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Research Article

Quantification of intestinal bacteria, operating cost and performance of fingerlings Nile tilapia subjected to probiotics

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ABSTRACT. The use of microorganisms of the genus *Bacillus* in aquaculture is a nutritional management practice that is rapidly expanding in regions with intensive fish farming. This study aimed to quantify the total bacteria and total coliforms from the intestinal microbiota and estimate the partial operating costs and growth performance of Nile tilapia (*Oreochromis niloticus*) of the GIFT strain. A total of 1,200 post-larvae (24.7 ± 0.50 mg) were distributed into 24 aquaria (0.03-m³ capacity) within a completely randomized design in 2 x 3 factorial (phase x bacteria), with four replications. Each aquarium, containing 50 post-larvae (sex reversal phase) or 30 fish (fingerlings phase), it was considered to be an experimental unit, consisting of three treatments (diet+*Bacillus subtilis* C-3102, diet+*Bacillus cereus* var. *toyoi* and diet without probiotic addition). The quantification of the total bacteria and total coliforms of the intestinal microbiota of tilapia were influenced ($P < 0.05$) by the stages of culture. The bacterial count was not influenced ($P > 0.05$) by adding probiotics in the diets and no effect of the interaction between phase and bacteria was observed. The weight gain, average daily weight gain, specific growth rate and apparent feed conversion were not affected ($P > 0.05$) by inclusion of probiotics as part of the diets. The inclusion of *B. subtilis* and *B. cereus* as part of diets for Nile tilapia promotes intestinal colonization and improves the survival rate without negatively influencing the feed intake, total biomass, gross revenue and partial operating costs and net revenue. Therefore it recommends the use of these probiotics to growth of tilapia fingerlings Nile, GIFT strain.

Keywords: economic analysis, microbiology, tilapia culture, probiotics, aquaculture.

Cuantificación de las bacterias intestinales, costo de operación y desempeño de alevines de tilapia del Nilo sometido a probióticos

RESUMEN. El uso de microorganismos del género *Bacillus* en la acuicultura es una práctica de manejo nutricional que se está expandiendo rápidamente en las regiones con la piscicultura intensiva. Este estudio tuvo como objetivo cuantificar las bacterias totales y coliformes totales de la microbiota intestinal y estimar los costos operativos parciales y comportamiento productivo de la tilapia del Nilo (*Oreochromis niloticus*) de la cepa GIFT. Un total de 1.200 post-larvas ($24,7 \pm 0,50$ mg) fueron distribuidas aleatoriamente en 24 acuarios (capacidad 0,03 m³) en un diseño completamente al azar en factorial 2 x 3 (fase x bacterias), con cuatro réplicas. Cada acuario, que contiene 50 post-larvas (fase de inversión sexual) o 30 peces (fase de alevines), se consideró como una unidad experimental, que consta de tres tratamientos (dieta+*Bacillus subtilis* C-3102, la dieta+*Bacillus cereus* var. *toyoi* y dieta sin adición de probiótico). La cuantificación de las bacterias totales y coliformes totales de la microbiota intestinal de tilapia fueron afectados ($P < 0,05$) por las etapas del cultivo. El recuento de bacterias no fue afectado ($P > 0,05$) mediante la adición de probióticos en la dieta y no se observó efecto de la interacción entre fase y bacterias. El aumento de peso, ganancia diaria de peso promedio, tasa de crecimiento específico y conversión alimenticia aparente no se vieron afectados ($P > 0.05$) por la inclusión de probióticos en las dietas. La inclusión de *B. subtilis* y *B. cereus* como parte de dietas para tilapia del Nilo promueve la colonización intestinal y mejora la tasa de supervivencia sin influir negativamente en el consumo de alimento, biomasa total, ingresos brutos y costos operativos parciales e ingresos netos. Por lo tanto, se recomienda el uso de estos probióticos para el crecimiento de alevines de tilapia del Nilo de la cepa GIFT.

Palabras clave: análisis económico, cultivo de tilapia, microbiología, probióticos, acuicultura.

INTRODUCTION

Probiotics are living microorganisms that are used as dietary additives; they live in the gastrointestinal tract of the host, adhering to the epithelial wall and proliferating in the intestine. Probiotics may act in the reduction and prevention of infection by pathogens by improving the intestinal bacterial flora, thus reducing the load of harmful bacteria by producing inhibitory substances, and assisting in digestion by producing digestive enzymes and further stimulating the immune system. If they will be used as dietary supplements, they should be able to survive for long periods of storage (Saad, 2006; Sahu *et al.*, 2008; Cutting, 2011; Cruz *et al.*, 2012; Mohapatra *et al.*, 2013).

