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RESEARCH ARTICLE

INVESTIGATION OF BOTANICAL ADDITIVES FOR GROWTH OF *Xiphophorus helleri* (Heckel.) AND IT'S GUT AUTOCHTHONOUS BACTERIAL FLORA

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Effect of Botanical Based Diet Supplementary Probiotic (BBD) and a mixture of fish gut hostile bacterial flora such as *Lactobacillus* sp. and *Bacillus* sp. (FG probiotic) on the growth of ornamental fish red orange sword tail fish of *Xiphophorus helleri* was investigated. The FM at a level of 5g / kg feed had no effect on growth rate rather it reduced the growth compared to control group. On the other hand, in FM probiotic feed fed *X. helleri* except FCR ($P<0.01$) the variations in growth parameters were statistically insignificant. Although, *X. helleri* there existed significant differences in the total wet weight gain and FCR of FM probiotic feed fed and control groups. The FM and SBM probiotic feed fed fishes did not show any significant protection ($P>0.05$). When *X. hellerei* were fed either a diet containing fishmeal (FM) as the crude protein source or a diet containing 50% replacement with soybean meal (BBD) for 10 weeks. The posterior intestine microvilli of BBD-fed fish were significantly shorter and the anterior intestine microvilli significantly less dense than the FM-fed fish. No significant differences in total viable counts of culturable microbial populations were found between the groups in any of the intestinal regions.

Key words: *Carassius auratus, Xiphophorus helleri*, probiotic, *Lactobacillus* sp., *Bacillus* sp., disease resistance

INTRODUCTION

Intensive aquafarming accompanies several disease problems often due to opportunistic pathogens as evident from general aquaculture. High stocking densities, high food inputs and other organic loads stimulate the selection and proliferation of opportunistic bacteria (Austin *et al.*, 1995). Due to this negative balance of the microbial community in rearing water as well as in fish gut, the aquaculturists often face mass mortality of their stocks (Aly *et al*., 2008). Aquafeeds are largely dependent on fishmeal (FM) supply, which places increasing pressure on wild fish stocks and is unsustainable as fish production continues to increase globally (Tacon, 2003; Kim *et al*., 2007). Plant feedstuffs are commonly utilized as key alternative protein sources because of competitive prices and relative availability. The fish farm health management has now become an integral part of ornamental fish Quality Assurance programme Shubhadeep *et al*., 2007 explained probiotic based food for typically suitable for all the metabolical activites. Soybean meal (SBM) and botanical based diets were is moderately rich in protein and is currently one of the major plant proteins frequently included in salmonid diets (Shubhadeep *et al.*, 2007**;** Wilson and Wurtele, 2007). However, SBM has been demonstrated to induce histological and functional changes of the fish gastrointestinal tract, which include enteritis, increased susceptibility to bacterial infection, changes in absorptive cells, increased presence of inflammatory cells and shortening of villi (Roed and Baeverfjord, 2000; Krogdahl, Bakke-McKellep and Baeverfjord 2003; Balcazar and Rojas, 2007). However, with changing scenario farmers are emphasizing on diagnosis and prevention of infection to promote health and production efficiency. The intestinal microbiota of fish responds both directly and indirectly to dietary changes (Ringo and Gatesoupe, 1998; Ringo and Birkbeck, 1999). While much effort has focused on evaluating the extent of SBM-induced histological damage, the effect on the gut microbiota is not so well documented. However, recent investigations have demonstrated SBM-induced changes in the gut microbiota of Atlantic cod, *Gadus morhua* L. (Ringo *et al.*, 2006a), and Atlantic salmon, *Salmo salar* L. (Bakke-McKellep *et al*. 2007; Ringo, Sperstad, Kraugerud and Krogdahl, 2008). Furthermore, Heikkinen *et al.* (2006) observed changes in allochthonous (transient digesta associated) bacterial populations, however, autochthonous (epithelium associated) populations were not investigated. Increased concern about antibiotic resistant micro-organisms has led to several alternatives including use of non-pathogenic micro -organisms as probiotic. India with a vast resource in the form of natural water bodies and species diversity has a great potential to uplift the production of ornamental fish (Jawahar *et al*., 2008). India shares only 0.007% of global trade in ornamental fish that can be raised to 0.1% in the next 5 years. The use of probiotics in aquaculture (Irianto and Austin 2002), and freshwater ornamental fish culture (Abraham *et al*. 2007a, b; Abraham 2008) is well documented. Bacteria belonging to the genus *Lactobacillus* are members of the lactic acid bacteria

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(LAB), a broadly defined group characterized by the formation of lactic acid as the sole or main end product of carbohydrate metabolism. They can be found in plants or material of plant origin, silage, fermented food (Jens, 2008). This communication reports the effect of commercial aquaculture probiotic and a mixture of fish gut antagonistic bacterial flora (*Lactobacillus* sp. and *Bacillus* sp.) on the growth and disease resistance of ornamental fish and *Xiphophorus helleri*.

