of titrating of samples

B = Reading of titrating of blank samples, N = Normality of HCl and

0.014 = Millieqivalent wt. of nitrogen (g)

Estimation of lipid

To determine crude lipid, the Soxhlet apparatus was used for the solvent extraction of lipid. Calculation of lipid was done by using the following formula:

% Crude lipid =

$$\frac{\text{Wt. of beaker with lipid after overn bry - Initial wt. of beaker}}{\text{Weight of sample}} \times 100$$

Estimation of carbohydrate (CHO)

Carbohydrate content of the samples was determined as a total carbohydrate by difference, that is, subtracting the measured protein, fat, ash, and moisture from 100 (Pearson 1970).

% Moisture content = [(X-Y)/X] × 100

Where,

X = Weight of sample (g) before drying and Y = Weight of samples (g) after drying

% Ash content = [(W1-W2)/W0] × 100

Where, W0 = Weight of sample

W1 = Weight of crucible with ash, W2 = Weight of empty crucible

% Fiber content = $[(W2-W3)/W_1] \times 100$

Where, W1 = Weight of sample (g)

W2 = Crucible weight with fiber and ashes, after drying at 130 °C for 90 minutes

W2 = Crucible weight with ashes, after muffle at 550 °C for 3 hours

Morphometric measurements of the fingerlings

Every week, fish were measured for wet body weight. After obtaining the data, wet weight gain was calculated using the following formula.

Wet weight gain (g) = Final weight (g) – initial weight (g)

Percentage (%) weight gain =

$$\frac{\text{Mean final weight (g) - mean initial weight (g)}}{\text{Weight of sample}} \times 100$$

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Growth Parameters and Rate of Feed Intake

The fish in each treatment were counted and weighed at the end of the experiment. Growth performance and feed efficiency were determined by evaluating some growth and nutrient utilization indices, including specific growth rate (SGR), feed conversion ratio (FCR), feed conversion efficiency (FCE), protein efficiency ratio (PER) and energy retention rate. The growth parameters and feed utilization were calculated as follows:

SGR = 100 (In W2 – W1) T-1

Where,

W1 and W2 are the initial and final weights and T is the number of days of feeding.

FCR = Total dry feed consumption (g)/ Live weight gain (g)

FCE = Live weight gain (g)/ Dry feed consumed (g)

PER = Live wet weight gain (g)/Crude protein intake (g)

Energy retention rate (%) = [(final biomass × final body energy) (initial biomass × initial body energy)]/ total energy intake

Isolation and Enumeration of Gut Bacteria from Experimental Fish

The probiotic treated *C. batrachus* were randomly sampled (3 fish/ treatment) for gut content analysis at the beginning and end of the experimental period. After surface sterilization, the entire gut was carefully removed and homogenized using sterile physiological saline (0.85%). The resultant aliquot was serially diluted, plated on TSA, and MRS agar, and incubated for 24h at 37ºC to recover total heterotrophic bacteria (THB) of gut samples. The bacterial populations of gut samples were expressed as the number of colony-forming units/ gram (CFU/ g).

Data Processing and Analysis

Fish weight gain, growth parameters, production, and water quality were determined and expressed as mean ± (standard deviation). Data analyses performed using Microsoft Excel 2010.

Results

Isolation of Gut Microbiota

To determine the total number of viable microorganisms from the gut, the total microbial load was measured. Average total microbial loads were ranged from 6.5×10^5 to 3.7×10^7 CFU/ g. About 16 isolates were selected based on their presumptive colony characteristics and light microscopic observations *viz.*, cocci or coccobacilli structure, Gram staining etc. Out of the 16 isolates, 3 isolates were selected for further analysis.

Phenotypic Analysis of Gut Bacteria

The optimum growth temperature was found 37°C for *Lactobacillus* sp., *Bacillus* sp., and *Lactococcus* sp. All these bacteria grew on NA, TSA and MRS agar media but were unable to grow on cetrimide agar. *Lactobacillus* sp. and *Bacillus* sp., showed positive reactions for methyl red, Voges-Proskauer, citrate utilization, lysine decarboxylase and glucose fermentation tests and negative reactions for indole production, coagulase test, ornithine decarboxylase, urease, H_2S production, fermentation of adonitol, sorbitol etc. Variations were observed in the cases of catalase production, nitrate reduction, nitrate reduction, fermentation of lactose and arabinose etc. (Table 1). On the other hand, *Lactobacillus* sp. showed positive results only for citrate utilization, fermentation of glucose, arabinose, sorbitol etc. and negative results for the remaining tests.

