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Evaluation of the presence and efficiency of potential probiotic bacteria in the gut of tilapia (*Oreochromis niloticus*) using the fluorescent *in situ* hybridization technique

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ABSTRACT

The Fluorescent *in situ* Hybridization (FISH) technique was employed to enumerate potential probiotic and putative pathogenic bacteria in the gut of tilapia (*Oreochromis niloticus*). Bacteria used in the study were isolated from water, sediment and intestines of tilapia (*Oreochromis niloticus*) raised in an aquaculture system. These isolates were tested *in vitro* on antagonism tests against putative pathogenic bacteria (*Aeromonas hydrofila*, *Enterococcus faecalis*, *Edwardsiella tarda*, *Pseudomonas fluorescens* and *Pseudomonas putida*), also isolated from the same aquaculture system. Two isolates that inhibited largest number of pathogenic bacteria were identified by sequencing as *Bacillus* sp. and *Enterococcus* sp. and were added to the commercial feed (10^6 cells g^{-1}) for *in vivo* tests. Treatments of the *in vivo* experiment were: 1) Control — fish fed with no added bacteria, 2) Bacil. — fish fed diets containing *Bacillus* sp.; 3) Enter. — fish fed diets containing *Enterococcus* sp., and 4) Bacil. + Enter. — fish fed diets containing *Bacillus* sp. and *Enterococcus* sp. (1:1). Each treatment consisted of four replicates with 15 juveniles of tilapia (*O. niloticus* — 16.74 ± 4.35 g e 9.82 ± 0.85 cm). The experiment lasted for 30 days and at the end of this period, three fish from each tank were killed, and the intestines were taken for microbiological analysis by FISH technique, where *Bacillus* and *Enterococcus*, as well as two putative pathogenic bacteria (*Aeromonas* and *Pseudomonas* sp.) were quantified. *Enterococcus* sp. and *Bacillus* sp. were present in high number in the gut microbiota of fish. However, *Bacillus* sp., showed an increase in its abundance, indicating a successful incorporation of this potential probiotic bacteria into the tilapia gut microbiota. Furthermore, in the Bacil. treatment it was observed a significant reduction of *Aeromonas* and *Pseudomonas* sp. abundances compared with the other treatments. These results indicate that the FISH technique is a potential tool to characterize the dynamics of potential probiotic bacteria and their efficiency in the control of pathogenic bacteria.

1. Introduction

Bacterial diseases are responsible for severe economic losses in aquaculture (Wang et al., 2008). The indiscriminate use of antibiotics to control pathogenic microorganisms brings important changes in the microbiota of the aquaculture systems and surrounding environment, creating bacterial resistance to commonly used antimicrobials (Resende et al., 2012) and even affecting natural beneficial bacteria (He et al., 2010, 2011, 2012). Therefore, it is important to seek and combat these pathogens with the development of alternative methods.

One alternative method for this is the use of microorganisms called probiotics that may restrict the growth of pathogens (Gatesoupe, 1999). Most commercial probiotics used in aquaculture were obtained from terrestrial animals (Nayak, 2010). Thus, aquaculture activity may be introducing exotic bacterial species or strains in aquatic environments, without knowing the consequences of this action. In this sense, there is a need to obtain autochthonous probiotic bacteria, originated from the raised organism or from the environment where they are produced (Aly et al., 2008a; El-Rhman et al., 2009; Jatobá et al., 2008). However, the process of isolation, identification and testing the potential probiotic bacteria is laborious and time consuming (Balcázar et al., 2006; Farzanfar, 2006; Kesarcodi-Watson et al., 2008; Verschuere et al., 2000b).

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A possible way to evaluate the efficiency of a probiotic candidate is to determine the probiotic and pathogenic bacterial abundances in the fish guts along the time. Many methodologies to count bacteria in fish gut have been developed based on selective growth media (Jatobá et al., 2011; Lallo et al., 2007; Meurer et al., 2007). However, many bacteria do not grow in the culture media normally used (Ray et al., 2010, 2012; Temmerman et al., 2004). The use of culture-independent molecular biology techniques is a more accurate tool to determine the abundance and efficiency of probiotic bacteria (Reid et al., 2006; Ringø et al., 2010). There are various molecular biology techniques that can characterize and quantify the extracted DNA from the bacterial communities. However, the Fluorescent *in situ* Hybridization (FISH) technique is more effective, since it allows a direct and precise quantification of the pathogenic and probiotic bacterial cells at species or genus level (Merrifield et al., 2010).

The main objective of this study was to test the Fluorescent *in situ* Hybridization (FISH) technique as a tool to enumerate potential probiotic and putative pathogenic bacteria in the gut of tilapia (*Oreochromis niloticus*). Furthermore, we want to demonstrate the feasibility in using endemic bacteria, isolated from aquaculture systems, as probiotic for the raised aquatic organisms.

2. Material and methods

2.1. Isolation of potential probiotic bacteria

Bacteria were isolated from the water and sediment of ponds, and from the intestines of 68 tilapias raised in the Fazenda Experimental de Leopoldina/Empresa de Pesquisa Agropecuária de Minas Gerais (FELP/EPAMIG) between May 2009 and January 2010. Fish for bacterial isolation were randomly sampled in six ponds with an area of 1,200 m^2 each. Fish were raised at a density of three fish per m^2. The average weight of the sampled tilapias was 638.8 ± 313.8 g. The fish were fed with commercial diet containing 28% crude protein (Soma ®). The amount of feed offered on a daily basis was ca. 2% of the total fish biomass in the pond. The cultivation system was semi-intensive, with the water flow estimated as 10 L s^{-1} ha^{-1}, representing a water exchange rate of 6% of the total volume per day.

