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Note

Feeding *Artemia* nauplii enriched with the probiotic bacterium *Bacillus subtilis* improved growth performance, survival and digestive enzyme activity of *Clarias batrachus* (Linnaeus, 1758) larvae

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ABSTRACT

Clarias batrachus (Linnaeus, 1758) larvae were subjected to different modes of probiotic (*Bacillus subtilis*) administration such as: T1: feeding *Artemia* nauplii enriched with *B. subtilis*; T2: feeding enriched *Artemia* nauplii + addition of *B. subtilis* in water and T3: addition of *B. subtilis* in water alone, for a period of 14 days. A control group (T0) with no probiotic administration was also maintained. On termination of the experiment, probiotic supplemented through enriched *Artemia* nauplii along with addition in water (T2) yielded significantly ($p<0.05$) higher length, weight, weight gain, specific growth rate and survival compared to control (T0) and T3 groups. All these parameters did not differ significantly ($p>0.05$) between T1 and T2. Similar trend was observed in the case of total gut bacterial count and digestive enzyme activity. The highest ($p<0.05$) digestive enzyme activity of protease, lipase and amylase were recorded in T2, while no significant ($p>0.05$) difference was observed between T2 and T1. The present study reiterated the advantage of *B. subtilis,* and its administration through enriched *Artemia* nauplii which could be a promising supplementation mode during *C. batrachus* larval rearing.

Keywords: Growth, Larval rearing, Magur, Probiotic, Survival

Asian catfish *Clarias batrachus* (Linnaeus, 1758) is one of the promising cultivable air-breathing catfish in Asian countries and considered as a candidate species for aquaculture diversification. Major attributes of this species are its hardy nature which can withstand adverse ecological conditions, response to high density culture and high yielding capacity. Thus it has great potential for grow-out culture in ponds (Thakur, 1986, Areerat, 1987, Sahoo *et al.*, 2008). But the major bottleneck is the lack of availability of seed in sufficient quantity for commercial farming. Early mortality of larvae in hatchery is the major hurdle which is attributed to improper nutrition, poor water quality and outbreak of diseases (Sinha *et al.*, 2014). Presently probiotics are increasingly being utilised in aquaculture for improving disease resistance, growth as well as feed utilisation efficiency (Balcazar *et al.*, 2006, Sahu *et al.*, 2008, Martinez Cruz *et al.*, 2012, Hai, 2015b). Accumulated evidence suggest that, probiotics aid to improve water quality, digestibility of nutrients, control of bacterial infections, tolerance to biotic/abiotic stress and reproduction (Sahu *et al.*, 2008, Ninawe and Selvin, 2009, Behera and Nayak, 2011, Martinez Cruz *et al.*, 2012). The commonly used probiotics in aquaculture sector belong to the genera, *Lactobacillus*, *Bacillus, Leuconostoc, Lactococcus*, *Aeromonas*, *Enterococcus*, *Carnobacterium*, *Shewanella*, *Pseudomonas*, *Enterobacter*, *Clostridium* and *Saccharomyces* (Balcazar *et al.*, 2006, Hai, 2015b, Kumar and Ghosh, 2016). Thus, probiotics are becoming a vital part in the aquaculture industry. Gut microbiota of fishes can be manipulated using probiotics *i.e.*, beneficial microbiota for enhancing nutritional efficiency as well as immunity against diseases (Balcazar *et al.*, 2006, Farzanfar, 2006, Akhter *et al.*, 2015, Hai, 2015a, b).

Various microbes have been investigated as probiotics for aquaculture of which several species belonging to the genus *Bacillus* have been found helpful to produce proteases and other enzymes that contribute to the natural digestion activity of the host (Ziaei-Nejad *et al.*, 2006; Cutting, 2011; Nimrat *et al.*, 2012a). *Bacillus* spp. have been found to act as source of micro and macro-elements in feed (Verschuere *et al.*, 2000) and have also been found to enhance survival, growth as well as improve water quality in terms of bioremediation (Cutting, 2011; Hai, 2015b). *Bacillus subtilis* is well known to prevent colonisation of potential pathogens in the gut of host organisms, through the production of antimicrobial compounds, or by actively competing for nutrients/space (Nayak, 2010; Sumathi *et al.*, 2014).

