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Effect of feeding bioencapsulated *Lactobacillus* sp. in live *Tubifex* sp. on the growth performance of gold fish *Carassius auratus* Linnaeus, 1758)

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Abstract
An attempt was made to feed bioencapsulate *Lactobacillus* sp in live fish food organism *Tubifex* for use in the culture of gold fish *Carassius auratus*. The *C. auratus* fries when fed with bioencapsulated *Lactobacillus* sp. in *Tubifex* showed significant improvement in total wet weight gain ($p<0.007$) and FCR ($p<0.01$) compared to control. The specific growth rate and mean survival were slightly higher, although insignificantly ($p>0.05$) in bioencapsulated *Tubifex* fed group. None of the bacteriological parameters of the fish gut between the experimental and control groups differed significantly ($p>0.05$). *Lactobacillus* sp. was recorded at a level of log 5.11/g on the 90th day of experimentation. When the experimental *C. auratus* fries were infected with *Pseudomonas fluorescens*, the bioencapsulated *Tubifex* fed group resisted the infection. The survival was significantly higher ($p<0.05$) in bioencapsulated *Tubifex* fed group (44%) than in control (22%). The *C. auratus* fed with bioencapsulated *Tubifex* showed less (55%) signs of tail/fin rot. Likewise, a significant improvement in total wet weight gain ($p<0.009$), FCR ($p<0.01$) and SGR ($p<0.04$) of *C. auratus* brooder fed with bioencapsulated *Tubifex* was seen compared to control group fed with depurated *Tubifex*.

Keywords: *Carassius auratus*, *Lactobacillus* sp, *Tubifex* sp, Bioencapsulation

Introduction

The use of probiotics in aquaculture is well studied with varying reports of their effect on growth, survival and disease resistance of different commercially important aquatic organisms (Douillet and Langdon 1993, Ringo *et al.* 1995, Gatesoupe 1999, Irianto and Austin 2002). In aquaculture sector, the ornamental fish breeding and trade provides excellent opportunities as a non-food fishery activity for employment and income generation. It is totally environmental friendly, socially acceptable, involves low investment with short gestation period for every cycle of breeding and growth, could be adapted as a small-scale backyard enterprise and ensures high profit. Application of

probiotics in ornamental fish rearing is also gaining importance. The beneficial effects of fish gut antagonistic bacterium as probiotic in ornamental fish culture have demonstrated in earlier reports (Mondal *et al.* 2003, Abraham and Banerjee 2007, Abraham *et al.* 2007a,b, Abraham 2008). The present study reports the bioencapsulation of probiotic in live fish food organism and its influence on the growth, survival and disease resistance of ornamental fish *Carassius auratus*.

Materials and methods

The live *Tubifex* sp. was procured from retail ornamental fish traders of Mohanpur, Nadia District as and when required. Before use, it was depurated for 2 days in running water with a flow rate of 6 L/h. An antagonistic bacterial strain, *Lactobacillus* sp. P21 isolated from *Cirrhinus mrigala* gut (Abraham and Banerjee 2007) was used as a probiont. For the purpose of standardization of optimum dose and time for the bioencapsulation of *Lactobacillus* sp. in *Tubifex*, a series of glass test tubes containing 10 ml each of de Man Rogosa Sharpe (MRS) broth were seeded with 24 h old probiont and incubated for 24 h. One gram each of depurated *Tubifex* was transferred in to the tubes containing 24h grown probiont for bioencapsulation. At regular intervals, the contents of the tubes were filtered through 60μ bolting silk cloth, sterilized by placing in boiling water for an hour, to screen out the broth from the *Tubifex*. The bioencapsulated *Tubifex* from each tube was then transferred aseptically into tubes containing 9 ml sterile saline separately. To determine the initial bacterial count of *Tubifex*, one gram of depurated *Tubifex* was transferred to a tube containing 9 ml sterile saline. Using a sterile glass rod, the *Tubifex* were macerated, vortexed and diluted by 10 fold serial dilution in sterile saline to appropriate levels. Aliquots (0.1 ml each) from appropriate dilutions were then spread plated on to MRS agar and incubated for 48 h. After incubation, the catalase negative colonies were counted along with total counts. For experimental purpose, bioencapsulation of *Lactobacillus* sp. in *Tubifex* was done for 45 min as above.

