Advances in biology through agronomy, aquaculture, coastal and environmental sciences

Leandris Argentel Martínez
Ofelda Peñuelas Rubio
Editors

Pantanal Editora
2023

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Pantanal Editora

2023

Editor Chefe: Prof. Dr. Alan Mario Zuffo
Editores Executivos: Prof. Dr. Jorge González Aguilera e Prof. Dr. Bruno Rodrigues de Oliveira

Diagramação: A editora. **Diagramação e Arte:** A editora. **Imagens de capa e contracapa:** Canva.com. **Revisão:** O(s) autor(es), organizador(es) e a editora.

Ficha Catalográfica

Catalogação na publicação
Elaborada por Bibliotecária Janaina Ramos – CRB-8/9166

A244

Advances in biology through agronomy, aquaculture, coastal and environmental sciences / Organizers Leandris Argentel Martínez, Ofelda Peñuelas Rubio. – Nova Xavantina-MT: Pantanal, 2023.
105p. ; il.

PDF book

ISBN 978-65-81460-82-2
DOI https://doi.org/10.46420/9786581460822

1. Agricultural sciences. I. Martínez, Leandris Argentel (Organizer). II. Rubio, Ofelda Peñuelas (Organizer). III. Title.

CDD 630

Índice para catálogo sistemático

I. Agricultural sciences

Pantanal Editora

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Prologue

Advances in biology through agronomy, aquaculture, coastal and environmental sciences is an electronic book, edited by Pantanal Editora, based on the compilation of research papers where the authors of the different chapters have used highly current scientific methodologies and research equipment.

The biological sciences as the main object of research in agriculture, aquaculture, coastal and environmental sciences generate every day an understanding of knowledge that allows raising the scientific level of society as part of universal access to knowledge.

This book mainly addresses issues related to the use of plants extracts as sustainable alternatives for biocontrol of pests and bacterial diseases. It also brings together information on viruses and other diseases in aquatic organisms. In addition, studies of mangroves structure and their contribution to carbon sinks in experimental sites in northwestern Mexico are presented. Finally, an analysis on educational strategies for environmental education based on plant biology is carried out.

Editors appreciate the participation of the authors who have come from higher education institutions and research centers of great scientific prestige in Mexico. The majority of them are members of the National Research System of CONACyT, Mexico.

The authors

Summary

Chapter 7

Probiotic effects in tilapia *Oreochromis niloticus* culture based on growth performance, survival and water quality

Recibida em: 12/03/2023
Aprobado em: 17/03/2023
10.46420/9786581460822cap7

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ABSTRACT

The intensive production systems in aquaculture have generated serious problems related to the occurrence of diseases and to a deterioration of the environment, resulting in huge economic losses for fish farmers. The objective of this study was to analyse the effect of a microbial consortium with probiotic potential on the growth, survival and water quality of the tilapia *Oreochromis niloticus* culture in Tabasco, Mexico. A microbial consortium *Rhodopseudomonas palustris*, *Lactobacillus plantarum*, *Lactobacillus casei* and *Saccharomyces cerevisiae* was applied every week by immersion in earthen ponds during a growing cycle. The experiment took place in three treatments: 1) ponds with no probiotcis, 2) ponds with a dose of 4l/ha and 3) ponds with a dose of 10l/ha. Each treatment was applied three times. The growth parameters and survival of the tilapia and physicochemical parameters of the water and sediment were recorded. The EM1 and EM2 treatments significantly ($p<0.05$) increased the survival (73.0±2.51% y 79.1±1.00%, respectively) compared with the (64.0±4.04%). However, it was no significant ($p>0.5$) effect on growth by the end of the culture. The water quality improved significantly ($p<0.05$) within recommended parameters for the specie with the EM1 and EM2 treatments; although only decreased significantly ($p<0.05$) the percentage of organic matter in the sediment with EM2 treatment (0.89±0.36%). The results of this study showed a beneficial effect of the microbial consortium in water quality and survival of tilapia culture. However, the dose represented an important factor in the response of fish.

Keywords: *Oreochromis niloticus*, Probiotics, Growth, Water quality.

