Lat. Am. J. Aquat. Res., 41(3): 490-497, 2013
DOI: 103856/vol41-issue3-fulltext-12

Research Article

The use of lactic acid bacteria isolated from intestinal tract of Nile tilapia (*Oreochromis niloticus*), as growth promoters in fish fed low protein diets

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ABSTRACT. In this study, the effect as growth promoter of five lactic acid strains (*Enterococcus faecium*, *E. durans*, *Leuconostoc* sp., *Streptococcus* sp. I and *Streptococcus* sp. II), isolated from intestinal tract of Nile tilapia (*Oreochromis niloticus*), was evaluated. Eight isocaloric diets were formulated: one containing 40% of protein as positive control, and seven with 27% protein. Five diets with 27% protein were supplemented with one of the isolated lactic acid bacteria in a concentration of $2.5x10^6$ cfu g^{-1} of diet. A commercial probiotic based on *S. faecium* and *Lactobacillus acidophilus* was added at the same concentration to one 27% protein diet as a comparative diet, and the last diet was not supplemented with bacteria (negative control). Tilapia fry (280 mg basal weight) stocked in 15 L aquaria at a density of two per liter were fed for 12 weeks with experimental diets. Results showed that fry fed with native bacteria supplemented diets presented significantly higher growth and feeding performance than those fed with control diet. Treatment with *Streptococcus* sp. I isolated from the intestine of Tilapia produced the best growth and feeding efficiency, suggesting that this bacteria is an appropriate native growth promoter.

Keywords: probiotics, Nile tilapia, *Oreochromis niloticus*, growth promoter, lactic acid bacteria.

Uso de bacterias ácido lácticas aisladas del tracto intestinal de tilapia nilótica (*Oreochromis niloticus*) como promotores de crecimiento en peces alimentados con dietas bajas en proteína

RESUMEN. Se evaluó el efecto como promotores de crecimiento de cinco cepas de bacterias ácido lácticas (*Enterococcus faecium*, *E. durans*, *Leuconostoc* sp., *Streptococcus* sp. I y *Streptococcus* sp. II) aisladas del tracto intestinal de tilapia nilótica (*Oreochromis niloticus*). Se formularon ocho dietas isocalóricas: una conteniendo 40% de proteína como control positivo y siete con 27% de proteína. Cinco dietas con 27% de proteína fueron suplementadas con cada una de las bacterias aislada a una concentración de $2,5x10^6$ ufc g^{-1} de alimento. Un probiótico comercial a base de *S. faecium* y *Lactobacillus acidophilus* a la misma concentración de inclusión bacteriana a una dieta con 27% de proteína como dieta comparativa, y la última dieta no fue suplementada con bacterias (control negativo). Juveniles de tilapia (280 mg de peso basal) fueron distribuidos en acuarios de 15 L de capacidad, a una densidad de dos juveniles por litro, alimentados durante 12 semanas con las dietas experimentales. Los resultados mostraron que los organismos alimentados con las dietas suplementadas con bacterias nativas presentaron crecimiento y asimilación del alimento significativamente mayor que las dietas control. El tratamiento con *Streptococcus* sp. I, aislada del intestino de la tilapia, produjo el mejor crecimiento y la mejor eficiencia alimenticia, sugiriendo que esta bacteria es apropiada como un promotor de crecimiento nativo de tilapia.

Palabras clave: probiótico, tilapia nilótica, *Oreochromis niloticus*, promotor de crecimiento, bacterias ácido lácticas.

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INTRODUCTION

Aquaculture is a fast-growing and rapidly expanding multibillion dollar industry. Marine capture fisheries and aquaculture supplied the world with about 104 million ton of fish in 2004 (FAO, 2007). Of this total, marine aquaculture accounted for about 18%, where shrimp from aquaculture continues to be the most important commodity traded in terms of value (2.4 million ton). Worldwide, the aquaculture sector has been expanding at an average compounded rate of 9.2% per year since 1970, compared with only 1.4% for capture fisheries and 2.8% for terrestrial-farmed meat production systems. During the last decades, antibiotics used as traditional strategy for fish diseases management but also for the improvement of growth and efficiency of feed conversion. However, the development and spread of antimicrobial resistant pathogens were well documented (Kim *et al.*, 2004; Cabello, 2006; Sørum, 2006).