MATERIALS AND METHODS

The experimental fish goldfish orange swordtail, *Xiphophorus helleri* (Heckel, 1848) were gained respectively from commercial goldfish breeders of Santragachi, Howrah district and swordtail breeders from Nagercoil Aquarium in K.K. District, India. A commercial probiotic for aquaculture application, which contained 2.82×108 cfu of live probiotic cells/g product, comprising *Lactobacillus sporogens* (LP21), *L. acidophilus, Bacillus subtilis, B. licheniformis, Streptococcus faecium, Saccharomyces cerevisiae* together with vitamins and minerals was procured locally for experiment-1. Two hostile bacterial strains, viz., *Lactobacillus* sp. and *Bacillus* sp. P3 isolated respectively from *Cirrhinus mrigala* gut and *Carassius auratus* gut as described in Abraham *et al*. (2007a) were used as probiotic strains in experiment 2. A commercial fish feed containing crude protein (Min 41%), crude fat (Min 6%), crude fibre (Min 3%) and moisture (Max 11%) was used for feeding the experimental fishes. The basic ingredients as per the manufacturer of the feed include: fishmeal, fish lipid oil, fish soluble, medicinal flower meal, lecithin, vitamin-C and vitamin and mineral premixes. The binder used was of the brand Trubind (Animal Health Nutritional Centre from Tirunelveli District). Each 10 g binder contained 100 mg protein, 25 mg cholesterol, 10 mg calcium, 20µg vitamin D3 and 50µg carotenoid.

The commercial aquaculture probiotic was admixed with the basal dry feed at a level of 500mg / kg feed using binder (FM probiotic feed). The probiotic stains LP21 (106 cells / g feed) and BP3 (105 cells / g feed) were added into the basal feed and admixed with binder (FG probiotic feed) as described earlier elsewhere (Abraham *et al*. 2007b). The binder was used at the rate 10-ml / 100 g feed. In control feed, binder alone was added as in test feeds. After admixing the ingredients using binder, the feeds were air dried for 1–2 days and placed in airtight plastic containers separately at room temperature (30 ± 1.5°C). Weighed twenty orange swordtail, *X. helleri* ranging from 1.37 – 1.52 g weight and 46.65 – 48.80 mm length of size introduced into each of six glass aquaria containing 35-liter tap water. During the study period of 30 days with continuous aeration *X. helleri* was fed with FM probiotic feed. The fishes of control tanks were fed with control feed in triplicate.

During the study period of 30 days (60 days for *X. helleri*) with continuous aeration, *C. auratus* and *X. helleri* were fed with FG probiotic feed containing a mixture of *Lactobacillus* sp. P21 and *Bacillus* sp. P3. The fishes of control tanks were fed with control feed in triplicate. In all the cases, feeding was done daily at the rate 5% of the body weight for the body weight for *X. helleri*, in two split doses. The wastes and faecal matter were siphoned out and 75% of the water was exchanged on every day. The fishes were observed for mortality daily and the dead ones removed immediately and weighed. The length and weight of the fish of all categories were noted at regular intervals. From these data, the survival percentage, wet weight gain, feed conversion ratio (FCR) and specific growth rate (SGR) were estimated. A pathogenic bacterium *Pseudomonas fluorescens* 58°C was used in the challenge experiment by immersion assay (Austin *et al.* 1995). Ten fishes each from FM probiotic feed fed and control groups of *X. helleri* from experiment-1 were introduced respectively into the tanks (X1 – X4) containing 20L bore well water as well, ten fishes each from FM probiotic feed fed and control groups of *X. helleri* from experiment -2 were introduced respectively into the tanks (X5 – X8) containing 20L tap water. To facilitate infection, two or three scales were removed from five fishes from each tank and reintroduced into the respective tanks. The cell suspension of *P. fluorescens* 58°C was inoculated into odd numbered tanks in such a way to get a level of 107 cells/ml rearing medium. The even numbered tanks served as control for both probiotic feed fed and control groups of experiment- 1 and 2. These experiments were carried out for a period of 30 days in duplicate and the fishes were fed daily with basal diet on demand. The dead fishes were removed immediately. The accumulated wastes and faecal matter were siphoned out on every 3rd day. Mortality, external signs of infection and behavioural abnormalities were recorded daily. Chi-square ($\chi 2$) test was followed to determine the significance of difference in the survival and disease resistance of the treatment and control groups (Snedecor and Cochran, 1974).