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INTRODUCTION

Aquaculture has become an important economic activity in many countries. However, the more intensive production systems have resulted in aquatic organisms being subjected to stressful conditions generated by a greater organism density and by feed and fertilizer inputs. Such conditions have caused serious problems related to the occurrence of diseases and to a deterioration of the environment, resulting in huge economic losses (Sahu et al., 2008; Al-Dohail et al*.,* 2011).

Veterinary medicines such as antibiotics and chemotherapeutics have long been applied to prevent and cure different types of diseases in fish farming (Aly et al*.,* 2008a). However, the abuse of these wide-spectrum antimicrobial agents has generated a variety of problems, including resistance to pathogenic agents, hormonal alterations in fish and negative effects (intoxication, cancer) on the health of consumers (De Schrijver; Ollevier, 2000; Al-Dohail et al*.,* 2011).

The use of clean technologies, including probiotics, in aquaculture is recognized as an alternative therapy in the management and control of fish health (Panigrahi et al*.,* 2010). Probiotics have been defined as "live microorganisms that benefit the host by changing the microbial community of the environment, or of that associated with the host, providing a better use of the feed or improving its nutritional value, stimulating a response to diseases, and improving the quality of the environment" (Verschuere et al*.,* 2000).

Using probiotics in fish farming has produced advantages such as a greater nutritional value (De Schrijver; Ollevier, 2000; Sahu et al., 2008), improved immune responses (Lallo et al*.,* 2008; Panigrahi et al*.,* 2010; Al-Dohail et al., 2011), an increase in survival (Aly et al*.,* 2008b; Ramakrishnan et al*.,* 2008), the inhibition of pathogenic bacteria (Skjermo; Vadstein, 1999; Huys et al*.,* 2001) and better water quality (Kristensen et al*.,* 1995; Makridis et al*.,* 2000; Balcázar et al*.,* 2006). However, most of the research carried out with probiotics and fish has taken place in the laboratory, for which reason its efficiency at the industrial level has been severely questioned (Gómez et al*.,* 2007; Kesarcodi-Watson et al*.,* 2008).

The tilapia *Oreochromis niloticus* is among the most successful freshwater species in fish farming, and this is due to its capacity to adapt to different production environments and to its high level of acceptance on the international market (Ingle de la Mora et al*.,* 2003). However, the presence of diseases and deficient water quality have caused the producers of this species to experience considerable economic losses. In view of this, the present study analyzed the effect of a microbial consortium of microorganisms (EM®) with probiotic potential on the quality of the water and sediment and on the growth and survival of the tilapia *O. niloticus* in a semi-intensive system located in a humid tropical area.

MATERIALS AND METHODS

Obtaining microorganisms and their culture. A microbial consortium (EM™ Technology, Japan) of worldwide distribution was used. The product contains three types of dormant efficient

microorganisms (EM): a photosynthetic bacterium (*Rhodopseudomonas palustris* at $2x10^3$ CFU ml^{-1}), lactic bacteria (*Lactobacillus plantarum* and *Lactobacillus casei* at $5x10^4$ CFU ml^{-1} each) and yeast (*Saccharomyces cerevisiae* at $4x10^3$ CFU ml^{-1}). The microorganisms were inoculated on a substrate of molasses and water to become active and were kept fermenting for seven days at a temperature of 36.5 to 37 °C, following the method suggested by the manufacturers (EMRO, 2008).

Tilapia production farm. The study took place in a commercial semi-intensive culture farm located in the municipality of Centro, Tabasco, Mexico (92°49'40" W, 17°57'34" N). The predominant climate in the area is humid warm with abundant summer rains (May-August) and a dry season (February-April). The field work started in February and ended in August 2019. The experiment was carried out in 0.25 ha earthen ponds, with a culture cycle of 180 days. The fry, all the same size, were obtained from a certified laboratory from one lot of breeders. They were placed in the ponds simultaneously at a density of 9 fish/m^2. The culture system was managed following the operational program of the farm technicians.