Table 1. Biochemical tests of *Lactococcus* sp. *Bacillus* sp. and *Lactobacillus* sp.

Characteristics	*Lactobacillus* sp.	*Bacillus* sp.	*Lactococcus* sp.
Pigment	Whitish	Slightly yellow	Slightly yellow
Gram stain	+	+	+
Cell morphology	Cocci	Cocci	Cocci
Growth on NA	+	+	+
Growth on TSA	+	+	+
Growth on MRS agar	+	+	+
Growth on cetrimide agar	-	-	-
Growth on TSA at:			
4ºC	-	-	-
37ºC	+	+	+
42ºC	-	-	-
Indole production	-	-	-
Methyl red	+	+	-
Voges-Proskauer	+	+	-
Catalase production	-	+	-
Coagulase test	-	-	-
Citrate utilization	+	+	+
Lysine decarboxylase	+	+	NT
Ornithine decarboxylase	-	-	-
Urease	-	-	NT
Nitrate reduction	-	+	-
H_2S production	-	-	-
Glucose	+	+	+
Adonitol	-	-	NT
Lactose	+	-	NT
Arabinose	+	-	+
Sorbitol	-	-	+

Determination of Bio-Safety Effects of Gut Bacteria

The bio-safety test by immersion technique using the *C. batrachus* revealed no clinical pathology or infection or mortality of the experimental fish. Fish were exposed to the gut bacteria *Lactobacillus* sp.: dose 5.2×10^7 CFU/mL, *Bacillus* sp.: dose 3.1×10^7 CFU/mL and *Lactococcus* sp.: dose 2.5×10^7 CFU/mL, respectively for 7 seven days (Table 2). Control fish received physiological saline (0.85%) only. Thus, experimental gut bacteria were proven safe for *C. batrachus*.

Table 2. Determination of bio-safety effects of candidate bacteria

Challenged fish	Challenged bacteria with a dose (CFU/ mL)	Treatment	No. of dead fish during the periods of (n=10)				Mortality (%)
			0-1 d	2 d	3 d	4-7 d	
C. batrachus	5.2×10^7 (*Lactobacillus* sp.)	Immersion	0	0	0	0	0
Control	Challenged with 0.85% PS		0	0	0	0	0
C. batrachus	3.1×10^7 (*Bacillus* sp.)		0	0	0	0	0
Control	Challenged with 0.85% PS		0	0	0	0	0
C. batrachus	2.5×10^7 (*Lactococcus* sp.)		0	0	0	0	0
Control	Challenged with 0.85% PS		0	0	0	0	0

Weight (g) of challenged *C. batrachus* (Ave. wt. ± S.D.), 2.9 ± 0.80 g
Water temperature (Ave. temp. ± S.D), 32.5 ± 0.6ºC, PS: Physiological saline (0.85%)

Proximate Compositions of Feed Samples

Commercial pelleted fish feed supplemented with probiotic *Lactobacillus* sp. (D1) contained 30.18% crude protein, 5.98% crude lipid, 33.87% carbohydrate, 11.03% ash, 13.44% moisture, and 5.5% crude fiber. Fish feed mixed with probiotic *Bacillus* sp. (D2) contained 29.55% crude protein, 6.40% crude lipid, 35.86% carbohydrate, 10.71% ash, 13.28% moisture, and 4.2% crude fiber. Feed supplemented with *Lactococcus* sp. (D3) contained 29.29% crude protein, 6.66% crude lipid, 35.38% carbohydrate, 10.7% ash, 13.47% moisture, and 4.5% crude fiber. Again, feed supplemented with the mixture of *Lactobacillus* sp., *Bacillus* sp., and *Lactococcus* sp. (D4) contained 29.98% crude protein, 6.35% crude lipid, 31.91% carbohydrate, 13.87% ash, 13.49% moisture, and 4.4% crude fiber. Basal diet (control diet without probiotic) (D5) contained 28.98% crude protein, 6.77% crude lipid, 36.43% carbohydrate, 11.09% ash, 12.45% moisture, and 4.28% crude fiber. The proximate composition of diets used for rearing *C. batrachus* fingerlings is shown and compared with the control diet in Table 3.