The tilapia culture generates jobs and income and it has been considered a strong sector in food production in Brazil and tropical places. The productions of large-scale commercial demand a high density of fish stocked, and therefore is common the emergence of diseases caused by stress during cultivation, entailing in a high mortality and economic losses to producer. The minimization of these problems has fostered several studies in last years, looking for changes of the antibiotics in animal production, especially in aquatic organisms culture (Nayak, 2010).

It is noteworthy that the use of probiotics in aquaculture deserves an important highlight on the economic evaluation, within view of the producer can get profitably environmental sustainably a quality fish. *Bacillus cereus* var. *toyoi* and *B. subtilis* C-3102 are products approved by the European Food Safety Authority (EFSA). These probiotics have been recently used in Nile tilapia (Albuquerque, 2011; Moura, 2011; Albuquerque *et al.*, 2013; Mello *et al.*, 2013; Nakandakare *et al.*, 2013; Wild *et al.*, 2014) and shrimp research (Silva *et al.*, 2011; Souza *et al.*, 2012).

The *B. subtilis* and *B. cereus* are great candidates probiotics used in aquatic organisms, because according to El-Haroun *et al.* (2006) this bacterial species can be lyophilized and thus are, capable to survive in high temperatures, such as; after the pelleting process, it can be stored at room temperature without any deleterious effect, and resisted to the low pH that can reach intact to the small intestine (Cutting, 2011), although further development of this technique is required.

This study aimed to quantify total bacteria and total coliforms from the intestinal microbiota and estimate the partial operating costs and productive performance of fingerlings of Nile tilapia of the Genetically Improved Farmed Tilapia (GIFT) strain, fed diets under inclusion of probiotic *B. cereus* var. *toyoi* and *B. subtilis* C-3102.

MATERIALS AND METHODS

The experiment was performed at the Aquaculture Laboratory of West Paraná State University, Uniãoeste/ Toledo, Paraná, Brazil, from March 2010 to June 2010. Microbiological analyses were carried out in the Laboratory of Microbiology at the Catholic University of Parana, PUCPR/Toledo, over 113 days of cultivation that was divided in the sex reversion and fingerling phases.

A total of 1,200 Nile tilapia post-larvae (*Oreochromis niloticus*) of the GIFT strain, with an initial average weight of 24.7 ± 0.50 mg were used. Post-larvae were distributed into 24 aquaria (0.03-m³ capacity) in a completely randomized design, comprising in 2 x 3 factorial (phase x bacteria), with four replications. Each aquarium, containing 50 post-larvae (sex reversal phase) or 30 fish (fingerlings phase) it was considered to be an experimental unit, consisting of three treatments (diet+*Bacillus subtilis* C-3102, diet+*Bacillus cereus* var. *toyoi* and diet without probiotic addition).

The fish were reversed sexually, adding in experimental diets the synthetic androgen 17 α -methyltestosterone, by volatilization of ethanol at a concentration of 60 mg kg⁻¹. The fish were fed *ad libitum* six times a day and at the end of the day, all aquaria were siphoned to remove any residues.

Initially, during the sex reversal phase was considered an experimental unit an aquarium (0.03-m³ capacity) containing 50 post-larvae and in the final period of sex reversal were removed 20% of majors and minors around the mean of the average individual weight of fish with intent to homogenize, the batch for the fingerlings phase. At the beginning of the fingerlings culture, there was a decreased of stocking density for an aquarium containing 30 fish.

A probiotic containing 500 million spores per gram, *B. subtilis* C-3102 (Comércio e Indústria Uniquímica Ltda.) or *B. cereus* var. *toyoi* (Sumitomo Chemical do Brazil Ltda.) was used as growth promoters of fish performance. In the diets, which contains probiotic it was added 1% of the product when the ingredients was mixed (Table 1).

It were collected randomly at the end of the sexual reversion and fingerlings phase, one gram of intestines of each experimental unit, in order to quantify the total number of bacteria, total coliforms bacteria, and colonization by probiotics per gram of intestine.

The intestines removed aseptically were weighed and diluted in 1 mL of sterile distilled water per gram of intestine in a test tube, and subsequently homogenized in vortex. Decimal dilutions were performed in sterile distilled water pipes (10^{-1} , 10^{-2} , 10^{-3} and 10^{-4}) from

Table 1. The percentage of composition and chemistry of experimental diets for Nile tilapia grown during the sex reversal and fingerlings culture.

