

# Antimicrobial Activities of Lactic Acid Bacteria Strains Isolated from Nile Tilapia Intestine (*Oreochromis niloticus*)

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## Abstract

The intestinal microflora of Nile tilapia (*Oreochromis niloticus*) fish was studied to isolate and identify lactic acid bacteria as new probiotic. A total of five lactic acid bacteria were isolated from intestine to evaluate with antibacterial properties. These strains were identified by conventional and molecular techniques as: *Enterococcus faecium*, *Leuconostocmesenteroides*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Enterococcus durans*. *Leuc.mesenteroides* present more ability to inhibit growth of fish pathogens bacteria and selected as possible probiotic bacteria to use in aquaculture.

**Keywords:** Probiotic, Antibacterial activity, Nile tilapia, *Oreochromis niloticus*

## 1. Introduction

Aquaculture is a fast-growing and rapidly expanding multibillion dollar industry. Marine capture fisheries and aquaculture supplied the world with about 104 million tons 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 tons). Worldwide, the aquaculture sector has been expanding at an average compounded rate of 9.2% per year since 1970, compared to 1.4% only for capture fisheries and 2.8% for terrestrial-farmed meat production systems (Lara-Flores, 2011).

With the increasing intensification and commercialization of aquaculture production, disease is a major problem in the fish farming industry (Bondad-Reantaso *et al.*, 2005). It has been well documented that the use of antibiotics develops drug-resistant microorganisms with antibiotic residues retained in fish flesh and environment. In addition, antibiotics can affect the normal microflora of the digestive tract which is beneficial to host and may be inhibited by treatment with the antibiotics (Aly *et al.*, 2008). In this respect, use of probiotic bacteria is a new approach, which is gaining acceptance in aquaculture to control potential pathogens (Aly *et al.*, 2008; Kim and Austin, 2008; Lara-Flores, 2011).

Lactic acid bacteria (LAB) are known microorganisms that have probiotic properties. They can produce inhibitory compounds such as lactic acid, hydrogen peroxide, diacetyl, acetaldehyde and bacteriocin. These compounds are able to inhibit the growth of harmful microorganisms (Ringø and Gatesoupe, 1998; Gatesoupe, 1999). According to many reports, lactic acid bacteria are normal flora in gastrointestinal (GI) tract of healthy animals like mammals and aquaculture animals (Nikoskelainen *et al.*, 2001) with no harmful effects (Ringø *et al.*, 1998). Probiotics improve intestinal microflora and support good health for host by protection against infections by stimulating the immune system, alleviating lactose intolerance, reducing blood cholesterol levels, improving weight gain and feed conversion ratio (Salminen *et al.*, 2004; Lara-Flores and Aguirre-Guzman, 2009; Lara-Flores, 2011).

The present experiment is aimed to isolate lactic acid bacteria as new probiotic from intestine of Nile tilapia (*Oreochromis niloticus*). Antagonism properties were also investigated to find out high potential probiotic of selected strain to use in fish production.

## 2. Materials and Methods

### 2.1 Collection the Samples

A total of 30 live juvenile Nile tilapia fish were collected from three different farms from Campeche State. The surface of fish bodies were disinfected by alcohol (70%); dissected under antiseptic conditions; intestines taken out and washed three times with normal saline (NaCl 0.85%). The intestines were then homogenized in 10ml on sterile saline solution (NaCl 0.85%) with a mechanic homogenizer (Rengpipat *et al.*, 2008).

### 2.2 Isolation of Lactic Acid Bacteria (LAB)

Using serial dilution, 0.1 ml of homogenized intestine samples were spread on tryptic soy broth (TSB) (DIBICO™) followed by 48 hrs incubation at 36° C (Paludan-Müller *et al.*,

1999). After pipetting, 0.1 ml of the cultured broth was transferred to KF(DIFCO™) and Rogosa agar (DIBICO™) (Badis *et al.*, 2004; Rengpipat *et al.*, 2008). The plates were incubated at 36° C for 48 hrs. Selected colonies that demonstrated specific morphology to a LAB were sub-cultured three times on new KF and Rogosa agar to obtain single colonies (Rengpipat *et al.*, 2008). Gram staining, catalase reaction by 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and microscopic morphology were used for primary identification of the isolates (Kopermsub and Yunchalard, 2010).