There is a risk associated with the transmission of resistant bacteria from aquaculture environments to humans, and risk associated with the introduction in the human environment of nonpathogenic bacteria, containing antimicrobial resistance genes, and the subsequent transfer of such genes to human pathogens (FAO, 2005). Considering these factors, as well as the fatal effect of residual antibiotics of aquaculture products on human health, the European Union and USA implemented bans on, or restricted the use of antibiotics (Kesarcodi-Watson *et al.*, 2008).

In connection with the ban of antibiotic growth promoters new strategies in feeding and health management in fish aquaculture practice have received much attention (Balcázar *et al.*, 2006). In addition, the global demand for safe food has prompted the search for natural alternative growth promoters to be used in aquatic feeds. There has been heightened research in developing new dietary supplementation strategies by promoting various health and growth compounds as probiotics (Denev, 2008).

The importance of probiotics in human and animal nutrition is widely recognized (Fuller, 1992; Rinkinen *et al.*, 2003), in recent years, the role of probiotics in nutrition and health of certain aquaculture species have also been investigated (Gatesoupe, 1999; Verschuere *et al.*, 2000; Kesarcodi-Watson *et al.*, 2008; Ringo *et al.*, 2010; Merrifield *et al.*, 2010). It appears that probiotics provide benefits by establishing favorable microbial communities, such as lactic acid bacteria and *Bacillus* sp. in the gastrointestinal track, which may alter gut morphology and produce certain enzymes and inhibitory compounds causing improved digestion and absorption of nutrients, as well as enhanced immune response (Verschuere *et al.*, 2000). Several studies have demonstrated that use of probiotics improves health of larval and juvenile fish, disease resistance, growth performance and body composition, however, the mode of action in fish species may vary between farmed fish species cultured in freshwater and marine environments.

The use of probiotic in feeds to improve growth of different fish species including African catfish, (Al-Dohail *et al.*, 2009); Senegalese sole (Sáenz de Rodrigáñez *et al.*, 2009), Nile tilapia (Lara-Flores *et al.*, 2003, 2010; El-Haroun *et al.*, 2006), Japanese flounder (Taoka *et al.*, 2006), gilthead sea bream and sea bass (Carnevali *et al.*, 2006) has been investigated. The effects of probiotics have been linked to modulation of gut microbiota and establish-ment of the beneficial microorganisms, higher specific and total digestive enzyme activities, in the brush border membrane, which increases the nutrient digestibility and feed utilization (Verschuere *et al.*, 2000; Balcazar *et al.*, 2006; Kesarocodi-Watson *et al.*, 2008). In addition, the production of vitamins by these gut microbiota could also increase vitamin synthesis and improve fish health (Holzapfel *et al.*, 1998). This study was carried out to find the effect of isolated acid lactic bacteria from intestinal tract of Nile tilapia (*Oreochromis niloticus*), on feed efficiency and growth of fry Nile tilapia fed with low protein diets.

MATERIALS AND METHODS

Bacterial strains

Five strains of lactic acid bacteria isolated from Nile tilapia intestine were characterized on the basis of morphological, physiological and biochemical test by Bergey´s Manual of Systemic Bacteriology (Holt *et al.*, 1993). Axenic cultures of the purified bacteria were tentatively identified, using Mini-API System Bio-Merieux, as *Enterococcus faecium, E. durans, Leuconostoc* sp., *Streptococcus* sp. I and *Streptococcus* sp. II. Commercial probiotic containing mixture of *Lactobacillus acidophilus* and *S. faecium* was used as control.