Ingredient	Bacteria		
	Probiotic-free	<i>Bacillus subtilis</i>	<i>Bacillus cereus</i>
Soybean meal	44.91	44.25	44.25
Fish meal	24.50	24.65	24.65
Corn bran	24.13	23.07	23.07
Probiotic	-	1.00	1.00
Mineral and Vitamin Premix ⁽¹⁾	1.00	1.00	1.00
DL-methionine	0.21	0.21	0.21
Antioxidant	0.02	0.02	0.02
Soybean oil	3.71	4.28	4.28
Dicalcium phosphate	2.12	2.13	2.13
Limestone	-	0.01	0.01
Salt	0.30	0.30	0.30
Total	100	100	100
Nutrient	(%)		
Starch	15.04	16.29	16.29
Calcium	1.55	1.44	1.44
Digestible energy (tilapia)	3,296.00	3,272.00	3,272.00
Crude fiber	3.22	2.96	2.96
total phosphorus	1.11	1.00	1.00
Ether extract	6.565	5.92	5.92
Total lysine	2.35	2.39	2.39
Methionine total	0.87	1.05	1.05
Crude protein	37.53	37.24	37.24

⁽¹⁾Mineral and Vitamin Premix: Composition per kg of product: iron: 10.312 mg, Mn: 6.875 mg, cooper: 1.100 mg, zinc: 16.500 mg, iodo: 137 mg, cobalt: 82.50 mg, selenium: 124.00 mg, chrome: 68.75 mg, vit. A: 1,100.000 UI; vit. D₃: 344.000 UI; vit.: E: 27.500 mg; vit. K: 1.375 mg; vit. C: 41.250 mg; biotin: 130.50 mg; choline: 75.625 mg; vit. B₉: 825.00 mg; inositol: 27.500 mg; niacin: 13.750 mg; vit. B₅: 6.875 mg; vit. B₆: 2.063 mg; vit. B₂: 2.750 mg; vit. B₁: 2.750 mg and vit. B₁₂: 4.120 mg.

the homogenized material. The agar medium pattern count (PCA) and Mc Conkey agar (MAC) were used to quantify total bacteria and coliforms bacteria, respectively.

The agar medium selective differentiator for *Bacillus* was used in Petri dishes with 1 mL of dilute solutions of 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴ for verify the presence of *B. subtilis* C-3102 and *B. cereus* var. *toyoi*. The duly prepared Petri dishes were incubated in a greenhouse microbiological at 28°C for 24 h. The colonies of bacteria were quantified with the aid of electronic colony counter marks Phoenix CP 600 Plus Electronic, using the official analytical methods for microbiological analysis for control of animal products and water, proposed by the Ministry of Agriculture, Livestock and Supply (Brasil, 2003).

It was used an adaptation of the mathematical model described by Silva *et al.* (2003) for the economic analysis of the production of tilapia fed with probiotics that considers the costs of acquiring fingerlings and feed prices and gross commercialization of fish. 1) OCP

= [(FI × PR) + (NF × UPF)], 2) GR = PP × TB, 3) IC = OCP/TB; 4) = NIP = GR - OCP, wherein: OCP: operating costs partial, FI: feed intake, PR: price per kg diet⁻¹; NF: initial number of fingerlings, UPF: unit price of fingerlings; GR: gross revenue, TB: total biomass; PP: purchase price of the fish, IC: incidence of cost; NIP: net income partial.

The normality of experimental errors and homogeneity of variance for each trait were previously evaluated using the Shapiro-Wilk and Levene tests, respectively. The microbiological data, it is partially operating costs and productive performance were subjected to analysis of variance (ANOVA) and Tukey's test ($\alpha = 0.05$) when significant differences were detected (StatSoft, 2005).

RESULTS

It is observed on the Table 2 that the quantification of the total bacteria and total coliforms of intestinal microbiota of tilapia were influenced ($P < 0.05$) by the

stages of culture. The number of colony forming units was lower in the sex reversal phase compared to the fingerlings phase.

The count (log CFU mL⁻¹) of total bacteria ranged between 4.85 for the fish fed without addition of probiotic in the diet formulation and 5.54 for diet with *B. cereus*. The total bacteria and total coliforms of intestinal microbiota of Nile tilapia were not influenced ($P > 0.05$) by adding probiotics in the diets. There was not observed effect of interaction between phase and bacteria during tilapia fingerlings culture (Table 2).