Carassius auratus fries of size 0.48–0.55 g weight and 29.10-33.40 mm length and brooders of size 7.71-7.89 g weight and 91.00–96.00 mm length were used. Forty *C. auratus* fries were introduced into each of the two 500L capacity fiberglass reinforced plastic (FRP) tanks containing 400L water. Nine brooders (5♀ and 4♂) were introduced into each of 500L capacity FRP tanks containing 400L water. The experiment was carried out for a period of 90 days. Both groups were fed with commercial pelleted feed at 5% of the body weight daily in two split doses. Bioencapsulated *Tubifex* were fed to the experimental fish at 5% of body weight on every 3rd day as against the 2nd dose of pellet feed. Simultaneously, the control fish were fed with depurated *Tubifex* at 5% of the body weight. The wastes and faecal matter were siphoned out and 50% of the water was exchanged on every 3rd day. The fishes were observed for mortality daily and the dead ones removed immediately and weighed. The length and weight of the fishes of all categories were noted at regular intervals. From these data, the survival percentage and

growth parameters such as wet weight gain, feed conversion ratio (FCR) and specific growth rate (SGR) were estimated.

Bacteriology was performed only for *C. auratus* fries. Two fish each were scooped out from experimental and control tanks and killed by placing them in separate glass beakers containing ice cubes. Gut was dissected out aseptically, macerated, serially diluted and enumerated the total plate count (TPC), total MRS count, *Lactobacillus* sp. count, motile aeromonads, presumptive pseudomonads, total coliforms, lactose fermenters and lactose non-fermenters as per APHA (1992).

A pathogenic strain *Pseudomonas fluorescens* 58C, from the collection of Kolkata University was used to test the disease resistance of the experimental *C. auratus* fries by immersion assay (Austin *et al.* 1995). From each of the bioencapsulated *Tubifex* fed and control *C. auratus* fries tanks, 10 each of fishes were scooped out and introduced into two glass aquaria namely B1 and B2. In a similar manner, 10 each of control *C. auratus* fries were introduced into two aquaria namely C1 and C2. To facilitate infection, two or three scales were removed from five fishes from each aquarium and reintroduced into the respective aquaria. All the aquaria contained 20L sand filtered water and the cell suspension of *P. fluorescens* 58C was added into B1 and C1 tanks in such a way to get a level of $2.0x10^6$ cells/ml of rearing water. The aquaria B2 and C2 received no bacterial inoculum and served as control, respectively for bioencapsulated *Tubifex* fed and control groups. The test animals were maintained in their respective aquaria for 30 days and fed daily with pellet feed on demand. The accumulated faecal matter and other wastes were siphoned-out on every 5^{th} day. Mortality, external signs of infections and behavioural abnormalities were recorded daily. Statistical analyses (χ^2-test and student-t test) were as per Snedecor and Cochran (1974).

Results and discussion

The pre-weighed depurated *Tubifex* were kept in 10ml MRS broth culture of *Lactobacillus* sp. P21 for pre-determined time and the count of *Lactobacillus* sp. on MRS agar as confirmed by catalase test (i.e., catalase negative) were assessed at regular intervals. The results of four different trials made to standardize the bioencapsulation of *Lactobacillus* sp. in *Tubifex* are presented in Table 1. The counts were consistently the same (log 9.08-9.16/g) for up to 60 min, and thereafter, showed a decline to about log 8.88/g in 90 min. The depurated *Tubifex* had TPC in the range of log 7.08-8.18/g.