Though the beneficial effects of environment friendly probiotics in aquaculture are well documented, there is no information on the probiotic effects of *Bacillus* spp. in the larvae of Asian catfish *C. batrachus*. Therefore, the present study aimed to determine the effects of *B. subtilis* and its mode of administration on the growth, survival and digestive enzyme of *C. batrachus* larvae.

Pure bacterial strain of *B. subtilis* (MTCC 441) was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Bacterial culture was inoculated into conical flask containing nutrient broth (HiMedia) and incubated at 30°C for 24 h (APHA, 1998). For enrichment of *Artemia* nauplii, the broth culture was centrifuged (Eppendorf, 5810R) at 2500 *g* for 30 min at 4°C. The pellet was washed thrice with sterile phosphate buffered saline (PBS, pH 7.2) and re-suspended in PBS. The bacterial concentration was adjusted to desired level using serial dilution method (Kumar *et al.*, 2013) and measuring optical density (OD600) by spectrophotometry (Lamda 25 UV-VIS spectrometer, Perkin Elmer). Hatching of *Artemia* cysts and its enrichment with probiotics was carried out as suggested by Sorgeloos *et al.* (1986). After hatching, nauplii were transferred to fresh saline water of 12‰ in 6 l capacity glass jar for enrichment. The daily requirement of *Artemia* nauplii were calculated and the probiotic bacteria (*B. subtilis*) were added to the enrichment medium at a cell density of 1.5 x10^6 cfu ml^{-1}. Enrichment was done for a period of 6 h. Vigorous aeration was provided in the glass jar to ensure thorough mixing and to keep *Artemia* cysts/nauplii in suspension. After enrichment, *Artemia* nauplii were collected using bolting silk cloth and fed to the larvae in the experimental groups.

C. batrachus larvae were obtained by induced breeding techniques in the catfish hatchery of ICAR-Central Institute of Freshwater Aquaculture (ICAR-CIFA), Bhubaneswar, India. Twelve uniform sized glass aquaria (30 l capacity) were used for the study. Total volume of water in the glass aquaria was maintained at 10 l to maintain a water height of 6 inches throughout the experimental period, The glass aquaria were uniformly filled with 10 l water and were provided with continuous aeration. Four days old larvae of 9±0.2 mm length and 4.68 ± 0.08 mg weight were stocked @11 nos. l^{-1} randomly into the 12 aquaria. Larvae of *C. batrachus* were subjected to different modes of probiotic (*B. subtilis*) feeding with three replicates each, such as: T0 - control with no probiotic administration; T1: *Artemia* nauplii enriched with *B. subtilis*; T2: *Artemia* nauplii enriched with *B. subtilis* + addition of *B. subtilis* in water and T3: addition of *B. subtilis* in water. Enrichment of *Artemia* nauplii with above mentioned probiotic bacteria was done at a concentration of 1.5 x 10^6 cfu ml^{-1} of enrichment medium while application of probiotic bacteria in water was done at concentration of 1.5 x 10^6 cfu ml^{-1} of rearing water per day. The larvae were fed *ad libitum* as per the treatments while larvae in control and T3 were fed with normal *Artemia* nauplii over a period of 14 days. The experimental tanks were cleaned once daily and 75% water was exchanged (before feeding and addition of probiotic bacteria) without much disturbance to the larvae.