Table 3. Proximate composition of the diets (% moisture basis)

Proximate composition	D1	D2	D3	D4	D5
Crude protein	30.18	29.55	29.29	29.98	28.98
Crude lipid	5.98	6.40	6.66	6.35	6.77
Carbohydrate	33.87	35.86	35.38	31.91	36.43
Ash	11.03	10.71	10.70	13.87	11.09
Moisture	13.44	13.28	13.47	13.49	12.45
Crude fiber	5.50	4.20	4.50	4.40	4.28

D1: basal diet + suspension of *Lactobacillus* sp. + cooled rice starch
D2: basal diet + suspension of *Bacillus* sp. + cooled rice starch
D3: basal diet + suspension of *c* + cooled rice starch
D4: basal diet + mixed bacterial suspension (*Lactobacillus* sp., *Bacillus* sp. and *Lactobacillus* sp.) + cooled rice starch
D5: basal diet only (control)

Water Quality Parameters

All the water quality parameters were within a suitable range. The water quality parameters of different rearing aquaria are shown in Table 4. Water temperature ranged from 27.9˚C to 30.5˚C during the study period. The maximum temperature was 30.5˚C on May 28, while the minimum was 27.9˚C on May 12, 2018. Water pH ranged from 7.2 to 7.4 during the study period. The highest pH value was 7.4 on May 28 while the lowest pH value was 7.2 on May 21, 2018. The values of ammonia varied from 0.03 to 0.009 mg/L. The highest ammonia value was 0.03 mg/L on May 12 and the lowest value was 0.009 mg/L on May 21 and May 28, 2018, respectively. Total alkalinity varied from 180 to 200 mg/L. The highest total alkalinity value was 200 mg/L on May 12 and the lowest value was 180 mg/L on May 21 and May 28, 2018, respectively.

Table 4. Mean water quality parameters in *C. batrachus* rearing water

Sampling date → Parameters ↓	12.05.18	21.05.18	28.05.18
	Mean ± SD	Mean ± SD	Mean ± SD
Temperature (˚C)	27.9 ± 0.28	29.9 ± 0.31	30.5 ± 0.39
pH	7.3 ± 0.13	7.2 ± 0.18	7.4 ± 0.17
Ammonia	0.03	0.009	0.009
Total alkalinity	200	180	180

Growth Parameters and Rate of Feed Intake of *C. batrachus*

Net weight gains of *C. batrachus* fingerlings were found to be 2.87 g, 3.72 g, 2.99 g, 2.92 g, and 3.41 g in D1, D2, D3, D4, and D5, respectively. The significantly highest net weight gain was recorded in D2 than other treatments (Table 5). The percent weight gains of the fingerlings were found to be 105.9%, 138.3%, 104.18%, 113.62%, and 110.71% in D1, D2, D3, D4, and D5 respectively. The highest percent weight gain was also recorded in D2 than the other treatments. The Specific growth rates (SGR) were calculated as 3.77, 4.69, 3.91, 3.83, and 4.38 respectively in D1, D2, D3, D4, and D5. The highest SGR was recorded in D2 than other treatments (Table 5). The Feed conversion ratio (FCR) of the experimental fish was calculated 1.24, 1.2, 1.2, 1.17, and 1.3 in D1, D2, D3, D4, and D5, respectively (Table 5). The lowest FCR was recorded in D4 than other treatments. Again, feed conversion efficiencies (FCEs) were found 0.81, 0.83, 0.83, 0.85, and 0.76 in D1, D2, D3, D4, and D5 respectively (Table 5). The highest FCE was recorded in D4 than other treatments. At the end of the experiment, D1, D3, D4, and D5 showed 100% survival of *C. batrachus* fingerlings but D2 exhibited 91.67% survival.