Table 1. Bioencapsulation of *Lactobacillus* sp. P21 in live fish food organism *Tubifex* sp

Time in min	Total MRS count/g	*Lactobacillus* sp. P21 count/g
0	7.67 ± 0.52	<5.00 ± 0.00
15	9.13 ± 0.06	9.12 ± 0.06
30	9.11 ± 0.04	9.08 ± 0.05

45	9.17 ± 0.06	9.16 ± 0.06
60	9.16 ± 0.06	9.15 ± 0.07
90	8.90 ± 0.22	8.88 ± 0.24

The *C. auratus* fries when fed with bioencapsulated *Tubifex* showed significant improvement in total wet weight gain ($p<0.007$) and FCR ($p<0.01$) compared to control. On the other hand, the SGR and mean survival were slightly higher, although insignificantly ($p>0.05$) in bioencapsulated *Tubifex* fed group than that of the control (Table 2). A significant improvement in total wet weight gain ($p\leq0.009$), FCR ($p<0.01$) and SGR ($p<0.04$) of *C. auratus* brooder fed with bioencapsulated *Tubifex* was seen compared to control fed with depurated *Tubifex*. The mean survival, however, showed no variation (Table 2). The results demonstrated the beneficial effect of feeding *Lactobacillus* sp to *C. auratus* brooder. In general, higher the weight gains, the higher the fecundity.

Table 2. Growth performance of *Carassius auratus* fries and brooder fed with bioencapsulated *Tubifex*

Growth parameters	*Carassius auratus* fries		*Carassius auratus* brooder	
Fries	Bioencapsulated *Tubifex* fed	Control	Bioencapsulated *Tubifex* fed	Control
Total wet weight gain (g)	41.23 ± 0.46[a]	33.93 ± 0.38[a]	84.27 ± 2.23[c]	33.93 ± 0.38[c]
Mean survival (%)	93.75 ± 3.75	86.25 ± 1.25	100.00 ± 0.00	86.25 ± 1.25
Food conversion ratio	4.38 ± 0.04[b]	4.91 ± 0.04[b]	5.28 ± 0.04[d]	5.96 ± 0.06[d]
Specific growth rate	1.30 ± 0.06	1.18 ± 0.02	0.91 ± 0.01[e]	0.66 ± 0.05[e]

Values sharing common superscripts within rows are significantly different. a: $p<0.0067$, $t= 12.19$, $df=4$; b: $p<0.011$, $t= -9.28$, $df=4$; c: $p<0.009$, $t= 10.20$, $df=4$; d: $p<0.01$, $t= -9.27$, $df=4$; e: $p<0.038$, $t=4.99$, $df=4$;

The results of all the above experiments were fairly uniform, probably as a result of supply of unknown growth factors or growth stimulators needed for the growth of *C. auratus*. The results of Gildberg and Mikkelsen (1998) also revealed that the specific growth rates of fish given different diets containing lactic acid bacteria at 10^8/g feed were fairly uniform. It can be inferred from the results of the present study that the bioencapsulation of *Lactobacillus* sp. play an important role in improving the dietary value of *Tubifex*, which favourably influenced the growth and survival of *C. auratus*. Likewise, feeding turbot *(Scophthamous maximus)* larvae with bioencapsulated lactic acid bacteria and *Bacillus toyoi* significantly improved the weight of turbot larvae (Gatesoupe 1999). The observed growth improvement in the present study could probably be attributed to the supply of essential nutrients and enzymes important in digestion process (Douillet and Langdon 1993) or due to alteration in host mechanism (Deeth

1984). de la Banda *et al.* (1995) reported increased enzyme activity in turbot larvae when fed with disabled lactic acid bacteria. According to them, supply of lactic acid bacteria seems to be an effective supplement to counter enzymatic deficiencies. Increased growth may also be attributed to production of vitamins by lactic acid bacteria (Goldin and Gorbach 1992).