It was observed the presence of *Bacillus* in all treatments. Whereas *Bacillus* are bacteria Gram positive cosmopolitan which can be isolated from various habitats as water, soil air, then, possibly the fishes which there were not fed with probiotic, it may have exposed by another transmission, such as within ingredient in the diet it contained the genus *Bacillus*, external manipulation or even water in which the fish were cultured.

The addition of probiotic *B. cereus* var. *toyoi* or *B. subtilis* C-3102 in the diet did not influence ($P > 0.05$) weight gain (WG), daily weight gain (DWG), specific growth rate (SGR) and apparent feed conversion (AFC) of Nile tilapia fingerlings (Table 3).

The gain in biomass was significantly higher ($P < 0.05$) in the fish group fed a diet containing *B. subtilis* than in the group lacking probiotics, however, no differences were observed when compared with fish fed with *B. cereus*. There were no differences of gain in biomass between fish fed with *B. cereus* and treatment without probiotics (Table 3).

The survival values were greater ($P < 0.05$) in the fish group fed, adding probiotic *B. cereus* var. *toyoi* or *B. subtilis* C-3102 in relation to the group fed probiotic-free (Table 3). It is noted that the fish fed *B. cereus* or *B. subtilis* resulted a higher biomass of fingerlings compared to those that not fed with probiotics ($P < 0.05$). The feed intake was not affected by the addition of probiotic *B. cereus* var. *toyoi* or *B. subtilis* C-3102 in diet for Nile tilapia fingerlings ($P > 0.05$).

Gross revenues of the fingerlings cultivation of Nile tilapia was highly influenced ($P < 0.01$) by use of probiotics in food, where the revenues from the sale of the fish ranged between 64.18 and 95.41, respectively, for the fish group lacking probiotic and fed adding *B. subtilis* (Table 3).

The partial operational costs arising with feed costs showed that, the price of the post-reversed fingerlings was affected significantly ($P < 0.05$) by treatment which in its formulation contained *B. subtilis* in relation to fishes did not fed with probiotics. However, the partial operational costs of fishes fed with *B. cereus* did

not differ significantly from the group fed with *B. subtilis* and probiotic-free. The values in this economic parameter did not promote a return satisfactory. Probably this is due to the final biomass together with survival of the fingerlings obtained at the end of cultivation. Wild *et al.* (2014) evaluating the economic viability of adding probiotic in the Nile tilapia diet found that use of *B. cereus* showed higher return on investment that the use of *B. subtilis* or the combination *B. cereus* and *B. subtilis*, as part of diets for tilapias.

Body weight and length of fish at the end of fingerlings culture showed geometrical behavior that was expressed by the regression equations ($y = 0.1078x^{4.1817}$; $R^2 = 0.82$; $y = 0.209x^{3.1023}$; $R^2 = 0.69$ and $y = 0.1018x^{4.2136}$; $R^2 = 0.92$), respectively for fish diets that did not contain probiotic *B. subtilis* and *B. cereus* (Fig. 1). The determination coefficient (R^2) of the weight and length relation represents the proportion the correlation resulting of casual factors, ranged between 0.69 and 0.92 for the fishes that were not fed by probiotics and *B. cereus*. These results are important and can interfere in a productive performance, aiming to provide fishes more uniforms and get a better use at the time of marketing, because the interaction of the fishes larger about the smaller promotes a hierarchy resulting in a variation of the cultivated lots.

According to Fox *et al.* (1997), elevated cortisol levels may induce alterations in the production of growth hormone (GnRH), therefore, explains the difference in the weight and size between individuals of the same lot. Therefore, the dominant fishes tend to form feeding zones the incumbent subordinates to feed, therefore increasing the range of fish size.

DISCUSSION

Jatobá *et al.* (2008) while studying the isolation of lactic acid bacteria of the intestine of tilapia through probiotics potential, they observed values of total bacteria different to those found in the present study in which varied between 6.35 and 8.06 log CFU mL⁻¹.

The addition of probiotic *B. cereus* var. *toyoi* and *B. subtilis* C-3102 did not influence ($P > 0.05$) the weight gain, average daily weight gain, specific growth rate and feed conversion of Nile tilapia juvenile (Table 3). Like the present study, Marengoni *et al.* (2010), using *B. subtilis*, *B. pumilus* and *B. licheniformis* as probiotic in diets for red tilapia fingerlings, found similar results whose probiotics did not influenced the production performance and survival. Albuquerque (2011) and Moura (2011) also found no differences in the addition of probiotics in diets on performance of Nile tilapia fingerlings of GIFT strain.