### 2.3 Identification of the Selected Isolate

Polymerase Chain Reaction (PCR) method was used to confirm the conventional identification method (Nguyen *et al.*, 2007). DNA of the isolates was extracted according with the method proposed by Karp (1998). PCR was used to amplify the extracted DNAs using primers pAF 5'AGA GTT TGA TCC TGG CTC AG 3' as forward and phR 5'AAG GAG GTG ATC CAG CCG CA 3' as reverse primers (Kopermsub and Yunchalard, 2010) and the products were sequenced (Institute of Biotechnology (IBT-UNAM) using the specific primers, pAF and phR). The sequencing, around 1500 bp, were analyzed using MEGA Blast 5 nucleotide sequence software and then compared with the published sequences in GenBank using BLAST software (<http://www.ncbi.nlm.nih.gov/blast/>) from National Center for Biotechnology Information (NCBI).

### 2.4 Pathogens Used to Study Antibacterial Activity of the Isolated LAB

Four freshwater fish pathogens, *Pseudomonas aeruginosa*T3, *P. putida*T4, *Vibrio harveyi*T34 and *Mycobacterium marinum* T217 were used to determine the antibacterial effect of the candidate strains by diffusion and double-layer techniques and commercial LAB for human (*L. acidophilus*) were used as positive control, obtained from the Microbiology and Molecular Laboratory of Ecology, Fisheries and Oceanography of Gulf of Mexico Institute.

#### 2.4.1 Diffusion Method

The pathogenic bacteria were cultured in TSB and incubated at 36° C for 24 hrs. Thereafter, 10 µl of the cultures were spread on tryptic soy agar (TSA) by swab. At the same time, the selected strain was cultured in TSB at 36° C for 24 hrs. The bacteria were harvested by centrifugation at 13,000 rpm for 2 min and the supernatants were used for antibacterial test using well diffusion methods (Cappuccino and Sherman, 2002; Balcázar *et al.*, 2008).

#### 2.4.2 Double-layer Method

The antagonism activity was detected using the double-layer method of Dopazo *et al.* (1988). Briefly, 50 µl of overnight cultures of the LABs in TSB were grown on TSA for 24 hrs at 36° C. Then, 10 µl of overnight culture of the pathogenic strains in TSB were mixed with 5 ml of soft agar at 45° C (1% of TSA) and were poured over the dish surface; according with Dopazo *et al.* (1988) at this temperature the bacteria were not affected. The double-layer dishes were incubated at 36° C and the inhibition of pathogen growth around and/or over the macrocolony were recorded after 24 hrs.

### 3. Results and Discussion

#### 3.1 Isolation and Identification of LAB

A total of five of lactic acid bacteria designated as 165, 178, 222, 249 and 252, were isolated from juvenile Nile tilapia fish intestine. The isolates were gram-positive, catalase-negative and short rod or cocobasilli shaped. These strains were selected for identification and further antagonism analysis. The results from the laboratory of IBT-UNAM identified 165 as *Leuconostocmesenteroides*, 178 as *Enterococcus faecium*, 222 as *Lactobacillus fermentum*, 249 as *L. plantarum* and 252 as *E. durans*.

These strains were obtained from a previous study (Lara-Flores *et al.*, 2008), which determined the total population and groups of bacteria in Nile tilapia. The authors observed that the lactic acid bacteria were a minor part of microflora in Nile tilapia with less than 7% of the total count. The same result has been reported by Ringo (1993) and Allameh *et al.* (2012). For example Ringo (1993) found that 10% population level of gut microbiota in Arctic charr (*Salvelinus alpinus* L.) was lactic acid bacteria. Though, Ringo *et al.* (2005) reported the gut microbiota of fish was less diverse than in terrestrial animals, the gastrointestinal tract of fish is not as simple as believed.