All bacteria were grown aseptically in 10 mL of MRS broth for 24 h at 35 ± 2°C. Five mL were transferred under aseptic conditions into 250 mL of MRS broth and held on a shaker at 150 rpm for 24-48 h at 35 ± 2°C. The cells of each isolate were harvested by centrifugation at 10,000 rpm for 15 min and washed twice with phosphate buffer (PB) having pH 7.0, then dispensed in 5 mL PB.

Experimental diets

Eight isocaloric diets were formulated: one containing 40% protein, and the other seven with 27% of protein level. The lower protein inclusion in the latter diets was used as a stress factor since that the optimum protein level for fry tilapia is 40% (Tacon, 1984). Each one of the lactic acid bacteria isolates was added to lower protein diets in a concentration of $2.5x10^6$ cfu g^{-1} of diet. The commercial probiotic was added to one diet with 27% protein in a concentration of $2.5x10^6$ cfu g^{-1} of diet for comparison. Finally, positive and negative control diets were formulated with 40 and 27% of protein level, respectively, both diets without bacterial supplements. To all diets, 0.5% chromic oxide was added for determining digestibility. Tables 1 and 2 shows diet formulation and proximate composition respectively.

Experimental setup

Population density was also used as a stress factor, under the assumption that overpopulation is one of the main growth-inhibiting factors in intensive aquaculture systems. To this end, 32 glass aquaria of 15 L capacity were stocked at a 30 organisms per aquaria (2 fry per liter). All fry had similar average initial weights (280 ± 10 mg). The different diet formulations were assigned within the aquaria. The animals were allowed to adapt to the experimental system for a week, and fed with a conventional diet, after which the different treatments were randomly assigned to the aquaria, with four replicates per treatment.

Feed was manually administered *ad libitum* four times a day, for 12 weeks. A daily record was kept of feed offered. Bulk weight was measured weekly to follow growth in weight and calculate survival and feeding ration. Briefly, the fish were taken from each tank using a net previously disinfected with a 1% benzalkonium chloride solution. Initial mean weight (IMW), final mean weight (FMW), specific growth rate (SGR), Feed conversion ratio (FCR), survival, protein efficiency ratio (PER), apparent nitrogen utilization (ANU), apparent organic matter digestibility (AOMD) and apparent protein digestibility (APD) were measured using the following equations:

SGR = 100[(log. final body weight-log initial body weight)/time (days)]

FCR = individual food intake/individual weight gain

PER = individual protein intake/individual weight gain

ANU = 100(carcass nitrogen deposition/N intake)

Beginning in the third week of the experiment, feces were collected by siphoning the aquaria 30 min after the second daily feeding, to minimize leaching. Scales were removed from the collected feces, the feces were oven dried at 105°C for 24 h, and then stored in hermetic containers under refrigeration to preserve them until analysis.

Table 1. Formulation of experimental diets.

	Diet							
Ingredients (g kg^{-1})	CON 40	CON 27	ED	B_2	B_3	A_1	A_2	ALL 27
Anchovy fish meal	542.3	366.0	366.0	366.0	366.0	366.0	366.0	366.0
Cod liver oil	0.0	18.5	18.5	18.5	18.5	18.5	18.5	18.5
Soybean oil	32.6	64.0	64.0	64.0	64.0	64.0	64.0	64.0
Yellow corn starch	345.0	470.4	462.6	468.1	468.3	462.2	460.4	469.4
Mineral premix[1]	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Vitamin premix[2]	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Chromic oxide	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Microorganisms	0.0	0.0	7.8	2.5	2.3	8.2	0.6	0.1

CON 40: Positive control, CON 27: Negative control, ED: Diet supplemented with *E. faecium*, B_2: Diet supplemented with *E. durans*, B_3: Diet supplemented with *Leuconostoc* sp., A_1: Diet supplemented with *Streptococcus* sp. I, A_2: Diet supplemented with *Streptococcus* sp. II; ALL 27: Diet supplemented with commercial probiotic. [1]Jauncey & Ross, (1982). [2]Tacon, (1984).