To study the efficacy of *B. subtilis*, the total bacterial count in gut of the larvae was examined on day 1, 7 and 14 of the experiment. At each sampling, five larvae from each aquaria were randomly selected and surface disinfected with alcohol, washed in sterile distilled water and dissected to collect gut. Gut samples collected form each treatment were pooled, weighed and homogenised in 5 ml of sterile normal saline solution (0.85% w/v NaCl). Enumeration of total bacterial count was performed by serial dilution and plating on brain heart infusion (BHI) agar (Himedia) plates. The plates were incubated at 28°C for 24-48 h in BOD incubator followed by counting of bacterial colonies.

Growth performance of the larvae in terms of percentage length and weight gain, specific growth rate (SGR) and survival rate were estimated using the following formulae:

Length gain %	= (Final length - Initial length)×100 / Initial length
Weight gain %	= (Final weight - Initial weight) × 100 / Initial weight
Specific growth rate (SGR)	= 100 × ($\log_e$ Average final weight -$\log_e$ Average initial weight) /No. of culture days
Survival (%)	= (Total No. of animals harvested/Total No. of animals stocked) × 100

For assay of the digestive enzyme activity, 55 fry per aquaria were collected on termination of the experiment and then immediately frozen at -70°C until enzyme assays were performed. Approximately 1.0 g of whole fry were homogenised in chilled 10 mMTris HCl buffer at pH 7.5 and enzyme extracts were obtained after centrifugation at 10,000 *g* for 30 min at 4°C. The supernatant of each sample was assayed in triplicate. Bradford (1976) method was used to measure total soluble protein using bovine serum albumin as a standard. Amylase activity was assayed by the method of Bernfeld (1955) using

3,5-dinitrosalicylic acid with soluble starch as the substrate. Total protease activity was assayed according to the method of Anson (1938), using Folin's reagent with casein as the substrate. Lipase activity was determined following Cherry and Crandell (1932). Enzyme activities were measured as the change in the absorbance using a PerkinElmer, Lamda25 UV-VIS spectrometer.

Water samples from each aquaria were taken periodically between 07.00 and 08.00 hrs every alternate days before cleaning. The water quality parameters such as pH, total alkalinity, free CO_2, ammonia nitrogen, nitrite-nitrogen and nitrate-nitrogen were analysed following APHA (1998). Water temperature and dissolved oxygen were recorded daily with a dissolved oxygen meter (MERCK, Germany).

The data on the growth performance and survival were subjected to statistical analysis using SPSS v.16. One-way analysis of variance was used to compare all the treatments. Duncan's multiple range test was performed at 95% significance level to compare the treatment means for different parameters.

There was no significant difference in the physico-chemical parameters of water between control and treatment aquaria (p>0.05). Water temperature (°C), dissolved oxygen (mg l^{-1}), pH, total alkalinity, ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen ranged from 27.5-28.8^{0}C, 6.2 to 7.1 mg l^{-1}, 7.48 to 8.19, 110 to 130 mg l^{-1}, 0.16 to 0.37 mg l^{-1}, 0.001 to 0.007 mg l^{-1} and 0.003 to 0.023 mg l^{-1} respectively. Values observed in this study were within the optimum range for *C. batrachus* as reported by Rao *et al*. (1994).

