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INTESTINAL MICROFLORA OF KUTUM *RUTILUS FRISII KUTUM* UNDER DIETARY SUPPLEMENTATION WITH PROBIOTIC AND VITAMIN C

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Abstract

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The purpose of this study was to evaluate the effect of dietary probiotic and vitamin C on intestinal microflora of kutum *Rutilus frisii kutum* juveniles. The juveniles were fed to apparent satiation the diet supplemented with probiotic Primalac® (0, 0.1 and 0.3%), vitamin C (0, 200 and 400 mg kg^{-1}) or their combination for 8 weeks. Result indicated that fish fed with the control diets without supplementation exhibited statistically ($P<0.05$) higher mortality than other treatments. On the basis of carbohydrate utilization profiles using API 50 CHL kits (fermentation pattern), the intestinal detected strains were divided into the three groups (*Lactobacillus brevis*, *L. plantarum*, and *L. lactis*). With increasing the culture period, number of total bacterial counts and lactic acid bacteria increased.

Key words: Probiotic, Vitamin C, Intestinal microflora, Kutum, *Rutilus frisii kutum*

Introduction

Probiotics are used as dietary supplementations in aquaculture and their role in intestinal microbial balance, growth, nutrition, health status and resistance against infectious agents are already established (Gatesoupe, 1999). Previous applications of probiotics have shown to improve growth, survival and feed efficiency (Lara-Flores et al., 2003; Yanbo et al., 2006; Balcazar et al., 2007; El-Dakar et al., 2007; Reid, 2008; Hai and Fotedar, 2009; Denev et al., 2009; Li et al., 2009).

It is well known that intestinal microflora play an important role in the health and nutrition of the host

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(Burr and Gatlin, 2005). Lactic acid bacteria (LAB) are a major commensal bacteria of the intestinal tract of mammals including humans in whom they are used as probiotic (Yan and Polk, 2002; Tuohy et al., 2003). Several species of these bacteria are part of the natural intestinal microflora of healthy fish (Ringo and Gatesoup, 1998). Lactic acid bacteria have probiotic activity and have shown to produce bacteriocins and other chemical compounds that may inhibit the growth of pathogenic bacteria (Nousiainen and Setala, 1993).

Vitamin C or l-ascorbic acid (AA) is important in fish due to its dietary essentiality, rapid degradation in feeds and metabolic functions such as antioxidant activity (Dabrowski, 2001). There have been several studies on the role of vitamin C associated with promotion of growth, survival, feed efficiency, immune response , disease and stress resistance (Sobhanai et al., 2002; Moe et al., 2004; Ai et al., 2006; Falahatkar et al., 2006; Nayak et al., 2007; Eicher et al., 2006).

Kutum aquaculture is aimed at restocking the natural environment (southern Caspian Sea) and every year up to 200 millions of 1 g juveniles are released into the natural environment (Abdoli and Naderi, 2009). Since the lack of information about the synergistic effect of probiotic and vitamin C in fish especially in kutum gut microflora, the objective of the present study was to present information on this topic and survival ratio. This information can be used to identify the predominant LAB strains and assist with the identification of candidates for use as probiotics.

Materials and Methods

Experimental materials and fish

Kutum juveniles were provided from a local hatchery (Rajaee Fish Farm Center, Sari, Iran) and fed with a grinded basal diet of trout (40% protein, 16% fat,12% ash, 3% fiber, and 11% moisture) (Isfahan Mokamel, Iran). The juveniles acclimatized in the two 2000-L fiberglass storage tanks and fed basal diet (4 times daily) for two weeks prior to the commencement of the experiment. The probiotic Primalac® (Star Labs, Clarksdale, MO, USA) was used in this study and comprised of $2.5x10^7$ CFU/g of each the four bacterial groups including *Lactobacillus acidophilus*, *Lactobacillus Kazei, Streptococcus faecium, Bifidobacterium thermophilum*, which totally reached to $2.5x10^8$ CFU/g. The vitamin C (L-ascor-

Table 1

Total mesophilic and LAB counts in the intestine of kutum fed different diets at days 30 and 60