Table 2. Mean values and standard deviation (log CFU mL⁻¹) of total bacteria, coliforms, presence (+) or absence (-) of *Bacillus* in Nile tilapia fed with different probiotics during periods of sex reversal and fingerlings phases. F: variable test, P: critical values.

Parameter	Total Bacteria	Bacterial count	<i>Bacillus</i>
Phase cultivation	F = 12.59; P = 0.00097	F = 18.83; P = 0.00009	
Sex reversal	4.59 ± 0.10b	3.05 ± 0.06b	+
Fingerlings culture	5.97 ± 0.39a	4.66 ± 0.36a	+
Bacteria	F = 1.23; P = 0.30304	F = 0.05; P = 0.94724	
Probiotic-free	4.85 ± 0.53	3.79 ± 0.48	+
<i>Bacillus subtilis</i> sususubtilis	5.54 ± 0.29	3.93 ± 0.31	+
<i>Bacillus cereus</i>	5.44 ± 0.28	3.84 ± 0.33	+
Bacteria	Phase	F = 2.48; P = 0.09607	F = 1.54; P = 0.22666

Table 3. Mean values and standard deviations of economic variables in the cost of production of Nile tilapia fed different probiotics. F: variable test, P: critical values.

Parameter	Bacteria			(F; P) ^(#)
	Probiotic-free	<i>Bacillus subtilis</i>	<i>Bacillus cereus</i>	
WG (g) ⁽¹⁾	8.53 ± 0.48	8.99 ± 0.64	8.46 ± 0.48	0.28; 0.75576
DWG (g day ⁻¹) ⁽²⁾	0.11 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.28; 0.75576
SGR (% day ⁻¹)	4.58 ± 0.07	4.85 ± 0.10	4.59 ± 0.08	3.02; 0.07060
GB (g) ⁽⁴⁾	155.5 ± 25.85b	242.25 ± 21.27a	221.48 ± 13.78ab	4.70; 0.02060
AFC ⁽⁵⁾	1.63 ± 0.41	1.02 ± 0.06	1.11 ± 0.05	1.87; 0.17855
SUR (%) ⁽⁶⁾	76.43 ± 0.08b	94.58 ± 0.01a	93.76 ± 0.02a	5.46; 0.01233
TFC (kg) ⁽⁷⁾	7 001.63 ± 909.90	8 897.51 ± 690.78	8 981.59 ± 399.80	2.57; 0.10059
PR (R\$) ⁽⁸⁾	1.72	1.91	1.85	-
UPF (R\$) ^{(9)(##)}	0.08	0.08	0.08	-
SPF (R\$) ⁽¹⁰⁾	102.00	106.50	102.00	-
BT (kg) ⁽¹¹⁾	5 465.04 ± 865.34b	8 315.33 ± 707.78a	7 659.96 ± 463.19ab	4.57; 0.02257
GR (R\$) ⁽¹²⁾	64.18 ± 10.53b	95.41 ± 2.64a	90.10 ± 4.48a	5.60; 0.00869
OCP (R\$) ⁽¹³⁾	91.88 ± 1.63b	96.82 ± 1.12a	96.02 ± 0.80ab	4.63; 0.02159
NIP (R\$) ⁽¹⁴⁾	-27.71 ± 9.32b	-1.41 ± 2.44a	-5.92 ± 4.48ab	5.27; 0.01410
IC (R\$ kg ⁻¹) ⁽¹⁵⁾	0.03 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	1.77; 0.19412

(##)The price according to weight/thousand (g) data Atoaqui – Associação Toledana de Aquicultura haverst/2010-2011 in Toledo, Paraná, Brazil; ⁽¹⁾Weight Gain (WG), ⁽²⁾Daily Weight Gain (DWG), ⁽³⁾Specific Growth Rate (SGR), ⁽⁴⁾Gain in Biomass (GB), ⁽⁵⁾Apparent Feed Conversion (AFC), ⁽⁶⁾Survival Rate (SUR), ⁽⁷⁾Total Feed Consumption (TFC), ⁽⁸⁾Price per kg diet⁻¹ (PR), ⁽⁹⁾Unit Price of Fingerlings (UPF), ⁽¹⁰⁾Selling Price of Fingerlings (SPF) ⁽¹¹⁾Biomass Total (BT) ⁽¹²⁾Gross Revenue (GR) ⁽¹³⁾Operating Cost Partial (OCP), ⁽¹⁴⁾Net Income Partial (NIP) ⁽¹⁵⁾Incidence of cost (IC).