Table 2. Proximate composition of experimental diets (% dry matter).

	CON 40	CON 27	ED	B_2	B_3	A_1	A_2	ALL 27
Moisture	7.30	6.63	6.48	7.27	7.43	7.51	6.89	7.30
Crude protein	39.90	27.09	27.90	27.66	27.56	28.02	28.65	27.68
Ether extract	8.62	8.65	7.95	10.25	9.42	10.42	8.01	9.25
Crude fiber	1.66	3.87	3.83	3.94	4.33	4.01	3.75	3.98
Ash	10.27	10.90	10.39	9.86	10.02	10.00	9.84	10.23
Nitrogen-free extract	32.25	42.86	43.45	41.06	41.24	40.04	44.86	41.56
Gross energy (MJ kg^{-1})	19.95	19.75	20.09	19.83	19.92	20.67	20.82	19.65

For water quality control, temperature and dissolved oxygen were measured daily, and weekly analyses were done of total ammonium, nitrite, nitrate and pH levels, using standard methods (APHA, 1989). The following values (±SD), appropriate for tilapia cultivation, were used: temperature, 28.83 ± 0.45°C; dissolved oxygen, 5.71 ± 1.16 mg L^{-1}; pH 7.98 ± 0.45; ammonia, 0.09 ± 0.04 mg L^{-1}; nitrite, 0.08 ± 0.02 mg L^{-1} and nitrate, 5.93 ± 0.61 mg L^{-1}. Every third day, each aquaria was partially cleaned and the water partially changed (1:l). Once a week, the same day bulk weight measurement was done, the aquaria were completely cleaned and a total change of water in the system carried out.

Chemical analysis

Proximate chemical analyses of diet ingredients were made and a sample of fish, at the beginning and end of the experiment, according to standard methods (AOAC, 1995). Gross energy in the feed was determined by combustion in a Parr adiabatic calorimeter. To evaluate digestibility, the chromic oxide content of each diet and the collected feces were analyzed using the acid digestion method (Furukawa & Tsukahara, 1966). Protein content was also determined for the feces, to assess protein digestibility.

Statistic

Growth performance and feed utilization efficiency parameters were statically compared using one-way ANOVA ($P < 0.05$), and differences among means were identified using Duncan Multiple Range Test. Analyses were carried out with the StatGraphics Plus Version Centurion XV computer software. Arcsin transformation of raw data were made when necessary.

RESULTS

The growth performance including IMW, FMW, SGR, FCR, PER, ANU, AOMD, APD and survival rate of Nile tilapia are shown in Table 3. No significant differences were observed in IMW among treatments. Fish fed with CON 27 diet showed significantly lower survival (66.7%) than those fed with bacteria-supplemented and positive control diets ($P < 0.05$). The highest survival was recorded for CON 40 and diet supplemented with *E. durans* (100%). The treatment CON 27 presents the lower FMW (5.95 g). Fish fed with diets supplemented with native bacteria exhibited higher FMW compared to controls diets.

The ALL 27 treatment resulted with the significantly higher FCR (2.02) among the bacteria-supplemented diets, thought all the other bacteria-containing diets showed FCR significantly lower than those for the controls diets ($P < 0.05$). The best FCR recorded for the A_1 treatment (1.19).

The PER was significantly higher in treatment A_1 (2.53) than in the others treatments. The lower PER was recorded for the CON 40 treatment (1.36). Fish from A_1 treatment presented ANU significantly greater (48.4%), in comparison with the other treatments. The lowest biological value was observed in control diets.

In general, AOMD and APD were variable among treatments. The maximum value were obtained in the A_2 treatment (AOMD = 95.08%; APD = 94.28%), which was statically different from the rest of the treatments.

Whole body composition data are presented in Table 4. The moisture content showed no significant difference among fish fed with the experimental diets, and it ranged from 72.9 to 76.4%. The uppermost two values (18.7 and 18.4%) of crude

Table 3. Growth and feeding performance of fish feed with diets supplemented with bacteria.