As seen in Table 3, none of the bacteriological parameters of the fish gut between the experimental and control groups differed significantly ($p>0.05$). Feeding *C. auratus* fries with bioencapsulated *Tubifex* did not affect the dominant Gram-negative non-beneficial bacteria such as motile aeromonads, total coliforms, lactose fermenters and lactose non-fermenters. Although the presumptive pseudomonads were reduced in the gut of bioencapsulated *Tubifex* fed fish, the difference between this group and the control was observed only at $p<0.06$ level. The fact is that the probiotic strain *Lactobacillus* sp. was recorded at a level of log 5.11/g gut on the 90th day of experimentation, may be because of their inability to withstand peristaltic movement in the gut or lack of attachment site to effect colonization. This probably indicated that the population of *Lactobacillus* sp. was not sufficient enough in the gastrointestinal tract of *C. auratus* fries that could significantly affect the growth or exclude the non-beneficial bacteria. The results contradict with that of the *in-vitro* study of Abraham and Banerjee (2007), which demonstrated that *Lactobacillus* sp. was capable of inhibiting motile aeromonads, coliforms and pseudomonads. This, however, could not be transferred to the *in-vivo* situation when administered via live fish food organism *Tubifex*. Such inability could probably be attributed to the poor colonization of the *Lactobacillus* sp. in the gut of bioencapsulated *Tubifex* fed group. Bogut *et al.* (2000) observed reduction in the incidence of *Escherichia coli* in the gut of sheat fish, *Silurus glanis*, when fed with *Enterococcus faecium* for 58 days. It has been stated that by colonizing the intestinal mucous layer lactic acid bacteria may serve as first defense barrier against invading pathogenic bacteria (Ringo and Gatesoupe 1998). Once colonized, the probiont alter the microbial matabolism by the increase or decrease of relevant enzyme levels, competitively exclude the potential pathogen by the production of inhibitory compounds or competition for space or oxygen (Irianto and Austin 2002).

When the experimental fishes were challenged with *P. fluorescens* 58C, the bioencapsulated *Tubifex* fed group resisted bacterial infection. The survival was significantly high ($p<0.05$) in bioencapsulated *Tubifex* fed group (44%) than in control (22%). Further, the bioencapsulated *Tubifex* fed *C. auratus* showed less (56%) signs of tail/fin rot compared to control (78%, Table 4). Likewise, earlier studies on *Salmo salar* and *Onchorynchus mykiss* (Robertson *et al.* 2000), *C. auratus* (Mondal *et al.* 2003) also presented less evidence of minor health problem such as fin/tail rot in probiotic fed group. The results of the present study demonstrated that *Lactobacillus* sp. P21 was capable of improving the disease resistance and reducing the bacteria induced mortalities in *C. auratus*, besides improving the dietary value of *Tubifex*. Presumably, the

Table 3. Log counts of bacteria in the gut of *Carassius auratus* fries fed with bioencapsulated *Tubifex*

Bacteria	Bioencapsulated *Tubifex* fed				Control			
	Days of culture				Days of culture			
	0	30	60	90	0	30	60	90
Total plate count /g	9.93	9.25	9.45	9.30	9.93	9.38	9.36	8.66
MRS count / g	9.29	8.92	9.02	9.23	9.26	8.79	8.98	8.61
Lactobacillus P21 count / g	<5.16	<5.23	<5.26	5.11	<5.16	<5.32	<5.26	<5.39
Motile aeromonads / g	9.004	8.76	9.01	9.11	9.004	8.83	8.89	8.29
Presumptive pseudomonads / g[a]	3.16	3.09	3.19	3.13	3.16	3.24	3.46	3.34
MPN total coliforms / g	5.12	5.54	6.31	6.58	5.12	5.76	7.86	7.52
Lactose non-fermenters / g	8.94	8.79	8.65	8.91	8.94	8.56	8.41	7.55
Lactose fermenters / g	7.56	5.62	5.81	<5.39	7.56	5.69	5.97	<5.39

a: $P \leq 0.06$; t= -2.31; df= 6

Lactobacillus sp. P21 might have activated the cellular and humoral responses of the ornamental fish as has been suggested in earlier (Irianto and Austin 2002).

Table 4. Disease resistance in *Carassius auratus* fries fed with bioencapsulated *Tubifex*

Treatment	Survival (%)		Infectivity* (%)	
	Infected stock	Uninfected stock	Infected stock	Uninfected stock
Bioencapsulated *Tubifex* fed	44.44[a]	88.89	56.56[b]	11.11
Control	22.22[a]	77.78	77.78[b]	33.33

*: Percentage of fish exhibited tail / fin rot in 30 days of experimental infection. Infected with *Pseudomonas fluorescens* 58C at a level of 1.85×10^6 cells / ml. The pathogenic bacterial cells were inoculated into the rearing water on the 1st, 5th, and 20th days of experiment.

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