#### 3.2 Antibacterial Activity

##### 3.2.1 Diffusion Method

Results obtained from diffusion method demonstrated that the LAB isolates do not presents any inhibitory effect against fish pathogen bacteria. This could have been because the lactic acid bacteria did not produce inhibitory substances in the culture medium probably because the growing conditions were not appropriate or because there was no competition or a stimulating factor for the production of inhibitory substance. Filho-Lima (2000) mentioned that in many cases the inhibitory substances are produced only in the presence of the pathogen and without this stimulation, inhibitory substances are not produced. Furthermore, Sugita *et al.* (2007) mentioned that the antibacterial activity can not be studied using only the supernatant of the culture, since the inhibition process involves a variety of factors that are activated at the same time.

##### 3.2.2 Double-layer Method

The antimicrobial activity of LAB isolates were tested against fish pathogenic bacteria are summarized in (Table 1) by using double layer assay, and Figure 1 illustrates the zones of inhibition against fish pathogenic bacteria under study. The diameters of the inhibition zones were standardized as Light (L) if halo covers less than 10% of the Petri dish, Medium (M) if halo covers less than 30% and High if halo covers more than 30%. *L. mesenteroides* and the commercial probiotic inhibited all the fish pathogenic bacteria with the highest level of inhibition towards *Mycobacterium* T217 and *Pseudomonas* T3 (Table 1). A similar frequency of inhibitory bacteria was observed for isolates from halibut larvae (Bergh, 1995), rainbow trout (Brunt and Austin 2005), turbot (Hjelm *et al.*, 2004), shrimp (Rengpipat *et al.*, 1998) and Indian major carps (Ghosh *et al.*, 2007). Allameh *et al.* (2012) observed a significant

inhibitory effect of *L. mesenteroides* against *A. hydrophila* and *S. putrefaciens*, all freshwater fish pathogens. In this study we observed a significant inhibitory effect of *L. mesenteroides* to four freshwater fish pathogens different that Allameh *et al.* (2012) used, thereby confirming the effectiveness of the bacteria to inhibit the growth of fish pathogenic bacteria, and this demonstrates the potential for use as probiotic in fish.

Table 1. Antimicrobial activity of isolates LABS against indicator strain by Dopazo’s method in percentage coverage of Petri plate by the LAB

Fish Pathogens Strains	LABS Strains					
	Control	<i>Ent. faecium</i>	<i>Leuc. Mesenteroides</i>	<i>L. fermentum</i>	<i>L. plantarum</i>	<i>Ent. durans</i>
<i>Pseudomonas</i> T3	50	50	30	0	0	10
<i>Pseudomonas</i> T4	90	0	30	8	0	5
<i>Vibrio</i> T34	80	0	90	0	0	5
<i>Mycobacterium</i> T217	40	50	50	0	0	10