Gastrointestinal microbiology
Conventional culture-based identification

After aseptic dissection, the intestine was divided into two sampling regions with two samples taken per region: the anterior digesta (AD), posterior digesta (PD), anterior mucosa (AM) and the posterior mucosa (PM). The anterior section was determined as the region between the distal most pyloric caeca and the proximal border of the posterior section and the posterior section determined as the region from the increase in diameter of the intestinal tract to the anus. After cutting at the proximal border between the sections, digesta from the anterior and posterior regions was removed by gentle squeezing. The anterior and posterior intestinal mucosal tissue was then excised, cut open and washed thoroughly three times with phosphate-buffered saline (PBS) before homogenization with the help of macerator (MSE). The resulting material from three fish per tank was pooled into one sample, thus yielding three samples per treatment. Samples were then appropriately diluted with PBS and 100 µL was spread onto duplicate tryptone soy agar plates (TSA) after Nielsen and Gram (2004). Plate counts were performed after 7 days aerobic incubation at 20°C and colony forming units (CFU) per gram were determined for viable microbial populations. Twenty-five colonies were randomly taken from plates containing between 30–300 colonies and sub-cultured on TSA until pure cultures were achieved. A total of 1500 isolates were then tentatively placed into groups or genera, according to standard methods (Holt and Bergey, 1994) based on the colony morphology, cell morphology and Gram stain, production of catalase, oxidase, glucose fermentation, motility and endospore formation. Dominant colonies from Gram-negative groups/genera isolated were identified to species level using Microbact™ 24E test kits.

Statistics

Data were transformed where necessary and an independent samples two-tailed *T*-test was carried out using SPSS 15.0

(SPSS Inc.) to evaluate the effect of SBM on intestinal microbiota. Significance was accepted at the $P < 0.05$ level.

RESULT

Log Total Viable Counts (TVC) of allochthonous and autochthonous bacteria isolated from the anterior and posterior intestine of rainbow trout under different dietary regimes are shown in (Tables 2, 3), respectively. Mean log TVC were in the range 5.7–5.8 CFU g^{-1} on the anterior mucosa, 6.0–6.1 CFU g^{-1} on the posterior mucosa, 6.6–6.7 CFU g^{-1} in the anterior region and 6.9 CFU g^{-1} in the posterior region. No significant differences of total viable populations between the dietary groups were found in any of the intestinal regions investigated. Clear differences in microbial populations between the treatments are evident (Tables 2 and 3). Common groups identified belong to the Proteobacteria phylum, in particular *Pseudomonas* spp., *Aeromonas* spp., *Vibrio* spp. and *Enterobacteriaceae*. There was a clear reduction of the level of *Aeromonas* spp. isolated from the BBD -fed fish. This was particularly true with allochthonous populations whereby no *Aeromonas* spp. were recovered from the BBD-fed fish, however, *Aeromonas* spp. accounted for 37.3% (6.2 CFU g^{-1}) in the anterior intestine and 32.0% (log 6.4 CFU g^{-1}) in the posterior intestine of fish fed the FM-based diet. Similarly, autochthonous *Aeromonas* spp. accounted for 23% of the total viable populations of the FM group, but < 5% of the SBM group.