Evaluation of the probiotic in tilapia culture. The experimental design consisted of adding different doses of the microbial consortium with probiotic potential to the water during a culture cycle. The product was added weekly after changing the water, as follows: treatment 1 (C, control) ponds with no product, treatment 2 (EM1) ponds with a dose of 4 L ha^{-1} and treatment 3 (EM2) ponds with a dose of 10 L ha^{-1}. Each treatment was applied three times. Feeding of the fry started on the first day they were placed in the ponds and continued until they reached a commercial size (180 days), following the feeding program established for the gray tilapia (El Pedregal Silver Cup, Mexico). The farm technicians adjusted the feeding rate weekly in accordance with estimations of body weight increase and survival rate.

Environmental parameter data. Water quality was monitored every two weeks during the day, from 7:00 to 9:00 am. The parameters pH, temperature, dissolved oxygen and salinity were recorded at a depth of 50 cm with Hanna HI 95928 equipment (USA). The concentrations of total ammonium nitrogen (TAN) and nitrate (NO_3-N) were estimated using a Hanna HI 9828 (USA) ammonium recorder. Water transparency and depth were recorded *in situ* with a Secchi disk. Sediment was collected at a depth of 15 cm with a PVC corer (Kristensen et al*.,* 1995). The samples were taken to the laboratory where the variables pH, organic matter, total nitrogen and extractable phosphorus were recorded (NOM-021-RECNAT, 2000).

Growth parameter data. Fifty fry were collected randomly every 30 days during the culture cycle to record the weight (g) with an electronic scale (±5 g) and the total length (cm) with an ichthyometer (±1 mm). The fish were returned to the tank after the biometrical data were recorded. A total of 3,150 fish were weighed and measured [measured organisms x number of replicates per treatment x (days of culture measuring $frequency^{-1}$)]. Weight gain (g $tilapia^{-1}$), weight gain (%), daily weight gain (g day^{-1}), specific growth rate (SGR, % day^{-1}) and survival (%) were estimated at the end of the culture cycle (Ali et al*.,* 2010).

One-way analysis of variance (ANOVA) was applied to the environmental, growth and survival parameters of the tilapia. The $p<0.05$ values were considered significantly different. When the means of the treatments presented significant differences, an *a posteriori* analysis and a Tukey test were applied to identify the nature of the differences ($p<0.05$) (Daniel, 2008). A multiple discriminant analysis (MDA) was carried out to determine whether the treatments were related to the environmental parameters (Zar, 2010). All statistical analyses were carried out with STATISTICA version 7.0 for Windows (StatSoft, 2010).

RESULTS AND DISCUSSION

Environmental parameters in the tilapia culture. Significant among-treatment differences ($F=33.38$, $p<0.05$) were estimated for six of the seven environmental parameters in the water column (Table 1). The lowest concentration of dissolved oxygen was recorded in treatment C (4.63 ± 2.84 mg L^{-1}), while no significant differences (Tukey, $p>0.05$) were recorded for treatments EM1 and EM2. The temperature values in treatments C and EM1 were significantly (Tukey, $p<0.05$) greater than those in treatment EM2, whereas the transparency values in treatment EM2 were significantly smaller (Tukey, $p<0.05$) than those in treatments C and EM1 (Table 1).

Table 1. Environmental parameters in the control and treatment tanks during farming of the tilapia *O. niloticus*.

	Treatment		
Parameters	**C**	**EM1**	**EM2**
Water			
Dissolved oxygen (mg L^{-1})	4.63 ± 2.84^{a}	4.80 ± 3.19^{a}	5.18 ± 1.76^{a}
Temperature (°C)	29.9 ± 1.74^{a}	30.5 ± 2.22^{a}	28.7 ± 2.26^{b}
pH	4.24 ± 3.58^{a}	7.84 ± 0.62^{b}	8.05 ± 1.04^{b}
NO_3-N (mg L^{-1})	0.30 ± 0.05^{a}	0.37 ± 0.06^{b}	0.28 ± 0.14^{a}
Total ammonium nitrogen (mg L^{-1})	0.35 ± 0.20^{a}	0.04 ± 0.03^{b}	0.12 ± 0.19^{b}
Transparency (cm)	12.5 ± 11.5^{a}	9.00 ± 4.34^{a}	29.6 ± 28.9^{b}
Depth (cm)	75.2 ± 45.6^{a}	95.5 ± 24.9^{b}	105 ± 27.5^{b}
Sediment			
pH	7.38 ± 0.19^{a}	7.02 ± 0.71^{b}	7.29 ± 0.42^{a}
Extractable phosphorus (mg kg^{-1})	9.98 ± 6.18^{a}	9.17 ± 4.62^{a}	25.6 ± 10.6^{b}
Total nitrogen (%)	0.16 ± 0.03^{a}	0.07 ± 0.02^{b}	0.08 ± 0.02^{b}
Organic matter (%)	1.60 ± 0.56^{a}	0.89 ± 0.36^{b}	1.38 ± 0.23^{a}
n	3	3	3