Table 5. Growth responses of *C. batrachus* fed with gut microbiota supplemented feed for 3 weeks

Parameters	D1	D2	D3	D4	D5 (Control)
Initial BW (g)	2.71	2.69	2.87	2.57	3.41
Final BW (g)	5.58	6.41	5.86	5.49	6.49
Net weight gain (g)	2.87	3.72	2.99	2.92	3.08
Weight gain (%)	105.9	138.3	104.18	113.62	110.71
Specific growth rate (SGR) (% day)	3.77	4.69	3.91	3.83	4.38
Food conversion ratio (FCR)	1.24	1.2	1.2	1.17	1.30
Food conversion efficiency (FCE)	0.81	0.83	0.83	0.85	0.76
PER (Protein energy ratio)	2.67	2.92	3.41	2.85	2.65
Survival (%)	100	91.67	100	100	100

Growth Parameters and Rate of Feed Intake of *C. batrachus* Supplemented with Gut Microbiota and Prebiotic

Net weight gains of *C. batrachus* fingerlings were found to be 3.43 g, 4.46 g, 3.38 g, 3.86 g, and 3.41 g in D1+prebiotic, D2+prebiotic, D3+prebiotic, D4+prebiotic, and D5+prebiotic, respectively. The significantly highest net weight gain was recorded in D2+prebiotic than other treatments (Table 6). The percent weight gains of *C. batrachus* fingerlings were found 114.72%, 165.19%, 120.28%, 123.72%, and 110.71% in D1+prebiotic, D2+prebiotic, D3+prebiotic, D4+prebiotic, and D5+prebiotic respectively. The highest percent weight gain was recorded in D2+prebiotic. The Specific growth rates (SGR) of *C. batrachus* fingerlings were calculated as 4.40, 5.34, 4.35, 3.83, and 4.38, respectively in D1+prebiotic, D2+prebiotic, D3+prebiotic, D4+prebiotic, and D5+prebiotic. The highest SGR was recorded in D2+prebiotic. FCR were calculated as 1.28, 1.06, 1.16, 1.08, and 1.11 in D1+prebiotic, D2+prebiotic, D3+prebiotic, D4+prebiotic, and D5+prebiotic respectively and D4+prebiotic revealed the lowest FCR. Additionally, the highest FCE was recorded in D4+prebiotic than the other treatments. The survival of the fingerlings was found 100% in all the treatments except in D1+prebiotic (91.67%) (Table 6).

Table 6. Growth responses of *C. batrachus* fed with gut microbiota supplemented feed and prebiotic supplementation in water for 3 weeks

Parameters	D1 + Prebiotic	D2 + Prebiotic	D3 + Prebiotic	D4 + Prebiotic	Control
Initial BW (g)	2.99	2.70	2.81	3.12	3.08
Final BW (g)	6.42	7.16	6.19	6.98	6.49
Net Weight Gain (g)	3.43	4.46	3.38	3.86	3.41
Weight Gain (%)	114.72	165.19	120.28	123.72	110.71
SGR (% day)	4.40	5.34	4.35	4.82	4.38
Food conversion ratio (FCR)	1.28	1.06	1.16	1.08	1.11
Food conversion efficiency (FCE)	0.87	0.94	0.86	0.93	0.9
Protein energy ratio (PER)	2.6	3.18	2.95	3.08	3.1
Survival (%)	91.67	100	100	100	100

Effects of *Lactobacillus* sp., *Bacillus* sp., and *Lactococcus* sp. in the gut content and growth of C. *batrachus*

The effects of gut bacteria (*Lactobacillus* sp., *Bacillus* sp., and *Lactococcus* sp.) supplementation as probiotic in the experimented fish with and without prebiotic is represented in Table 7. Colonies that appeared from the gut content of *C. batrachus* were counted both on TSA and MRS agar. TSA plates gave total counts of bacteria and the MRS agar only grows the Gram-positive bacteria. The initial gut microbiota of the experimented fish supplemented without prebiotic in MRS agar was highest in D3 (*Lactococcus* sp.) (Table 7). The final gut microbiota counted in MRS agar was also highest in D3 (Table 7) (Figure 5). Among the prebiotic supplementation group, Gram-positive bacteria (beneficial one) was counted highest finally in D1+prebiotic supplementation (Table 7). The final total gut microbiota was also found highest in the same dietary treatment.