Treatments		Initial	Total count (Day 30)	Total count (Day 60)	LAB count (Day 30)	LAB count (Day 60)
Probiotic, %	Vit. C, mg	Probiotic cells				
(1) 0	0	0	ND[a]	1.1×10^8	ND	8.1×10^6
(2) 0.1	0	2.5×10^8	ND	3.2×10^8	ND	5×10^7
(3) 0.3	0	7.5×10^8	1×10^7	3×10^8	7.5×10^5	4.3×10^7
(4) 0	200	0	$3.1\times^6$	2.4×10^8	3.6×10^5	4.2×10^6
(5) 0.1	200	2.5×10^8	6×10^6	2.4×10^8	4.9×10^5	5.9×10^6
(6) 0.3	200	7.5×10^8	ND	2.2×10^8	ND	6.5×10^6
(7) 0	400	0	1.9×10^6	1.9×10^8	2.8×10^4	6.1×10^6
(8) 0.1	400	2.5×10^8	3.2×10^7	4.1×10^8	9.4×10^5	8.1×10^6
(9) 0.3	400	7.5×10^8	1.1×10^7	4.3×10^8	8.6×10^5	7.3×10^7

[a] ND= Not determined

bic acid) employed in this study was from Science Laboratories (Qazvin, Iran).

Experimental setup

The nine treatments including probiotic (0, 0.1%, and 0.3%) and vitamin C (0, 200, and 400 mg kg^{-1}) in a factorial design (3×3) has been assigned for the experiment (Table 1). Acclimatized fish were distributed randomly in twenty seven 2000-L fiberglass tanks (with three replicates for each treatment and control) which were filled each with 1000 L of well water. During the experimental period, aeration was continuously supplied and different physic-chemical parameters of the rearing water such as temperature, dissolved oxygen and pH were routinely monitored. The pH and temperature varied in the range of 8-8.4 and 17-23°C, respectively. Water flow was 10 $Lmin^{-1}$ and the dissolved oxygen content was more than 7.5 mgL^{-1}. The fish were stocked at 200/tank and cultured for 60 days. The proper amount of probiotic, vitamin C or their mix (Table 1) were added to basal diet and mixed by means of a mixer. Then the feed was air dried for 12 h and stored at 4^0C. The control treated with only basal diet. The feed was given four times daily at 10% body weight per day.

Survival

Survival was measured by counting the number of juveniles at the end of the culture period. Survival rate calculated as follows: Survival (%) = (N_t-N_o) x100, where N_t and N_o are the number of juveniles at the end (t) and beginning (o) of the study.

Microbial analysis

The intestine samples (1 g) of 10 juveniles collected from each tank after 30 and 60 days of feeding and homogenized with 9 ml sterile salt solution in stomacher bags (Stomacher lab-Blender 400). Dilution series were prepared from the homogenates. Mesophilic acterial counts and the presumptive number of lactic acid bacteria (LAB) were examined in duplicate by spread plate technique on plate count agar (Oxide, Basingstoke, UK) and MRS agar (Difco, Detroit, MI, USA) respectively. After 24 h of incubation at 37°C, the number of bacterial colonies was counted and the amount of bacteria was calculated as colony forming unit (cfu). Bacterial counts (BC) in each juvenile were calculated by the following formula: BC (cfu per juvenile) = Number of bacterial colonies on plate x dilutes multiple x volume of the homogenized liquid/ number of juvenile.

Phenotypic characterization

The initial identification of bacteria was based on colony and cell morphology determined by light microscopy, gram staining and catalase reaction. Carbohydrate fermentation profiles were examined with API Rapid CH fermentation strips (bioMerieux, marcy l'Etoile, France) in duplicate at 37°C, in lactobacillus identification medium (CHL broth, API 50 CHL; bioMerieux). The identification software API LAB 50 CHL version 5.0 (bioMerieux) was used to facilitate the interpretation of fermentation patterns.

Data analysis

A 3x3 factorial design using the general linear models (GLM) was used for investigation of main and interaction effects of probiotic and vitamin C on mortality. Comparison of differences for mean values was performed by Duncan's multiple range test ($P < 0.05$) by SPSS 16.