Means on a same line with a different superscript are significantly different ($p<0.05$).
Average ± standard deviation.
C: Control, EM1: Probiotic dose 4 L ha^{-1}, EM2: Probiotic dose 10 L ha^{-1}.

Treatments EM1 and EM2 recorded the greatest depths during the culture cycle and were significantly different (Tukey, $p<0.05$) from treatment C, which presented the minimum depth (Table 1). Regarding pH, treatment C had the lowest value and was significantly different (Tukey, $p<0.05$) from treatments EM1 and EM2.

The NO_3-N concentrations presented no significant differences (Tukey, $p>0.05$) in treatments C and EM2, and these were significantly different from treatment EM1, where the greatest concentration was recorded (0.37 ± 0.06 mg L^{-1}). In the case of the TAN, the greatest concentration was recorded in treatment C (0.35 ± 0.20 mg L^{-1}) and was significantly different (Tukey, $p<0.05$) from those in treatments EM1 and EM2, where the lowest TAN concentrations (0.04 ± 0.03 mg L^{-1}; 0.12 ± 0.19 mg L^{-1}) were recorded.

Regarding the physicochemical parameters in the sediment (Table 1), treatments C and EM2 presented no significant differences (Tukey, $p>0.05$) with respect to the pH values, whereas the lowest value was recorded in treatment EM1 (7.02 ± 0.71) and was significantly different (Tukey, $p<0.05$) from those recorded in the other treatments. The extractable phosphorus content was significantly different (Tukey, $p<0.05$) in treatments C and EM1 compared with treatment EM2, which had the greatest content (25.6 ± 10.6 mg kg^{-1}). Treatment C presented the greatest percentage of total nitrogen ($0.16\pm0.03\%$) and was significantly different (Tukey, $p<0.05$) from treatments EM1 and EM2 ($0.07\pm0.02\%$; $0.08\pm0.02\%$). Finally, treatment EM1 recorded the lowest percentage of organic matter ($0.89\pm0.36\%$) and was significantly different from treatments C and EM2 ($1.60\pm0.56\%$; $1.38\pm0.23\%$).

According to the MDA, the environmental parameters recorded in the water were not significant, while the sediment presented two significant variables ($\lambda=0.05$; $F_{20.210}=35.9$; $p<0.05$; $R^2=0.93$): total nitrogen ($\lambda=0.08$; $p<0.05$) and extractable phosphorus ($\lambda=0.08$; $p<0.05$). Figure 1 shows the distribution of the corresponding observations between the first and second functions based on the discriminant space. Treatments EM1 and EM2 overlapped in comparison with treatment C, which remained isolated. Additionally, 95% of the canonical variables were correctly classified. According to the values of the standardized coefficients for the variables in the first discriminant function, the discriminant effect among the three treatments in the tilapia culture indicated that the total nitrogen had a canonical load of 73% ($\lambda_p=0.57$; $F_R=39.5$; $p<0.05$; $T=92\%$; $R^2=8\%$). while in the second discriminant function, the extractable phosphorus presented a canonical load of 65% ($\lambda_p=0.58$; $F_R=36.5$; $p<0.05$; $T=55\%$; $R^2=45\%$). The other variables did not present a significant canonical load. The use of probiotics in aquaculture has increased in recent years as a result of their potential benefits on fish health and on improved environmental quality (Balcázar et al*.,* 2006; Gómez et al*.,* 2007). The use of efficient microorganisms (EM1 and EM2) in this study produced a positive effect on water quality, as the water parameter values lay within the recommended range of values for the semi-intensive culture of the tilapia *O. niloticus* (Ingle de la Mora et al*.,* 2003), in comparison with the control treatment (C). However, optimum growth conditions for this

species were maintained by using the greatest dose of the commercial mixture of microorganisms (EM2) and not the dose recommended by the manufacturers (EM1).