Table 7. Total plate count of *C. batrachus* gut content fed with *Lactobacillus* sp., *Bacillus* sp., and *Lactococcus* sp. in agar media (CFU/ g) after 21 days of rearing

Diet	TSA		MRS agar	
	Initial	Final	Initial	Final
D1	2.7×10^2	4.1×10^5	<10	2.0×10^5
D2	3.2×10^2	2.5×10^5	<10	1.0×10^3
D3	1.8×10^2	5.2×10^6	2.2×10	1.1×10^4
D4	2.0×10^3	6.2×10^5	<10	1.0×10^3
D5 (Control)	2.6×10^2	5.0×10^5	5.6×10	9.0×10^3
D1 + Prebiotic supplementation	1.2×10^3	7.9×10^6	1.2×10	4.0×10^4
D2 + Prebiotic supplementation	8.1×10^2	8.7×10^6	<10	1.0×10^3
D3+ Prebiotic supplementation	5.6×10^2	3.3×10^5	<10	1.0×10^3
D4+ Prebiotic supplementation	3.4×10^2	6.9×10^6	<10	1.0×10^3
D5 (Control) + Prebiotic supplementation	1.0×10^2	2.7×10^4	<10	1.4×10^2

Discussion

The application of probiotics in aquaculture is one of the most promising ways to ensure improved growth rate, enhance disease prevention capacity of fish, and improve water quality. Studies have already demonstrated that probiotics modulate the non-specific immune responses which may increase disease resistance ability in fish against bacterial infections in the aquatic environment (Del'Duca *et al.*, 2013; Eissa *et al*., 2014). Besides, few studies described an important role of probiotics in feed efficiency and growth promotion (Gatesoupe 2002; Lara-Flores *et al*., 2003, 2010). In the present study, primarily, we have isolated and identified three potential Gram-positive gut bacteria *i.e.*, *Lactobacillus* sp., *Bacillus* sp., and *Lactococcus* sp. from *C. batrachus* intestine through gram staining and a series of phenotypical and biochemical analysis, which has been demonstrated in Figure 1 and Table 1. The biochemical test ensured that the isolates were belonging to autochthonous microflora, which has been described in many studies (Holt *et al.*, 2000, Ghosh *et al.*, 2010, Cantas *et al.*, 2012). Safety evaluation of probiotics is very important to understand the possible virulence or pathogenicity, adhesion, translocation, colonization, and survivability of probiotics in the host gut region (Huys *et al*., 2013). In the present study, we have tested the isolated gut bacteria for bio-safety assessment through the immersion technique with the bacterial

suspensions. The study showed no mortality or any clinical signs of infection or disease in *C. batrachus* fingerlings during the bio-safety test, which determined the potentiality of these isolates as an applicable probiotic. Similarly, Mukherjee and Ghosh (2014) reported results about biosafety assessment, while the gut probiotics were experimentally injected (intraperitoneal injection) in *Catla catla* fingerlings and showed no mortality or any clinical signs.

Furtherly, the growth-promoting effects of these three gut bacteria were analyzed by a feeding trial with probiotics supplementation, wherein three of these gut bacteria and a combination of them were treated with a commercial basal diet, individually (D1, D2, D3, D4, respectively). These four diets were also fed along with prebiotic supplement (molasses, yeast, and rice bran mixed with water and fermented for 72 hrs). Regarding the growth effects of bacteria supplemented feed, higher levels of growth assessing parameters were found for the fishes fed with experimental diets as compared to the control diet that demonstrated the potentiality of the reported species. Irianto and Austin (2002), and Denev *et al*., 2009 have been demonstrated the potential probiotic properties of these bacteria for different kinds of fish and shellfish species culture.

In the present study, better growth performance and SGR were observed in *C. batrachus* fingerlings with the gut microbiota supplemented diet *viz.*, D1, D2, D3, and D4 compared with the control diet. Similar observations have been reported on African catfish, *C. gariepinus* (Al-Dohail *et al*., 2009; Ayoola *et al.*, 2013), and *L. rohita* (Mohamed *et al*., 2007). The authors reported that growth performance in the fish was significantly ($P<0.05$) higher in the probiotic treated groups than the control when *Lactobacillus acidophilus* and *L. delbrueckii* were used as probiotics as feed additives in their formulated diets. Besides, excellent growth result was revealed by feeding the fish supplemented with autochthonous gut bacteria along with prebiotic (D1+prebiotic, D2+prebiotic, D3+prebiotic, and D4+prebiotic). Fish fed with bacterial supplementation and prebiotic shows even better growth performance than the fish fed with only the same gut microbiota. For example, the experimental diet, D2 shows better results in percent weight gain and SGR than from control and the diet supplemented while D2 + prebiotic shows even better SGR and percent weight gain than that of D2. These indicates an improvement in the health and growth performance of fish despite the differences in the methods and species used in the present study. The improvement in growth may, however, be related to the improvement in the intestinal microbial balance as reported by Fuller (1989). The variations between probiotics supplemented diets with or without prebiotics can be better understood by explaining the beneficial outputs of prebiotics application. Several studies described the beneficial role of prebiotics in growth promotion, disease resistance, and survivability of fish. The beneficial effects of prebiotics such as innate immune response upregulation, immune system stimulation, phagocytic and neutrophilic activation, modification of gut microflora, and boosting their functional activities; all may contribute to show better growth performance, higher survival of experimental fish (Ringø *et al.*, 2010; Akhter *et al.*, 2015; Carbone and Faggio, 2016). Moreover, the supplemented prebiotics are subjected to fermentation, and it may release several nutrients, functional compounds, which may provide extra energy, nutrients, and essential elements to improve the host animal health (Peng *et al.*, 2020).