Probably the time of use of probiotics has influenced in the total bacteria count during cultivation. In addition, the probiotics used in vivo were isolated from gut of the fishes of the same population that would be subsequently subjected to a challenge, as opposed to what was done in this study. Together with this hypothesis, these values may have been different due to the inhibition mechanisms by competitive exclusion for nutrients, space or specificity relationship probiotic-host, as reported by Gomez-Gil *et al.* (2000).

In relation to incidence of cost it was found that, it was no significant differences between the treatments containing probiotic or not in formulation ($P > 0.05$). The values of incidence of cost are not in accordance with those observed by Marengoni *et al.* (2008) and when evaluated the different stocking densities upon performance and economic viability of juvenile Nile tilapia in western Paraná, it were obtained in this parameter a variation of values between 0.67 and 2.00, resulting this, due to the higher biomass obtained at the end of cultivation.

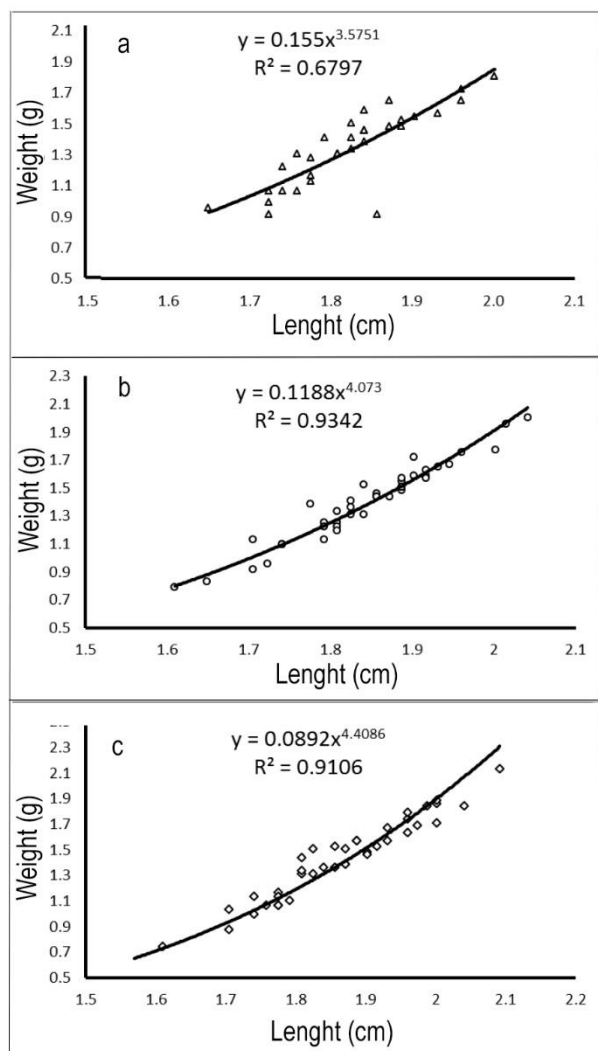


Figure 1. Weight curves (g) and length (cm) of fingerlings Nile tilapia probiotic-free (a), *Bacillus subtilis* (b), and *Bacillus cereus* (c).

Similarly, Olvera-Novoa *et al.* (2002) which studying the utilization of *Candida utilis* as protein source for tilapia fingerlings they obtained a minimum value of 2.24 whereas that, within this study they showed values of 1.09 and 2.64, respectively for maximum and minimum incidence of cost (Table 3).

Taking into consideration that there were no significant differences ($P > 0.05$) in feed consumption of fingerlings that contained *B. subtilis* in the formulation and, more expressive values of total biomass and gross income ($P < 0.05$), generating a lesser of cost incidence it is possible recommend the utilization of this probiotic for fingerlings the Nile tilapia, with particular emphasis on environmental sustainability of fish farming (Wild *et al.*, 2014).

CONCLUSIONS

The inclusion of *Bacillus subtilis* C-3102 and *Bacillus cereus* var. *toyoi* in diets for Nile tilapia fingerlings of the GIFT strain promotes intestinal colonization and can be recommended in the cultivation of this species during sex reversal and fingerlings phases.

The inclusion of *B. subtilis* and *B. cereus* as part of diets for Nile tilapia promotes intestinal colonization and improves the survival rate without negatively influencing the feed intake of the fingerlings, total biomass, gross revenue and partial operating costs and net revenue. Therefore it recommends the use of these probiotics in growth of fingerlings Nile tilapia, GIFT strain.

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