Table. 1. Formulation and nutrient analysis of experimental diets (FM, fish meal; SBM, soybean meal)

Ingredients (g kg^{-1})	Diet	
	FM	BBD
Fishmeal	640	322
Marine fish oil	150	170
Soybean meal[b]	04	60
Wheat flour	199	37
Vitamins[c]	5	5
Minerals[d]	5	5
Tapioca powder	8	8
Rice braun	7	5.2
Nutrient analysis (%) Moisture	8.1	7.2
Protein	46.9	45.1
Lipid	21.8	22.0
Ash	10.2	8.3
NFE*	13.0	17.4
Gross energy[f] (MJ kg^{-1})	22.0	22.4

1. All dietary ingredients produced with naturally available botanical based supplementary feed (Laboratory manual)
2. [a]LT-fishmeal,
3. [b]HiPro soybean meal (48% protein).
4. [c]Vitamin premix,
5. [d]Mineral premix.
6. [e]Nitrogen free extracts = dry matter (DM) − (crude lipid + crude ash + crude protein).
7. [f]Gross energy calculated using factors of 23.62, 39.5 and 17.56 kJ g^{-1} for protein, lipid and carbohydrate, respectively

Use of probiotics in aquaculture began with the commercial preparation meant for terrestrial animals. With increasing intensification in commercial aquaculture, many products are being made available for aquaculture purpose with varying success rate. The results of the present study (Table-1) revealed that the commercial aquaculture probiotic at a level of 5g / kg feed had no effect on the growth rate of *X. helleri*. Moderately, it reduced the growth rate of *X. helleri* compared to control group. The FM probiotic feed also had no significant effect on the total wet weight gain, FCR, SGR of *X. helleri* ($P>0.05$). There survival significant differences in the total wet weight gain and FCR of FM probiotic feed fed and control groups of *X. helleri*. When compared with the autochthonous bacterial population on anterior mucosa possessed viable populations were decreased when compared with posterior mucosa. Similarly CFU count also maximum obtained on posterior mucosa than anterior mucosa region (Table 4). Furthermore among the five dominant isolates *Bascillus subtitlis* Table 5. Composition of *X. hellerei* culturable allochthonous intestinal microbiota from fish fed fishmeal (FM) as protein source and soybean meal (BBD) as 50% protein replacement. Expressed as percentages and log CFU g^{-1} (as determined from percentage of total viable load). $n = 10$, pooled.

Considerably elevated numbers of yeast were observed in the SBM group. This was most evident with regard to the allochthonous populations, with the relative abundance increasing from 14% in the FM to 50% in the SBM group. These isolates were presumptively identified as *Saccharomyces* spp. (smooth butyrous colony, oval/circular cell morphology, no pseudo mycelium and positive glucose fermentation). According to Microbact™ *Aeromonas hydrophila* and *Aeromonas caviae*, *Vibrio* spp. were *Vibrio alginolyticus*, *Pseudomonas* spp. were *Pseudomonas stutzeri* and *Pseudomonas putida* and members of the *Enterobacteriaceae* were *Enterobacter hormaechei* and *Citrobacter* spp. The group of 'Gram-negative cocci', which were only isolated from SBM-fed fish, were identified as *Psychrobacter* spp. The group 'other Gram-positive rods' were identified as *Arthrobacter aurescens*, *Janibacter* spp. and *Streptomyces coelicolor*. *Bacillus* spp. was identified such as SG-1, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus cereus* and *Bacillus pumilus*.

Table 2. Growth performance of *Xiphophorus helleri* fed with commercial aquaculture probiotic, and fish gut probiotic feed containing *Lactobacillus* sp. and *Bacillus* sp. Growth parameters

	Xiphophorus helleri			
Experiment 1	FM probiotic feed	Control	BBD probiotic feed	Control
Total wet weight gain (g)	15.26 ± 0.77a	16.57 ± 0.08a	6.30 ± 3.94	5.67 ± 1.37
Mean survival (%)	100.00 ± 0.00	100.00 ± 0.00	93.33 ± 2.36	96.67 ± 2.36
Food conversion ratio	2.65 ± 0.05	2.44 ± 0.21	3.21 ± 1.16	3.59 ± 0.86
Specific growth rate	1.83 ± 0.53	1.96 ± 0.14	1.03 ± 0.36	0.91 ± 0.25
	FM probiotic feed	Control	BBD probiotic feed	Control
Bottanical Based Diet (BBD)				
Experiment 2	FM probiotic feed	Control	BBD probiotic feed	Control
Total wet weight gain (g)	15.26 ± 0.77a	16.57 ± 0.08a	6.30 ± 3.94	5.67 ± 1.37
Mean survival (%)	100.00 ± 0.00	100.00 ± 0.00	93.33 ± 2.36	96.67 ± 2.36
Food conversion ratio	2.65 ± 0.05	2.44 ± 0.21	3.21 ± 1.16	3.59 ± 0.86
Specific growth rate	1.83 ± 0.53	1.96 ± 0.14	1.03 ± 0.36	0.91 ± 0.25

Values sharing common superscripts within rows are significantly different. a: $P<0.04$, $t = -2.95$, $df = 4$; b: $P<0.04$, $t = -2.95$, $df = 4$; c: $P<0.0066$, $t = 12.22$, $df = 4$; d: $P<0.043$, $t = -2.91$, $df = 4$.