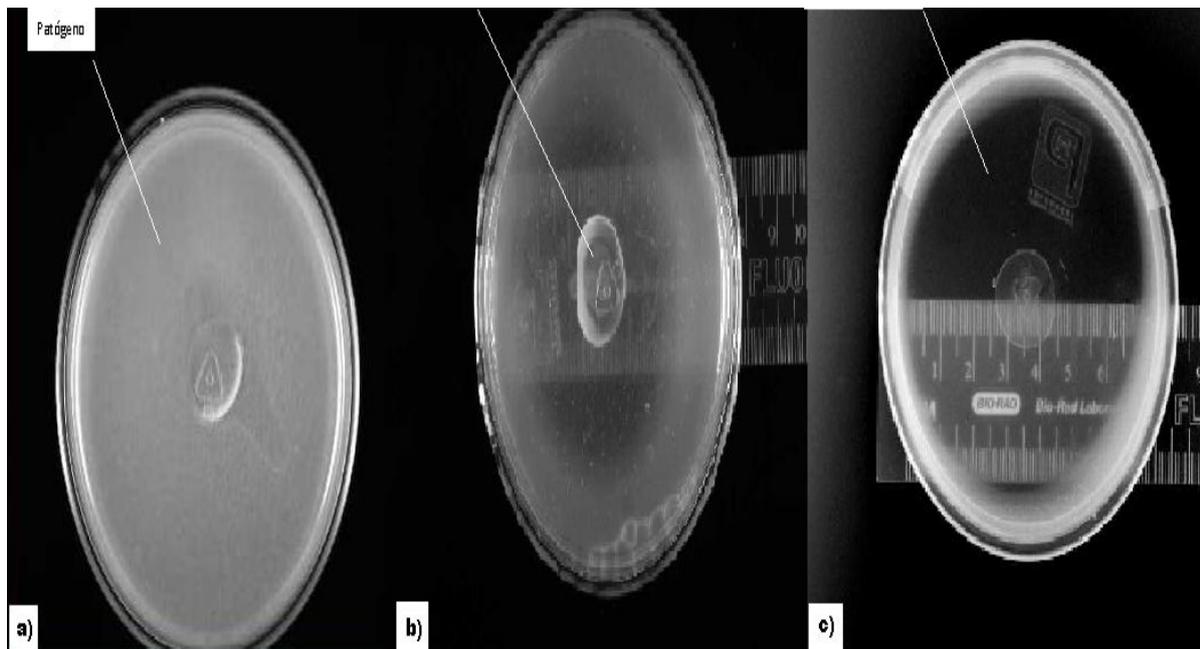


Figure 1. The antimicrobial activity of LAB isolates by using double layer assay. a) Inhibition of *Pseudomonas* T4 by *L. fermentum* b) Inhibition of *Mycobacterium* T217 by control strain c) Inhibition of *Vibrio* T34 by *Leuc. mesenteroides*

#### 4. Conclusions

The present study concluded that *L. mesenteroides* was normal in microflora in Nile tilapia gut. This LAB showed high ability to inhibit growth of freshwater fish pathogen particularly *Vibrio sp.* and *Mycobacterium sp.* Therefore, *Leuc. mesenteroides* has high potential probiotic to use in Nile tilapia culture.

In the opinion of Lara-Flores and Aguirre-Guzman (2009) these specific preparations with beneficial effects for preventing disease and other natural elements are for a stable health in

the intestinal environment and immune system. The establishment of a strong program of prevention of diseases, which include probiotics and good management, may result in increased production of aquatic organisms. This is one of the purposes of this work, as mentioned above was based on the search for a viable, reliable and low cost alternative to use for control diseases in aquaculture.