Several authors have mentioned that factors such as the dose, the frequency of probiotic use and the type of organism that is farmed are considered important for the product to be successful, as the concentration has an effect on the action mechanisms of the microorganisms (Balcázar et al., 2006), and it is possible to observe a positive effect on environmental quality or a negative effect on fish health (skin infection) (Gómez et al., 2007; Ali et al., 2010).

In this study, the increase in the concentration of oxygen and the decrease in nitrate and total ammonium nitrogen in treatment EM2 coincide with other results that have shown that phototrophic bacteria (*R. palustris*) produce oxygen from carbon dioxide, with hydrogen sulfide as an electron donor, and decrease the amount of this gas that is toxic to farmed fish (Çetinkaya et al., 1999). Additionally, these bacteria have the capacity to use ammonium and nitrate as a source of nitrogen, reducing the concentration of these compounds in the water of culture systems (Kyum et al., 2004). In addition, other studies have indicated that the use of the yeast *Saccharomyces cerevisiae* decreases nitrogenous compounds (ammonium and urea) (Kesarcodi-Watson et al., 2008) and improves water quality.

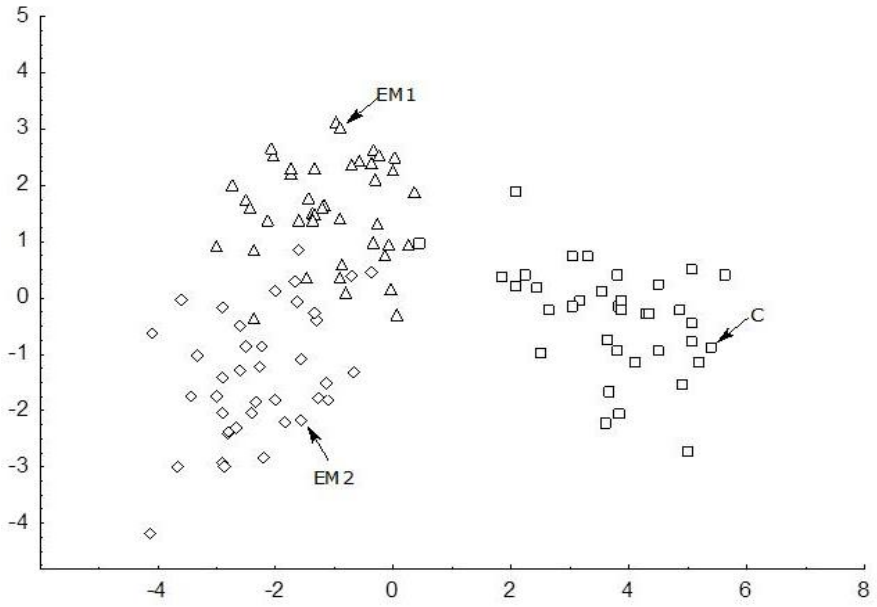

Figure 1. Discriminant analysis of the environmental parameters that explain the variability in the treatments (overlapping area: 95% confidence interval). C: Control, EM1: Probiotic dose 4 L ha^{-1}, EM2: Probiotic dose 10 L ha^{-1}. (X= Discriminant function 1; Y= Discriminant function 2).

Regarding the pH values, treatments EM1 and EM2 were observed to be moderately alkaline compared with treatment C, which was acidic. Some authors have mentioned the importance of controlling pH in culture systems due to its direct relationship with the synthesis of ammonium, nitrites and nitrates and to its effect on fish growth (Lallo et al., 2008). Previous studies have reported that efficient microorganisms such as those used in this study (*R. palustris*, *L. plantarum*, *L. casei* and *S. cerevisiae*) produce compounds such as quinones, biotin (photosynthetic bacteria) (Qi et al., 2009), lactic acid,

hydrogen peroxide, acetaldehyde, diacetyl, bacteriocins (lactic bacteria) (Verschuere et al., 2000), vitamins, glycerol, ethanol and carbon dioxide (yeast) (Irianto; Austin, 2002; Kesarcodi-Watson et al., 2008) that lower the pH in water due to their hydrophilic characteristics (presence of hydroxyl groups).