Table 3. Disease resistance *Xiphophorus helleri* fed with aquaculture probiotic and fish gut probiotic feed containing *Lactobacillus* sp. and *Bacillus* sp.

Name	Survival (%)			Infectivity (%)
	Infected stock (in %)	Uninfected stock (in %)	Infected stock (in %)	Uninfected stock (in %)
Experiment-1	*Xiphophorus helleri*			
FM probiotic feed	95.00.	100.0 ± 0.0	15.0 ± 5.0	10.0 ± 0.0
Control	95.00 ± 5.00	100.0 ± 0.0	25.0 ± 5.0	10.0 ± 0.0
SBM probiotic feed	100.00 ± 0.00	100.0 ± 0.0	0.00 ± 0.00	0.00 ± 0.0
Control	100.00 ± 0.00	100.0 ± 0.0	10.0 ± 0.0	5.00 ± 5.0
Experiment-2	FM probiotic feed	Control	BBD probiotic feed	Control
Total wet weight gain (g)	4.29 ± 0.27	3.75 ± 1.03	6.91 ± 1.26[c]	4.93 ± 1.1[c]
Mean survival (%)	55.53 ± 6.32	51.07 ± 3.16	71.67 ± 4.71	68.33 ± 4.7
Food conversion ratio	1.47 ± 0.0[b]	2.16 ± 0.40[b]	1.64 ± 0.21[d]	2.40 ± 0.4[d]
Specific growth rate	2.53 ± 0.02	2.25 ± 0.48	1.03 ± 0.36	0.91 ± 0.2

Table: 4. Composition of *X. hellerei* culturable autochthonous intestinal microbiota from fish fed fishmeal (FM) as protein source and Botanical Based Diet (BBD) as 50% protein replacement. Expressed as percentages and log CFU g^{-1} (as determined from percentage of total viable load). Pooled from ten fishes

Name of the Bacterial flora	FM Ant. mucosa %	FM Ant. mucosa CFU g−1	BBD Ant. mucosa %	BBD Ant. mucosa CFU g−1	FM Post. Mucosa %	FM Post. Mucosa CFU g−1	BBD Post mucosa %	BBD Post mucosa CFU g−1
possible microbial population	5.7	5.0	4.8	5.6	6.5	6.8	6.1	5.7
Enterobacteriaceae	4.5	5.2	-	-	8.4	10.5	10.6	6.7
Pseudomonas spp.[b]	4.6	6.4	2.5	-	9.5	14.6	5.9	9.4
Staphylococcus spp	3.9	5.3	4.6	-	-	8.5	8.7	8.6
Micrococcus varians	5.8	4.1	5.9	5.8	-	9.1	-	2.4
Actinobacter spp.,	6.4	8.2	8.	-	-	4.8	-	7.6
Bacillus subtilis	6.4	4.6	6.4	6.8		11.2	5.6	11.3
Gram positive Rods [g]	5.1	5.9	5.3	8.7	14.5	.68	-	18.6
Gram-Negative (*Proteus mirabilis*)		4.6	0.9	4.9	2.7	5.7	9.6	24.9
Aeromonas spp		4.8	11.9	-	-	7.9	4.5	-
Clostridium		5.1	14.3	20.5	5.6	-	6.51	-
Other		6.8	12.5	14.6	27.6	-	47.1	41.3
Yeasts		2.6	15.6	15.7	19.6	17.3	8.2	5.6
Total Isolates		125	125	125	125	125	6.3	6.5