Water samples (20 mL) were concentrated to 2 mL by centrifugation at 8000 ×*g* for 10 min at 4 °C. These concentrated samples, 2 g of homogenized sediment and 2 g of homogenized intestine tilapias samples were serially diluted (ten-fold dilutions were prepared to 10^{-6}) in 0.9% sterile (121 °C for 15 min) saline solution and plated on agar plates of Man, Rugosa and Sharpe (MRS – Difco®) before being incubated in a bacteriological incubator at 35 °C for 24 hours in microaerophilic conditions. After checking the growth, all bacterial colonies were characterized and differentiated by the Gram staining and re-isolated on Petri dishes with Tryptic Soy Agar (TSA – Difco®) to confirm the purity of the isolated bacteria. Subsequently, the pure bacterial isolates were stored in −20 °C in with 10% glycerol solution.

2.2. Selection of potential probiotic bacteria by in vitro antagonism

The bacterial isolates were tested by the double-layer method (Booth et al., 1977; Verschuere et al., 2000a) to check its ability to inhibit putative pathogenic bacterial strains. These putative pathogenic bacterial strains were isolated from the same aquaculture environment in previously study (Resende et al., 2012). Potential pathogens used for the *in vitro* tests were *Aeromonas hydrophila*, *Edwardsiella tarda*, *Enterococcus faecalis*, *Pseudomonas fluorescens* and *Pseudomonas putida*.

Search for the potential probiotics was performed with all bacterial isolates obtained from water, sediment and tilapia's gut. They were cultured in Tryptic Soy Broth (TSB – Difco®) at a density relative to 0.5 MacFarland. Later, they were inoculated with the Steer's replicator on Mueller-Hilton Agar (Difco®) and incubated at 35 °C for 24 hours. After the growth of the colonies, they were killed by exposed to chloroform for 30 minutes. Then, residual chloroform was allowed to evaporate for other 30 minutes. Afterwards, the putative pathogenic bacteria strains were grown in semi-solid tryptic soy medium and added to the plates with potential probiotic bacteria in a double-layer. The plates were immediately incubated at 35 °C for 24 hours. After that, the plates were checked for bacteria growth or inhibition halos, which indicated the antagonistic activity of the potential probiotic bacteria (Booth et al., 1977; Verschuere et al., 2000a).

The two bacterial isolates that inhibited the largest number of selected putative pathogenic strains in the *in vitro* tests were considered as the best candidates for probiotics (Ghosh et al., 2007; Nayak and Mukherjee, 2011) and were identified by genetic sequencing. For this, DNA from these isolated bacteria was extracted using the Fast DNA kit (Qbiogene®) according to the manufacturer's instructions. The DNA fragments were amplified by PCR using general bacterial primers (EUB338f, 5'- ACTCCTACGGGAGGCAGC-3' (Amann et al., 1990); 926Rr, 5'-CCCGTCAATTCMTTTGAGTTT-3' (Watanabe et al., 2001); with replicons length of approximately 550 bp. These were cloned and then sequenced by ABI 3730 DNA Analyser. The sequences obtained were compared with those present in the GenBank database using the tool Basic Local Alignment Search Tool for Nucleotide - BLASTN. Sequences showing more than 99% similarity were considered to belong to the same operational taxonomic unit.

2.3. Experimental Design (in vivo experiments)

The two potential probiotic bacteria obtained in the *in vitro* tests were then evaluated in *in vivo* experiments. For the *in vivo* tests, 240 tilapia juveniles (16.74 ± 4.35 g and 9.82 ± 0.85 cm) were employed. They were randomly divided into 16 tanks of 1,000 L, composing four treatments (see below), each one with 15 fish per tank.

These tanks are part of the recirculation system water of the FELP/EPAMIG; the water flux was estimated to be approximately 2.8 L per minute. Juveniles tilapias were acclimated for three days before the beginning of the feeding experiment with different diets, as described below. The animals were fed three times a day with their respective diets (see below) in the proportion of 8% of the total biomass of fish in the tank.

2.4. Incorporation of probiotic bacteria candidates in the feed

The potential probiotic bacteria were incorporated into the diet (Jatobá et al., 2008) and offered to juvenile tilapia along the 30 days of the experiment.

For this, the two strains were thawed in TSB after confirmation of the purification of each isolate and were incubated in a bacteriological incubator at 35 °C for 24 hours. When bacterial abundance were 4.5 . 10^8 cells per mL (direct counting by DAPI staining – Porter and Feig, 1980), the culture was sprayed on a commercial feed containing 36% crude protein (Max Peixe Tropical®). The experiment was composed of four treatments: 1) Control – diet only included sterile TSB; 2) Bacil. – feed was sprayed with *Bacillus* sp. culture; 3) Enter. – feed was sprayed with *Enterococcus* sp. culture; and 4) Bacil. + Enter. – feed was sprayed with *Bacillus* sp. and *Enterococcus* sp cultures in the same proportions (1:1). Subsequently, the different types of feed were placed in a bacteriological incubator at 35 °C for 24 hours. After checking the density of these bacteria in different types of diets (more than 10^6 specific cells added . g^{-1}). These feeds were stored at 4 °C and their bacterial density remained in the same order of magnitude during all experiment.