The probiotic potential of *B. subtilis* was evident from the higher survival and growth performance in terms of final length, final weight, % weight gain and SGR of *C. batrachus* fry in the *B. subtilis* supplemented treatments over non-supplemented group (Control) (p<0.05) (Table 1). Similarly, earlier studies demonstrated improvement in larval growth and survival with *B. subtilis* supplementation in *Macrobrachium rosenbergii* (Seenivasan, *et al*., 2012), *Penaeus vannamei* (Nimrat *et al*., 2012a), *Penaeus monodon* (Nimrat *et al*., 2012b), *Sparus aurata* (Nihan *et al*., 2013) and *Heteropneustes fossilis* (Ramakrishnan, *et al*., 2015). Supplementation through enriched *Artemia* nauplii and addition in water (T2) yielded significantly high survival and growth in terms of length, weight, % weight gain and SGR compared to control (T0) and T3. Statistically similar (p>0.05) results were obtained between *B. subtilis* supplemented through enriched *Artemia* nauplii (T1) and T2. Suzer *et al*. (2008) reported increased growth and survival of seabream, *S. aurata* larvae supplemented *Lactobacillus* spp. through live feed with water in the same environment. Similar results were reported in the Persian sturgeon, *Acipenser persicus* larvae (Faramarzi *et al*., 2011), seabream larvae (Avella *et al*., 2010) and *M. rosenbergii* larvae (Keysami *et al*., 2007). However, Ziaei-Nejadand *et al*. (2006) reported that growth performance and survival were not significantly different between *Bacillus* spp. administered through *Artemia* enrichment and addition to water. The improved growth performance and survival of *C. batrachus* larvae in T2 and T1 might be due to the colonisation of probiotic bacteria in the gut of *Artemia* nauplii and transition to larval fish intestine after digestion (Suzer *et al*., 2008). Moreover in the present work, larvae in group T3 administered with probiotics in the rearing water showed significantly higher growth and survival compared to control (T0), which is in line with the results from the earlier studies on *Clarias gariepinus* larvae (Ariole, 2012), tilapia, *Oreochromis niloticus* (Wang *et al*., 2008, Zhou *et al*., 2010), large yellow croaker *Larimichthys crocea* (Ai *et al.,* 2011), grouper *Epinephelus coioides* (Sun *et al*., 2010) and shrimp *P. vannamei* larvae (Zhou *et al*., 2009, Liu *et al*., 2010). Increased growth in larvae fed with *B. subtilis* could be attributed to improved digestive activity which is evident from the increased digestive enzyme activity in *B. subtilis* supplemented treatments (Keysami *et al*., 2012).

The functionality of probiotic strain depends on its gut colonisation potential (Walker and Duffy 1998; Balcazar *et al*., 2006). In the present study, total bacterial counts recorded in the gut of *C. batrachus*

Table 1. Yield attributes of *C. batrachus* fry supplemented with *B. subtilis* through different modes (means± S.E, n=3)

Treatment	Growth parameter				
	FL (mm)	FW (mg)	WG (%)	SGR (% d^{-1})	Survival (%)
Control (T0)	14.51±0.06^a	18.85±0.2^a	318.81±6.39^a	9.96±0.1^a	76.41±1.4^a
T1	16.00±0.08^c	27.52±0.30^c	511.49±6.62^c	12.83±0.07^c	86.92±1.33^c
T2	16.00±0.04^c	27.84±0.19^c	518.69±4.15^c	12.92±0.04^c	88.21±0.68^c
T3	14.93±0.10^b	21.94±0.14^b	387.54±3.13^b	11.10±0.03^b	81.79±1.36^b

Values in the same column sharing a common superscript are not significantly different (p>0.05)
FL: Final total length, FW: Final weight, WG: Weight gain, SGR: Specific growth rate

larvae supplemented with *B. subtilis* through different modes, on day 1, 7 and 14 of larval rearing are shown in Table 2. Significant increase in total gut bacterial count was observed in all the treatments during day 1 to 14. Except for the day 1, subsequent samplings on day 7 and 14 showed significantly high total gut bacterial count in group T2 supplemented with *B. subtilis* through enriched *Artemia* nauplii and addition in water compared to the control (T0) and group T3 supplemented with *B. subtilis* by addition in water. There was no significant difference in total gut bacterial count between T2 and T1 ($p>0.05$). Significant increase in total gut bacterial count in *B. subtilis* supplemented groups observed in the present study suggests that, *B. subtilis* successfully colonised in the gut of *C. batrachus* fry and might have led to improved survival by preventing colonisation of potential pathogens through the production of antimicrobial compounds, or by actively competing for nutrients or space (Nayak, 2010; Sumathi *et al.*, 2014). Similar results were reported in *M. rosenbergii* using *B. subtilis* as a probiotic (Keysami *et al.*, 2012).