1. not detected.
2. [a]Dominant isolates identified from Gut ant. Mucosal of FM intaken *X. hellerei* as *Enterobacter* spp. and *Salmonella enterica* and by Microbact™ as *Enterobacter hormaechei* and *Citrobacter* spp.
3. [b]Dominant isolates identified by Microbact™ 24E as *P. stutzeri* and *P. putida.*
4. [c]Dominant isolates identified by Microbact™ 24E as *A. hydrophila* and *A. caviae.*
5. [d]Dominant isolates identified by Microbact™ 24E as *V. alginolyticus.*
6. [e]Dominant isolates identified as *Bacillus* spp. SG-1, *B. subtilis, B. licheniformis, B. cereus* and *B. pumilus.*
7. [f]Dominant isolates identified as *Psychrobacter* spp.
8. [g]Dominant isolates identified as *Arthrobacter aurescens, Janibacter* spp. and *Streptomyces coelicolo*

DISCUSSION

Use of probiotics in aquaculture began with the commercial preparation meant for terrestrial animals. With increasing intensification in commercial aquaculture, many products are being made available for aquaculture purpose with varying success rate. The results of the present study (Table 1) revealed that the commercial aquaculture probiotic at a level of 500mg / kg feed had no effect on the growth rate of *C. auratus* and *X. helleri.* The FM probiotic feed also had no significant effect on the total wet weight gain, FCR, SGR of *X. helleri* (P>0.05) variations made in the in growth parameters were statistically insignificant. There existed significant differences in the total wet weight gain and FCR of FG probiotic feed fed and control groups of *X. helleri.* Many workers have used commercially available products to improve the growth performance of fish successfully. The commercial preparations of *Streptococcus faecium* and a mixture of bacteria and yeast improved the growth and food conversion efficiency of *Cyprinus carpio* (Bogut *et al.*, 1998).

and *Catla catla* (Mohanty *et al*., 1996), respectively. The results of Lara-Flores *et al*. (2003) also indicated that the *Oreochromis niloticus* fry subjected to diets with a probiotic supplement exhibited greater growth than those fed with the control diet. The recent reports on the use of *Lactobacillus* spp. and *Bacillus* spp. (Salinas *et al*. 2005; Balcazar and Rojas-Luna, 2007; Aly *et al*. 2008) also demonstrated the beneficial effects of stimulating the gut immune system and the growth improvements in the fish larvae. The results of the study with FG probiotic corroborate the observations of Carnevali *et al*. (2004), who recorded a significantly decreased larvae and fry mortality when *Lactobacillus fructivorans* (AS17B), isolated from sea bream (*Sparus aurata*) gut, was used a probiotic. The results of the CA probiotic of the present study, however, are in accordance with few of the earlier studies (Murthy and Naik, 2002) conducted on a variety of aquatic animals. For example, reduced growth due to poor digestion of oyster *Crassostrea virginica* fed with higher proportion of yeast and reduced growth due to catabolic effect at higher dose (Murthy and Naik, 2002) in *C. mrigala* have been amply documented. Uma *et al*. (1999) investigated the efficiency of commercial

Table 5. Composition of *X. hellerei* culturable allochthonous intestinal microbiota from fish fed fishmeal (FM) as protein source and soybean meal (BBD) as 50% protein replacement. Expressed as percentages and log CFU g^{-1} (as determined from percentage of total viable load). *n* = 10, pooled.

Name of the Bacterial flora	FM Ant.mucosa		BBD Ant. mucosa		FM Post.mucosa		BBD Post.mucosa	
	%	CFU g^{-}	%	CFU g^{-}	%	CFU g^{-}	%	CFU g^{-}
Viable Bacterial inhabitants		8.6		6.7		7.9		5.9
Enterobacteriaceae[a]	6.3	8.4	3.3	6.2	5.7	5.6	5.3	7.7
Pseudomonas spp.[b]	4.0	6.2	7.3	8.4	6.7	6.7	2.0	7.2
Staphylococcus spp.	–	–	–	–	–	–	–	–
Micrococcus variance	7.7	7.5	15.7	5.8	9.3	6.7	9.7	6.9
Actinotobacter spp.	–	–	0.9	3.5	5.0	6.2	–	–
Aeromonas hydrophila[c]	30.3	8.2	–	–	29.0	7.4	–	–
Vibrio spp.[d]	19.0	5.9	–	1.4	21.0	6.2	–	–
Bacillus spp.[e] (*B. subtilis)*	6.7	6.3	0.9	3.5	1.3	5.0	–	–
Gram-negative cocci[f,–]	–	–	5.3	6.4	–	–	4.7	4.6
Other Gram-negative rods	3.7	8.1	–	–	–	–	0.7	.8
Other Gram-positive rods[g]	9.7	7.6	1.3	6.0	1.0	6.0	34.7	9.4
Yeast	15.7	7.7	32.7	6.4	17.0	6.1	44.3	4.6
Total isolates	150		150		150		150	