Results and Discussion

Fish fed with the control diets exhibited statistically ($P < 0.05$) higher mortality (19.33±3.44%) than those in the treatments (Figure 1). The lowest mortality (9.33±3.1%) observed in fish fed with the diet supplemented with 0.3% probiotic (diet 3) while no significant differences was observed within the treatments 5, 6, 7 and 8 (between 13 and 14.66%) (Figure 1). Consequently, in the present study we observed significantly higher mortality in the fish fed with control diet, while in probiotic fed fish with high number of gut LAB bacteria, the overall mortality was low. Lower mortality in the juveniles fed the probiotics and vitamin C supplemented diet can improve the product yield resulting lower production costs. In rainbow

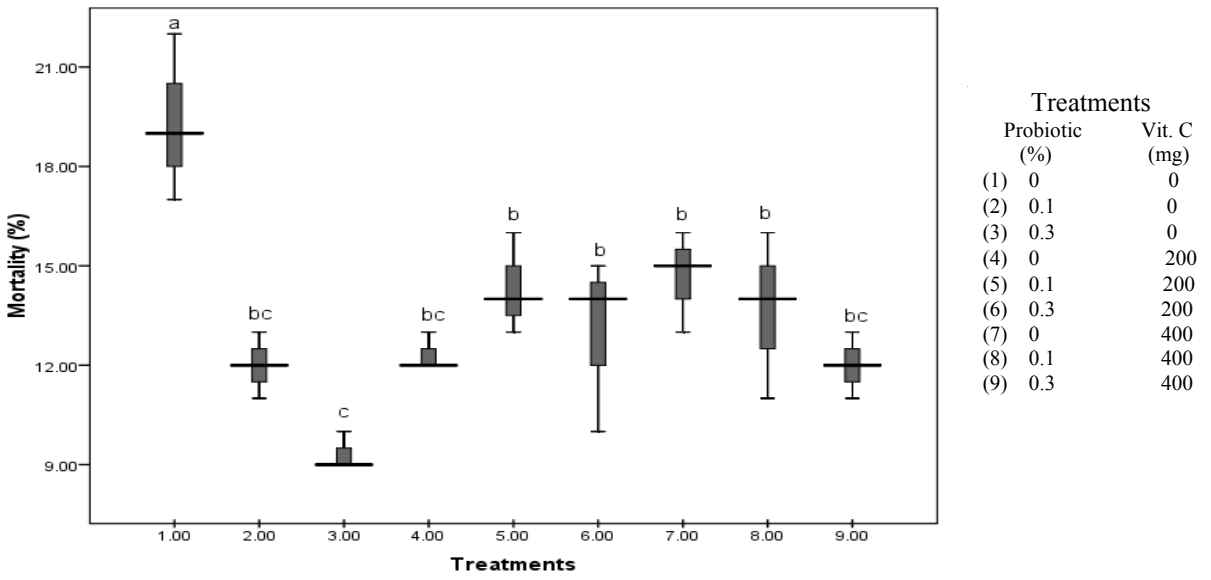

Fig. 1. Mortality of kutum fed diets processed with varied probiotic and vitamin C. Boxes with the same letter are not significantly different ($P > 0.05$) (using GLM and Duncan's test)

trout *oncorhynchus mykiss* (Bagheri et al., 2008), shrimp *Litopenaeus vannamei* (Li et al., 2009) and Western king prawns *Penaeus latisulcatus* (Hai and Fotedar, 2009) higher survival was observed when fed with the diet supplemented with probiotics compare to control group. Lactic acid bacteria reduced the adhesion of pathogenic bacteria in fish and are able to tolerate relatively low pH and bile concentration (Balcazar et al., 2008). In addition, beneficial role of LAB bacteria in disease resistance of fish has been proved. Atlantic cod *Gadus morhua* exposed to *Vibrio anguillarum*, lactic acid bacteria dominated the intestinal flora in surviving fish (Gildberg et al., 1997). Gatesoupe (1994) showed that turbot *Scophthalmus maximus* larvae fed with rotifers enriched with lactic acid bacteria had improved resistance against vibrio infection. High number of lactic acid bacteria (*Lactobacillus plantarum* L. *lactis* and

Table 2

Differentiation of LAB bacterial species living in the gut of fish in different treatments

Treatments		Initial			
Probiotic, %	Vit. C, mg	Probiotic cells	*Lactobacillus brevis*	*Lactobacillus plantarum*	*Lactococcus lactis*
(1) 0	0	0	-	-	-
(2) 0.1	0	2.5×10^8	+	+	-
(3) 0.3	0	7.5×10^8	+	+	+
(4) 0	200	0	-	-	-
(5) 0.1	200	2.5×10^8	-	+	-
(6) 0.3	200	7.5×10^8	+	-	+
(7) 0	400	0	-	+	+
(8) 0.1	400	2.5×10^8	-	-	-
(9) 0.3	400	7.5×10^8	-	+	-