Several researchers have demonstrated that one of the main modes of action and beneficial effects of probiotics in aquatic organisms is the enhancement of nutrition of host species *via* the production of supplemental digestive enzymes (Verschuere *et al.*, 2000; Martinez Cruz *et al.*, 2012). Bacteria particularly members of the genus *Bacillus* are known to produce a wide range of exoenzymes (Moriarty, 1996, 1998). In the present study, the activity of digestive enzymes such as protease, lipase and amylase were significantly elevated ($p<0.05$) in treatment groups supplemented with *B. subtilis* (Table 3). Earlier studies have also reported enhanced digestive enzyme activity with supplementation of *Bacillus* probiotics in *Cyprinus carpio* (Yanbo and Zirong, 2006), *Penaeus indicus* larvae (Ziaei-Nejad *et al.*, 2006), *P. vannamei* larvae (Zhou *et al.*, 2009, Nimrat *et al.*, 2012a) and seabream, *S. aurata* larvae (Nihan *et al.*, 2013). Digestive enzyme activities were enhanced in all the experimental groups supplemented with probiotics. But, the mode of administration of probiotics to the larval fish strongly affected the enzymatic activity (Suzer *et al.*, 2008). In the present work, increased digestive enzyme activities (protease, lipase and amylase) were significantly higher ($p<0.05$) in T2, compared to control (T0) and T3. However, no significant difference ($p>0.05$) was observed between T2 and T1. Significantly enhanced digestive enzyme activity ($p<0.05$) was observed in T2 and T1 compared to T3 and T0. However, the digestive enzyme activity in T3 was significantly higher than control T0. This is in agreement with earlier study in *P. vannamei* larvae, which received probiotic *Bacillus coagulans* directly in rearing water (Zhou *et al.*, 2009).

Table 2. Total bacterial count (CFU x 10^4) in the gut of *C. batrachus* fry supplemented with *B. subtilis* through different modes (Means± SE, n=3)

Treatment	Total bacterial count (CFU x 10^4) per fish		
	Day 1	Day 2	Day 14
Control (T0)	7.89 ± 0.73^{aA}	11.00 ± 1.01^{bA}	14.22 ± 0.80^{cA}
T1	8.11 ± 0.11^{aA}	23.33 ± 0.51^{bC}	35.89 ± 2.00^{cC}
T2	7.67 ± 0.66^{aA}	24.67 ± 0.33^{bC}	36.22 ± 1.32^{cC}
T3	7.33 ± 0.19^{aA}	19.22 ± 1.45^{bB}	24.00 ± 1.02^{cB}

Values in the same row sharing common lowercase superscripts are not significantly different (p>0.05) and values in the same column sharing common uppercase superscripts are not significantly different (p>0.05)

Table 3. Digestive enzyme activity of *C. batrachus* fry supplemented with *B. subtilis* through different modes (Means± S.E, n=3)

Treatment	Digestive enzymes (U mg Protein^{-1})		
	Protease	Lipase	Amylase
Control (T0)	0.751 ± 0.045^{a}	0.412 ± 0.033^{a}	0.339 ± 0.0137^{a}
T1	1.190 ± 0.032^{c}	0.623 ± 0.014^{c}	0.549 ± 0.0039^{c}
T2	1.279 ± 0.077^{c}	0.638 ± 0.007^{c}	0.563 ± 0.023^{c}
T3	0.919 ± 0.051^{b}	0.549 ± 0.002^{b}	0.482 ± 0.005^{b}

Values in the same column sharing common superscripts are not significantly different (p>0.05)

In conclusion, the present study while reiterated the advantages of *B. subtilis* supplementation in larval rearing of *C. batrachus*. The results also revealed that administration of *B. subtilis* through enriched *Artemia* nauplii to be better and economical over administration of only *B. subtilis* in water or application of enriched *Artemia* nauplii along with addition of *B. subtilis* in water.

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