1. –, not detected.
2. [a]Dominant isolates identified from Gut ant. Mucosal of FM intaken *X. hellerei Enterobacter* spp.
3. [b]Dominant isolates identified by Microbact™ 24E as *P. stutzeri* and *P. putida.*
4. [c]Dominant isolates identified by Microbact™ 24E as *A. hydrophila* and
5. *A. caviae*.
6. [d]Dominant isolates identified by Microbact™ 24E as *V. alginolyticus.*
7. [e]Dominant isolates identified as *Bacillus* spp. SG-1, *B. subtilis*,
   B. licheniformis, *B. cereus* and *B. pumilus*.
8. [f]Dominant isolates identified as *Psychrobacter* spp.
9. [g]Dominant isolates identified as *Arthrobacter aurescens*, *Janibacter* spp. and *Streptomyces coelicolor*.

probiotic (Lactosacc) containing organisms similar to SBM probiotic feed and observed a systematic reduction in the growth of *Penaeus indicus* when fed with higher dose of lactosacc due to poor digestion and assimilation of yeast and excessive faecal loss. Both probiotic feed fed fishes did not show any significant protection (P>0.05) against *P. fluorescens* 58C, although there was marked difference in the fishes exhibiting fin and tail rot (Table 2). Similarly, Robertson *et al.* (2000) and Abraham *et al*. (2007b) observed less evidence of minor health problems such as fin and tail rot in probiotic fed group. The fact is that the aquatic animals are quite different from the land animals for which the probiotic concept was developed. In finfish and shellfish, gram-negative facultative anaerobes prevail in the digestive tract and symbiotic anaerobes may be dominant in the posterior intestine of some herbivorous tropical fish. *Aeromonas, Plesiomonas* and Enterobacteriaceae are dominant in freshwater fish (Sakata, 1990). Most microbes are transients in aquatic animals and may change rapidly with the intrusion of microbes coming from water and food. A consequence of specificity of aquatic micro flora is that the most efficient probiotics for aquaculture may be different from those of terrestrial species (Steeve *et al*., 2001). Many of the earlier studies used commercial probiotic for land animals and also demonstrated the interest on the use of bacterial addition in aquaculture feeds. But, the survival of probiotic microbes is uncertain in the gastrointestinal tract of aquatic animals and so also the desired beneficial effect as has been observed in CA probiotic feed fed groups. After the pioneer studies by Maeda and Liao, (1992), attempts have been aimed at seeking autochthonous bacterial strains with probiotic properties. Although the results of the present study with antagonistic strains *Lactobacillus* sp. P21 and *Bacillus* sp. P3 isolated from fish gut are encouraging, further studies are required to elucidate their usefulness for commercial application in ornamental fish production. The results of the present study would form the basis for future research and development. As the intestinal microbiota of rainbow trout has been reported to be highly culturable (Spanggaard *et al*., 2000; Huber *et al.* 2004; Ringo *et al*. (2006a, 2008). Heterotrophic aerobic populations within the present study are within the range of values reported in other rainbow trout investigations (Heikkinen *et al*. 2006; Kim, Brunt and Austin 2007). Dietary SBM did not significantly alter viable microbial numbers in the intestinal tract in the present study; these results, together with those found by Ringo *et al*. (2008) suggest that quantitative changes of total viable populations of gut microbiota of salmonids may be less influenced by SBM than in other species, such as Atlantic cod (Ringo *et al*. 2006a) where it often resulted in higher populations. On the other hand, Heikkinen *et al*. (2006) and Bakke-McKellep *et al.* (2007) observed changes in total microbial populations in rainbow trout and Atlantic salmon, respectively. Heikkinen *et al*. (2006) demonstrated that SBM-fed rainbow trout displayed an initial increase of viable intestinal microbes, but after 8 weeks feeding these levels dropped below that of the control-fed fish. Furthermore, Bakke-McKellep *et al.* (2000) observed significant increases of TVC of autochthonous populations in both the mid and distal intestine of SBM-fed Atlantic salmon compared with the control group. Allochthonous populations in the distal intestine were also significantly higher in the SBM-fed fish.