	Diet								
Mean values[1]	CON 40	CON 27	ED	B_2	B_3	A_1	A_2	ALL27	±SE[2]
Survival (%)	100.00^{a}	66.66^{d}	91.66^{ab}	100.00^{a}	89.58^{abc}	91.66^{ab}	91.28^{abc}	87.11^{c}	8.125
Initial mean weight (g)	0.28^{a}	0.28^{a}	0.29^{a}	0.28^{a}	0.28^{a}	0.29^{a}	0.29^{a}	0.28^{a}	0.012
Final mean weight (g)	6.74^{ab}	5.95^{a}	7.22^{ab}	5.80^{a}	7.67^{ab}	9.89^{c}	6.99^{ab}	8.48^{bc}	0.611
SGR (% day^{-1})[3]	3.77^{ab}	3.65^{a}	3.82^{ab}	3.59^{a}	3.90^{ab}	4.18^{b}	3.80^{ab}	4.14^{b}	0.124
FCR[4]	1.84^{f}	2.00^{g}	1.57^{d}	1.64^{e}	1.51^{c}	1.19^{a}	1.42^{b}	2.02^{g}	0.007
PER[5]	1.36^{a}	1.71^{c}	2.27^{f}	2.05^{d}	2.23^{e}	2.53^{g}	2.29^{f}	1.66^{b}	0.010
ANU (%)[6]	21.46^{a}	30.48^{b}	42.08^{e}	31.64^{c}	39.66^{d}	48.45^{f}	41.61^{e}	31.31^{c}	0.033
AOMD (%)[7]	90.92^{c}	81.61^{a}	90.71^{c}	91.58^{d}	89.68^{b}	91.78^{d}	95.08^{e}	90.36^{c}	0.750
APD (%)[8]	91.57^{d}	76.62^{a}	89.19^{b}	91.00^{d}	89.06^{b}	90.60^{c}	94.28^{e}	90.17^{c}	0.830

[1]Values with the same superscript in the same row are not statistically different ($P > 0.05$), [2]Standar error, calculated from mean-square error of the ANOVA, [3]Specific Growth Rate, [4]Food Conversion Ratio, [5]Protein Efficiency Ratio, [6]Apparent Nitrogen Utilization, [7]Apparent Organic Mater Digestibility, [8]Apparent Protein Digestibility.

Table 4. Body composition of fish fed diets supplemented with bacteria.

		Diet								
Composition (% wet weight)	Initial	CON 40	CON 27	ED	B_2	B_3	A_1	A_2	ALL 27	± S.E.[1]
Moisture	81.09	74.93^{c}	73.47^{b}	74.42^{c}	76.44^{d}	72.93^{a}	73.76^{b}	74.33^{c}	73.68^{b}	8.13
Crude protein	11.80	15.22^{a}	17.08^{b}	17.88^{b}	14.79^{a}	17.27^{b}	18.72^{c}	17.50^{b}	18.37^{c}	0.01
Crude lipid	3.06	5.33^{b}	6.45^{e}	5.87^{c}	4.73^{a}	7.24^{a}	6.18^{d}	5.99^{cd}	6.75^{f}	0.61
Ash	2.46	3.49^{e}	3.76^{f}	2.56^{c}	3.54^{e}	2.72^{d}	1.81^{b}	2.73^{d}	1.25^{a}	0.12

[1]Values with the same superscript in the same row are not statically different ($P > 0.05$).

protein were achieved for fish fed diets A_1 and ALL27, with no significant difference. Fish from B_2 treatment showed lower lipid content (4.7%) in comparison with the other treatments. Statistical differences were observed also in the body ash content among fish fed with the different diets, with significantly lower content in fish from ALL 27 treatment (1.3%).