In aquaculture, productivity is a condition that is necessary to generate an ideal environment for fish development, but an increase in phytoplankton has been reported to cause problems of low dissolved oxygen concentration and mortality of farmed organisms (CNP, 2012). An increase in transparency was recorded in treatment EM2 compared with treatments C and EM1. This coincides with authors (Çetinkaya et al., 1999; Kyum et al., 2004.) who have reported that phototrophic bacteria may regulate phytoplankton growth through competition for nutrients (Qi et al., 2009), as is the case for the bacterium *R. palustris*, present in the commercial mixture of microorganisms that was used in this study.

The temperature and the depth of the water in the ponds have been associated with the environmental conditions and the operational conditions of the management of the farm (CNP, 2012). However, the significant changes recorded for these parameters did not influence the effect of the probiotics in the ponds of this study, as these variables did not have a considerable canonical load on which the efficiency of the product might have depended.

Farming systems characteristically generate high percentages of organic matter in the sediment. This material is closely related to the aerobic and anaerobic processes carried out by the microorganisms responsible for remineralizing nutrients in water and making them available to the microbial communities present in the ponds (Kristensen et al., 1995). In this study, the organic matter content in treatments EM1 and EM2 decreased compared with treatment C. It was also observed that the treatment with the greatest dose of microorganisms (EM2) had a greater percentage of total nitrogen and a lower content of extractable phosphorus in the sediment. These two variables were significant in the multiple discriminant analysis model and contributed to the behavior of the environmental parameters related to the treatments, as they had the greatest canonical loads. These results coincide with some authors' reports (Balcázar et al., 2006; Qi et al., 2009), which indicate that the use of lactic bacteria and microorganisms with probiotic potential in fish farming has produced significant decreases in the content of organic matter, as well as increases in labile substrates in the water. Studies have shown that organic phosphate is produced and organic nitrogen is available in ionic form during the fermentative processes of lactic bacteria (Lallo et al., 2008), which may be assimilated by the different microbial populations.

Growth and survival parameters in the tilapia culture. The three treatments in the semi-intensive tilapia culture were allowed the same time to harvest (180 days). The growth of the tilapia was significantly different in the three treatments ($F=20.96$; $p<0.5$) (Table 2). The lowest weight at the end of the culture cycle (harvest size) was recorded in treatment C, while the greatest values were obtained in treatments EM1 and EM2. However, the weights recorded in the three treatments presented no significant differences (Tukey, $p>0.05$).

The greatest total length at the end of the culture cycle was recorded in treatment EM2, followed by treatment EM1 and finally treatment C (Table 2). This last was significantly different (Tukey, $p<0.05$) from treatments EM1 and EM2, which presented no significant differences (Tukey, $p>0.05$).

In contrast, no significant differences (Tukey, $p>0.05$) were observed among the treatments (C, EM1 and EM2) with respect to the parameters weight gain (g), weight gain (%), daily weight gain (g day^{-1}) and specific growth rate (% day^{-1}). However, treatment EM2 recorded the greatest values (Table 2). Tilapia survival at the end of the culture cycle was significantly different ($F=20,57$, $p<0.05$) among the treatments (Table 2), as it increased significantly (Tukey, $p<0.05$) in treatments EM1 and EM2 compared with treatment C.

The use of probiotics in aquaculture has been associated with an efficient process of absorption and assimilation of farmed organisms' feed, as the microorganisms are capable of c76olonizing the gastrointestinal tract, where they secrete nutrients and digestive enzymes that improve the metabolic processes and immunological responses of the hosts (Balcázar et al*.,* 2006; Aly et al*.,* 2008a; Sahu et al., 2008).

Table 2. Growth parameters of the tilapia *O. niloticus* with respect to the treatments at the end of the culture cycle.