Table 3

Biochemical characterization and API 50 CH grouping of intestinal lactobacillus strains of kutum

	Intestinal lactobacillus strain groups		
	A	B	C
0	-	-	-
Glycerol	-	-	-
Glycerol	-	-	-
D-arabinose	-	-	-
L-arabinose	+	+	+
D-ribose	+	+	+
D-xylose	-	-	-
L-xylose	-	-	-
D-adonitol	-	-	-
Methyl-ßD-xylopyranoside	-	-	-
D-galactose	+	+	+
D-glucose	+	+	+
D-fructose	+	+	+
D-mannose	+	+	+
L-sorbosE	-	-	-
L-rhamnose	-	-	-
Dulcitol	-	-	+
Inositol	-	-	-
D-mannitol	-	+	-
D-sorbitol	-	-	-
Methyl-αD-mannopyranoside	-	+	+
Methyl-αD-glucopyranoside	-	-	-
N-acetylglucosamine	+	+	+
Amygdalin	+	-	+
Arbutin	+	+	+
Esculin	+	+	+
Salicin	+	+	+
D-cellobiose	+	+	+
D-maltose	+	+	+
D-lactose	+	+	+
D-melibiose	+	+	+
D-saccharose	+	+	+
D-trehalose	+	-	+
Inulin	+	-	-

(continued)

Table 3 (continued)

D-melezitose	-	-	-
D-raffinose	+	-	-
Amidon	-	-	-
Glycogen	-	-	-
Xylitol	-	-	-
Gentiobiose	+	+	+
D-turanose	-	-	-
D-lyxose	-	-	-
D-tagatose	-	+	+
D-fucose	-	-	-
L-fucose	-	-	-
D-arabitol	-	-	-
L-arabitol	-	-	-
Gluconate	-	-	-
2- ketogluconate	+	-	-
5-ketogluconate	-	-	-
Species affiliation	*Lactobacillus brevis*	*Lactobacillus plantarum*	*Lactococcus lactis*
Confidence	78%	99.70%	97.00%

- = Negative reaction, + = Positive reaction

L. fermentum) was observed in rainbow trout that did not exhibit signs of disease during furuncolosis infection (Balcazar et al., 2007). Enhanced survival and immune response was demonstrated in rainbow trout (Nikoskelainen et al., 2003) and gilthead seabream *Sparus auratus* (Salinas et al., 2005) fed with lactic acid bacteria.

The maximum total viable counts at day 60 were detected in the treatment 9 (0.3% probiotic and 400 mg kg^{-1} vitamin C) while the minimum was in the juveniles fed with diet 1 (Table 1). The total viable counts significantly showed the increasing tendency with increasing the culture period. The differences in intestinal lactic acid bacteria among juveniles generally showed the similar tendency to that in total viable counts (Table 1). Similarly, the maximum LAB counts at day 60 showed the same result in the treatment 9 (0.3% probiotic and 400 mg kg^{-1} vitamin C), while, the minimum was in treatment 4 (0% probiotic and 200 mg kg^{-1} vitamin C) (Table 1). In trout fed with the diet supplemented with probiotic, the counts of bacteria were higher than the control (Bagheri et al., 2008). He et al. (2009) indicated that with increasing the supplementation levels of DVAQUA (*Saccahromyces cerevisiae* as culture supplement), the counts of beneficial bacteria increased. In our study, isolates were classified into three groups (*Lactobacillus brevis*, *L. plantarum*, and *L. lactis*) (Table 2) according to the results of carbohydrate utilization testing using API 50 CHL systems (API LAB 50 CHL software) for the species identification (confidence interval, 78-99.7%) (Table 3). However, the initial combination of LAB strains of supplemented feed with probiotic was completely different from detected LAB bacteria of kutum gut. The LAB species *Lactobacillus plantarum* exhibited the more existence in the gut of kutum (treatments 2, 3, 5, 7, and 9) when compared to the two other LAB bacteria (Table 2). According to Table 2, treatments 1 (control with no supplementation) and 4 (0% probiotic and 200 mg

kg^{-1} vitamin C) showed nothing occurrence of the three detected LAB bacteria.

Conclusion

Addition of probiotic and vitamin C in kutum basal diets improved survival. The predominant LAB discovered in this study may be candidates for use as pobiotic bacteria in fish, thus probiotic properties of these LAB bacteria suggest to be characterized in further studies.

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