DISCUSSION

Many studies on probiotics in aquaculture have used *in vitro* models of specific bacteria as antagonists of pathogens (Vine *et al.*, 2004, 2006), measured the survival of probiotic in fish gut (Andlid *et al.*, 1998), or evaluated the beneficial effect of probiotic on health management, disease resistance and immune response of fish (Li & Gatlin III, 2004; Shelby *et al.*, 2006). Other important effect of the use of probiotic, that it is not extensively study, but demonstrated an important effect, is the feed efficiency and the growth promotion (Gatesoupe, 2002; Lara-Flores *et al.*, 2003, 2010).

In this study, groups administered diets with lactic acid bacteria showed similar and superior survival results when compared with positive and negative control groups. Similar results were observed by Suyanandana *et al.* (2002) when administered *Lacto-bacillus* sp. isolated from the intestine of Nile tilapia.

Probiotics are biopreparations containing living microbial cells that optimize the colonization and composition of the growth and gut micro flora in animals, and stimulate digestive processes and immunity (Bomba *et al.*, 2002). The results of the present study confirm the results from other studies that the incorporation of probiotic in the diets can improve growth performance in terms of SGR, FCR and PER. Gatesoupe (1991) reported increased weight gain in *Scophital mus* larvae fed a diet incorporating lactic acid bacteria and *Bacillus toyoi*. In the present study, fish fed lactic acid bacteria grew faster than those fed a control. It has been reported

that the improvement of growth by using probiotics is related to an enhancement of nutrition (El-Haroun *et al.*, 2006), as some probiotic strains may serve as a supplementary source of food and their activity in the digestive tract may be a source of essential nutrients (Balcazar *et al.*, 2006). According with Ghosh *et al.* (2007), most of this enhancement is reflected in the whole body proximal composition of fish. In the present experiment, and regardless of the treatments with lactic acid bacteria, the whole body composition of *O. niloticus* showed a trend of higher values of protein, which might indicates a better utilization of diet nutrient provided by the probiotic cells.

The mechanisms by which probiotic bacteria stimulate growth rate are not yet clearly. The improvement of feed utilization for fish fed diet, supplemented with probiotics, could be due to improvement in the intestinal microbial flora balance which, in turn, will lead to better absorption quality, increased enzyme activities (Tovar-Ramírez *et al.*, 2002; Balcazar *et al.*, 2006; Waché *et al.*, 2006; Al-Dohail *et al.*, 2009; Lara-Flores *et al.*, 2010), and more degradation of higher molecular weight protein to lower molecular weight peptides and amino acids (De Schrijever & Ollevier, 2000). Especially, the stimulating growth by probiotics containing LAB strains has been associated with improved feed conversion ratio and protein efficiency ratio attributed to an increase in lactic acid and cellulolytic and amylolytic enzyme production (Kesarcodi-Watson *et al.*, 2008). These contribute towards optimizing the digestion and use of protein for growth, that will result in more efficient protein in fish diets. The probiotic, after transit thought the stomach, they attach in the intestine and use a large number of carbohydrates for their growth and produce a range of relevant digestive enzymes (amylase, protease and lipase), that increase the digestibility of organic matter and protein, produce a higher growth, prevent intestinal disorders and produce or/and stimulate a pre-digestion of secondary compounds present principal in plant sources (El-Haroun *et al.*, 2006; Lara-Flores *et al.*, 2010). Moreover, the nutritional benefits of probiotic bacteria have been attributed to the synthesis of B vitamins and short chain fatty acids in the intestine, and the higher availability of trace elements (Holzapfel *et al.*, 1998; Lara-Flores & Aguirre-Guzmán, 2009). Our observation shows that a significant increase in body weight, and better efficiency, occur in fish fed with native bacteria supplemented specifically with the *Streptococcus* sp. I.

The present investigation showed that the addition of native bacteria in Nile tilapia fry diets improved animal growth and mitigated the effect of stress factors, such as the low protein level in diets. All native bacterial strains used in the present study were effective in stimulating fish performance. *Streptococcus* sp. I produced the best results, and it could be a good candidate for optimizing growth and feed utilization in intensive tilapia culture.

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Received: 13 November 2012; Accepted: 9 June 2013

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