## References

- Allameh, S. K., Daud, H., Yusoff, F. M., Saad, C. R., & Ideris, A. (2012). Isolation, identification and characterization of *Leuconostocmesenteroides* as a new probiotic from intestine of snakehead fish (*Channa striatus*). *African Journal of Biotechnology*, *11*, 3810-3816.
- Aly, S. M., Ahmed, YA-G., Ghareeb, AA-A., & Mohamed, M. F. (2008). Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of *Tilapia nilotica* (*Oreochromis niloticus*) to challenge infections. *Fish Shell Immunology*, *25*, 128-136. <http://dx.doi.org/10.1016/j.fsi.2008.03.013>
- Badis, A., Guetarni, D., Moussa-Boudjemaa, B., Henni, D. E., Tornadijo, M. E., & Kihal, M. (2004). Identification of cultivable lactic acid bacteria isolated from Algerian raw goat's milk and evaluation of their technological properties. *Food Microbiology*, *21*, 343-349. [http://dx.doi.org/10.1016/S0740-0020\(03\)00072-8](http://dx.doi.org/10.1016/S0740-0020(03)00072-8)
- Balcazar, J. L., Vendrell, D., de Blas, I., Ruiz-Zarzuola, I., Muzquiz, J. L., & Girones, O. (2008). Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish. *Aquaculture*, *278*, 188-191. <http://dx.doi.org/10.1016/j.aquaculture.2008.03.014>
- Bergh, Ø. (1995). Bacteria associated with early life stages of halibut, *Hippoglossus hippoglossus* L., inhibit growth of a pathogenic *Vibrio* sp. *Journal of Fish Diseases*, *18*, 31-40. <http://dx.doi.org/10.1111/j.1365-2761.1995.tb01263.x>
- Bondad-Reantaso, M. G., Subasinghe, R. P., Arthur, J. R., Ogawa, K., Chinabut, S., Adlard, R., Tan, Z., & Shariff, M. (2005). Disease and health management in Asian aquaculture. *Veterinary Parasitology*, *132*, 249-272. <http://dx.doi.org/10.1016/j.vetpar.2005.07.005>
- Brunt, J., & Austin, B. (2005). Use of a probiotic to control lactococcosis and streptococcosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases*, *28*, 693-701. <http://dx.doi.org/10.1111/j.1365-2761.2005.00672.x>
- Cappuccino, J. G., & Sherman, N. (2002). *Microbiology a Laboratory Manual* (Vol 1). New York: Benjamin Cummings.
- Dopazo, C. P., Lemos, M. L., Lodeiros, C., Bolinches, J., Baria, J. L., & Toranzo, A. E. (1988). Inhibitory activity of antibiotic-producing marine bacteria against fish pathogens. *Journal of Applied Bacteriology*, *65*, 97-101. <http://dx.doi.org/10.1111/j.1365-2672.1988.tb01497.x>
- FAO. (2007). *The State of World Fisheries and Aquaculture 2006*. Rome: Food and

Agriculture Organization of United Nations.

Filho-Lima, J. V. M., Vieira, E. C., & Nicoli, J. R. (2000). Antagonistic effect of *Lactobacillus acidophilus*, *Saccharomyces boulardii* and *Escherichia coli* combinations against experimental infections with *Shigella flexneri* and *Salmonella enteritidis* subsp. *typhimurium* in gnotobiotic mice. *Journal of Applied Microbiology*, 88, 365-370. <http://dx.doi.org/10.1046/j.1365-2672.2000.00973.x>

Gatesoupe, F. J. (1999). The use of probiotics in aquaculture. *Aquaculture*, 191, 147-165. [http://dx.doi.org/10.1016/S0044-8486\(99\)00187-8](http://dx.doi.org/10.1016/S0044-8486(99)00187-8)

Ghosh, S., Archana, S., & Chittaranjan, S. (2007). Isolation of putative probiotics from the intestines of Indian major carps. *Israeli Journal of Aquaculture*, 59, 127-132. <http://dx.doi.org/10.1128/AEM.70.12.7288-7294.2004>

Hjelm, M., Riaza, A., Formoso, F., Melchiorsen, J., & Gram, L. (2004). Seasonal incidence of autochthonous antagonistic *Roseobacter* spp. and *Vibrionaceae* strains in a turbot larva (*Scophthalmus maximus*) rearing system. *Applied Environmental Microbiology*, 70, 7288-7294. <http://dx.doi.org/10.1111/j.1472-765X.2008.02401.x>

Karp, G. (1998). *Biología Celular y Molecular*. Mexico: McGraw Hill-Interamericana.

Kim, D. H., & Austin, B. (2008). Characterization of probiotic carnobacteria isolated from rainbow trout (*Oncorhynchus mykiss*) intestine. *Letters in Applied Microbiology*, 47, 141-147.

Kopermsub, P., & Yunchalard, S. (2010). Identification of lactic acid bacteria associated with the production of plaasom, a traditional fermented fish product of Thailand. *International Journal of Food Microbiology*, 138, 200-204.