The study results also showed that FCR was better in fish fed with gut microbiota treated diets (D1, D2, D3, and D4) compared to the control diet. The results are in agreement with the findings on Nile tilapia (Lara-Flores *et al*., 2003; Mohamed *et al*., 2007), African catfish (Al-Dohail *et al*., 2009; Ayoola *et al*., 2013), and common carp (Noh *et al*., 1994; Yanbo and Zirong, 2006). Higher protein utilization, determined in terms of PER, increased to some extent in fish maintained with the gut microbiota supplemented diets than in the fish maintained with the control diet. This result also agreed with the findings of Lara-Flores *et al*., (2003) where better PER was found in Nile tilapia fed diets supplemented with commercial probiotics, *Streptococcus faecium* and *L. acidophilus*. Nourishment of probiotic bacteria growth and reproduction in gastrointestinal tracts through providing prebiotics and further actions of these gut microbiota such as increasing digestibility; nutrients and energy release; accelerating absorption rate may contribute to higher feed efficiency and nutrients utilization, which might led to lower the FCR and higher PER (Sheridan *et al.*, 2014).

Beneficial effects of probiotics in water quality improvement have been proven in aquaculture. Queiroz and Boyd (1998) applied Biostart, a commercial inoculum of *Bacillus* sp. was applied into three-channel catfish, *Ictalurus punctatus* ponds where the water quality parameters became very much suitable for fish that ultimately influenced the health condition, survival, and growth status than in controls. In the present study, water temperature varied from 27.6˚C to 30.2˚C during the present study. The temperature reported by Collins (1973) for the culture of *C. batrachus* was found similar to the present study. The slightly alkaline pH is most suitable for fish culture. Acidic pH of rearing water reduces the growth and metabolic rate and other physiological activities of fish (Swingle, 1967). Present study revealed, the range of pH from 7.2 to 7.4 that ultimately exhibited a similar range reported by Bhuiyan (1970) and Wahab *et al*. (1995). The consistency of these water quality parameters indicated that probiotics application had no adverse effects on water quality. Most importantly, probiotic supplemented diets significantly reduced ammonia content or lower ammonia excretion during the culture period (Table 4) which might be resulted from improvement in protein utilization or higher amount of non-digested carbohydrates of prebiotics in fish excreta. The beneficial role of probiotics supplementations in water quality improvement is supported by the findings of an experiment on *L. calbasu* gut probiotics (Bhatnagar and Dhillon, 2019) and the application of commercial probiotics on *Macrobrachium rosenbergii* (Ghosh *et al*., 2016).

Summary and Conclusion

The present research work was probably the first attempt in the Mymensingh region to evaluate the efficacy of autochthonous gut microbiota supplementation on the growth and survival of *C. batrachus.* Some intestinal autochthonous bacteria were isolated from adult *C. batrachus* followed by identified phenotypically upto genus level. The 3 weeks long feeding trial was investigated to determine survival and growth performance of *C. batrachus* fingerling using selected autochthonous gut bacteria supplemented diet under prebiotic added and without prebiotic condition. The specific functions of probiotics in aquaculture may not be denied. It could be concluded that both single and mixed gut

microbiota containing feed and prebiotic supplementation was found beneficial for the rearing of *C. batrachus* fingerlings. But the combination of these two supplements (gut bacteria and prebiotic) produced more beneficial effects by enhancing the better growth performance and survival of fish. More works are needed to assess the autochthonous microbiota and prebiotics supplementation on the growth and immune responses of *C. batrachus* and other catfish produced under intensive condition.

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