However, despite no change in viable counts in the present study, changes of the microbiota populations comprising the microbial community were observed, confirming previous findings (Heikkinen *et al*. 2006; Ringo *et al*. 2006a, 2008; Bakke-McKellep *et al*. 2007). The inclusion of dietary SBM had a pronounced effect on *Aeromonas* levels isolated from the intestinal tract in the present study. The reason for the large reduction within the SBM-fed fish is not clear, but is comparable with the findings of Heikkinen *et al*. (2006) who identified *Aeromonas* spp. as 19.6% of intestinal isolates from FM-fed rainbow trout but only 9.3% from SBM fish. However, these values should be viewed with caution as they are based on the identification of only 94 isolates. Members of the *Aeromonas* genus, such as *Aeromonas salmonicida* are potentially pathogenic and are responsible for destructive diseases, such as furunculosis (Austin and Austin 1993;

Dalsgaard and Madsen, 2000) and it has been suggested that the intestinal tract is a possible route of infection (Ringo *et al.* 2003, 2007; Birkbeck and Ringo, 2005). Taking this into consideration, the findings of the current study are particularly interesting and worthy of further consideration when conducting future research focusing on the effect of SBM on the intestinal microbiota of fish. Previously, supportive studies were made by Minouru and Takashi, 2001 production of short chain Production of short-chain fatty acids and gas from various oligosaccharides by gut microbes of carp (*Cyprinus carpio* L.) in micro-scale batch culture. Conspicuously elevated levels of autochthonous and allochthonous *Saccharomyces* spp. were identified in the SBM-fed fish in the present investigation. Yeasts have been isolated previously as part of the fish gut microbiota, including rainbow trout (Gatesoupe, 2007). Common strains from rainbow trout have been identified as *Debaryomyces hansenii*, *Saccharomyces cerevisiae*, *Candida* spp. and *Leucosporidium* spp; naturally proliferating yeasts in the fish digestive tract can generally be considered as commensal populations in healthy fish reared under good conditions (Gatesoupe, 2007). The reason for such a large increase in yeast populations observed in the present study may be a direct result of fermentable carbohydrates provided by SBM. Oligosaccharides typically constitute about 4–5% of SBM by dry weight (Obendorf *et al*., 1998). Raffinose and stachyose consists of fructose, glucose and galactose (Lan *et al*., 2007). Yeasts, including *Saccharomyces* spp., are able to ferment various sugars, including glucose and galactose (Barnett 2003); hence, an increase in yeast numbers may be a result of increased available sugars.

Indeed, *Arthrobacter* spp. has previously been identified from the digestive tract of rainbow trout (Huber *et al*. 2004; Kim *et al.* 2007). *Salmonella enterica* are water borne organisms and may have been acquired through the ingestion of food or water. It is not surprising that these aerobic bacteria survive in rich, organic intestinal contents. The group categorized as 'Gram-negative cocci' were identified as *Psychrobacter* spp., which have been previously isolated from the intestinal tract of salmonids (Ringo *et al*., 2006b; Hovda *et al*., 2007; Bakke-McKellep *et al*. 2007; Ringo *et al.* 2008), but interestingly were only isolated from the SBM-fed trout in the present study. This is rather similar to the findings of Ringo *et al.* (2006a, 2008), and Bakke-McKellep *et al.* (2007). Previously, Ringo *et al.* (2006a) only isolated *Psychrobacter* spp. from three intestinal samples mainly FM-based diet (out of six samples investigated); however, *Psychrobacter* spp. were isolated from all intestinal samples of fish fed either SBM or bioprocessed SBM rich diets. In particular, *P. glacincola* was identified from virtually all regions (11 of 12) with levels ranging from log 2.57 to 4.79 CFU g^{-1}. Bakke-McKellep *et al.* (2007) and Ringo *et al.* (2008) observed marginally higher levels of *Psychrobacter* spp. Quantitative analysis shows that the production of all organisms, autotrophic and heterotrophic, pelagic and benthic, large enough to be used directly by the fish (i.e., larger than 37 microns) is adequate to account for less than half of the measured fish growth. Production within the microbial community that flourishes on and rapidly digests the manure organic matter is adequate to produce the measured fish growth. The fish appear to harvest the microorganisms at the level of bacteria and protozoa, by ingesting the small straw-like particles which comprise much of the manure and serve as the substrate for the microbial growth.

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