Lara-Flores, M., Balan-Zetina, S. B., Ramos-Miranda, J., Cú-Escamilla, A. D., & del Río-Rodríguez, R. E. (2008). Determinación y prevalencia de *Mycobacterium* spp. en tilapia nilótica (*Oreochromis niloticus*) cultivada en el municipio de Champotón, Campeche. In: *Congreso de Investigación Formativa y Seminarios de Acuicultura y Zootecnia* (pp. 19-32). Ciudad Victoria.

Lara-Flores, M., Aguirre-Guzman, G. (2009). The use of probiotic in fish and shrimp aquaculture. A review. In: Perez-Guerra N. & Pastrana-Castro L. (Eds), *Probiotics: Production, evaluation and uses in animal feed* (pp. 75-89). Kerala: Research Signpost.

Lara-Flores, M. (2011). The use of probiotic in aquaculture: an overview. *International Research Journal of Microbiology*, 2, 471-478.

Nguyen, T. F. T., Kang, J. H., & Lee, M. S. (2007). Characterization of *Lactobacillus plantarum* PH04, a potential probiotic bacterium with cholesterol-lowering effects. *International Journal of Food Microbiology*, 113, 358-361. <http://dx.doi.org/10.1016/j.ijfoodmicro.2006.08.015>

Nikoskelainen, S., Ouwehand, A., Salminen, S., & Bylund, G. (2001). Protection of rainbow trout (*Oncorhynchus mykiss*) from furunculosis by *Lactobacillus rhamnosus*. *Aquaculture*,

198, 229-236. [http://dx.doi.org/10.1016/S0044-8486\(01\)00593-2](http://dx.doi.org/10.1016/S0044-8486(01)00593-2)

Rengpipat, S., Phianphak, W., Piyatiratitivorakul, S., & Menasveta, P. (1998). Effect of probiotics on Black tiger shrimp *Penaeus monodon* survival and growth. *Aquaculture*, 167, 301-313. [http://dx.doi.org/10.1016/S0044-8486\(98\)00305-6](http://dx.doi.org/10.1016/S0044-8486(98)00305-6)

Rengpipat, S., Rueangruklikhit, T., & Piyatiratitivorakul, S. (2008). Evaluation of lactic acid bacteria as probiotic for juvenile seabass (*Latescalcalifer*). *Aquaculture Research*, 39, 134-143. <http://dx.doi.org/10.1111/j.1365-2109.2007.01864.x>

Ringø, E. (1993). The effect of chromic oxide (Cr<sub>2</sub>O<sub>3</sub>) on aerobic bacterial population associated with the intestinal epithelial mucosa of Arctic charr, *Salvelinus alpinus* (L.). *Canadian Journal of Microbiology*, 39, 1169-1173. <http://dx.doi.org/10.1139/m93-177>

Ringø, E., Gatesoupe, F. J. (1998). Lactic acid bacteria in fish: a review. *Aquaculture*, 160, 177-203. [http://dx.doi.org/10.1016/S0044-8486\(97\)00299-8](http://dx.doi.org/10.1016/S0044-8486(97)00299-8)

Ringo, E., Schillinger, U., Holzappel, W., Naughton, P. J., Pierzynowski, S. G., Zabielski, R., & Salek, E. (2005). Antimicrobial activity of lactic acid bacteria isolated from aquatic animals and the use of lactic acid bacteria in aquaculture. *Biology of Growing Animals*, 2, 418-453. [http://dx.doi.org/10.1016/S1877-1823\(09\)70051-7](http://dx.doi.org/10.1016/S1877-1823(09)70051-7)

Salminen, S., Wright, A. V., & Ouwehad, A. (2004). *Lactic acid bacteria (Vol. 1)*. New York: Marcel Dekker Inc. <http://dx.doi.org/10.1201/9780824752033>

Sugita, H., Ohta, K., Kuruma, A., & Tomoko, S. (2007). An antibacterial effect of *Lactococcus lactis* isolated from the intestinal tract of the Amur catfish, *Silurus asotus* Linnaeus. *Aquaculture Research*, 38, 1-3. <http://dx.doi.org/10.1111/j.1365-2109.2007.01765.x>

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