	Treatment		
Parameters	**C**	**EM1**	**EM2**
Final weight (g)	393±28.6^{a}	409±5.49^{a}	414±54.8^{a}
Final total length (cm)	23.7±0.44^{a}	28.1±0.48^{b}	28.7±1.06^{b}
Weight gain (g tilapia^{-1})	346±26.5^{a}	353±7.51^{a}	366±54.3^{a}
Weight gain (%)	636±44.0^{a}	741±45.5^{a}	773±107^{a}
Daily weight gain (g day^{-1})	122±45.6^{a}	126±5.27^{a}	163±23.3^{a}
SGR (% day^{-1})	595±6.51^{a}	599±1.36^{a}	599±14.4^{a}
Survival (%)	64.0±4.04^{a}	73.0±2.51^{b}	79.1±1.00^{b}
N	3	3	3

Means on a same line with a different superscript are significantly different ($p<0.05$).
Average ± standard deviation.
SGR= Specific growth rate.
C: Control, EM1: Probiotic dose 4 L ha^{-1}, EM2: Probiotic dose 10 L ha^{-1}.

The present study proved that the addition of a commercial mixture of efficient microorganisms (*R. palustris*, *L. plantarum*, *L. casei* and *S. cerevisiae*) to the water increased the final weight of the tilapia, with treatment EM2 recording the greatest value. Studies carried out with commercial probiotics that include *L. acidophilus* and *S. cerevisiae* for *Cyprinus carpio* (Ramakrishnan et al*.,* 2008) and *Bacillus pumillus* for *O. niloticus* (Aly et al*.,* 2008b) recorded greater significant weights at the end of the culture cycle. However, the present study recorded no significant differences among the evaluated treatments. In contrast, the total lengths at the end of the culture cycle were significantly different in treatments EM1 and EM2 compared with treatment C, and the treatment with the greatest dose recorded the greatest total length

values. Several authors have reported that the use of probiotics in fish farming significantly improves growth due to an input of nutrients and sources of energy that lead to an increase in the size of the organisms (Mazurkiewicz et al., 2005; Ali et al., 2010). The results of the present study, however, showed a beneficial effect only with respect to the total length.

This study recorded no significant differences in the growth parameters of the tilapia (weight gain, daily weight gain and specific growth rate) with the addition of the commercial mixture to the water. However, the greatest values of these variables were recorded for treatments EM1 and EM2 compared with treatment C. Similar results were obtained for the growth parameters when using this same commercial probiotic for *Oreochromis* sp. under laboratory conditions (Ladino-Ojuela, Rodríguez-Pulido, 2009). The authors of that study discussed that it was possible that beneficial effects were not observed due to the time of evaluation (15 days). However, the experimental time in the present study was 180 days in a commercial farm. Previous studies have mentioned different methods for using probiotics in aquaculture and have indicated that the best method is with feed, as microorganisms can then enter, colonize and multiply in the digestive tract (Makridis et al., 2000; Irianto, Austin, 2002).

Survival at the end of the culture cycle increased in treatments EM1 and EM2 compared with treatment C. Various studies have confirmed an increase in survival with the use of probiotics (Ali et al., 2010; Al-Dohail et al., 2011). Of the different identified probiotic action mechanisms (Lallo et al., 2008; Panigrahi et al., 2010), two have been linked to an increase in farmed fish survival. The first is their capacity to produce antiviral effects, counteracting problems caused by pathogenic agents and improving the immune system.

Notwithstanding that the action mechanisms of the probiotics have not been completely explained and different responses are obtained in different culture systems, the results of this study indicate that the efficient microorganisms included in the commercial mixture had a positive effect on water quality and survival. However, this effect was recorded with the greatest dose and not with the dose recommended by the manufacturer. It is thus necessary to carry out studies to determine 1) the optimum dose to be provided, 2) an emergency dose in case of environmental changes, 3) the colonization of the efficient microorganisms in the digestive tract of the tilapia, 4) the presence of antagonistic effects, 5) whether there is an increase in the immune response and 6) the effect of providing EM® in the feed.

ACKNOWLEDGEMENTS

The authors thank Jesús Mercado, Aarón Jarquín and Juan Juárez for their technical support, the Fundación Produce Tabasco A.C. for financing the research through project 27-2007-0415, the owner of the fish farm and the technicians responsible for the farm for helping during the field work.

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