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Aquaculture 388–391 (2013) 115–121

Contents lists available at SciVerse ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

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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ARTICLE INFO

Article history:
Received 8 January 2013
Received in revised form 15 January 2013
Accepted 16 January 2013
Available online 24 January 2013

Keywords:
Bacillus
Enterococcus
FISH
Probiotic
Pathogenic

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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http://dx.doi.org/10.1016/j.aquaculture.2013.01.019

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.

2.5. Analysis of potential probiotic and putative pathogenic bacteria by Fluorescent in situ Hybridization (FISH)

After 30 days, three tilapia juveniles from each of the tanks were killed by thermal shock (ice bath for 30 minutes) and necropsied aseptically to remove the intestinal tract. These intestines were fixed in 2% paraformaldehyde (final concentration).

The samples of intestine were processed for analysis by Fluorescent *in situ* Hybridization (FISH) to identify and quantify four bacterial groups. For this, the samples were treated as described in the protocol proposed by Epstein and Rossel (1995). To each sample, 0.0001% Tween solution was added and then sonicated (Vibra Cell VCX 130PB, Sonics & Materials ®) three times (range 110.7 μm per 60 s). After sonication, the samples were centrifuged at 500 g for five minutes. The supernatant was removed and the remaining contents were washed twice with ultrapure water. The three supernatant fractions were placed in the same bottle and shaken vigorously. The material was then centrifuged as described before. Aliquots of each sample were filtered on polycarbonate filters (Nuclepore® - 0.2 μm) and stored in a refrigerator until the hybridization process.

Subsequently, the samples were subjected to FISH protocol (Cottrell and Kirchman, 2003), where oligonucleotide probes rRNA-targeted were used to identify potential probiotic added to diets (*Bacillus* and *Enterococcus*) and two putative pathogenic bacteria (*Aeromonas* and *P. fluorescens*) (Table 1). A negative control made with a probe without any specificity for bacteria was used to evaluate the efficiency of hybridization. All probes were labeled with the Cy3 fluorochrome. The abundance of bacteria was determined by direct counting at 1000× magnification using an epifluorescence microscope (Olympus® BX-60) equipped with Chroma U-N41007, U-MWU2, U-MWB2 and U-MWG2 optical filter set.

2.6. Statistical analysis

The data were tested for normality. The single criterion variance analysis (ANOVA - one way) and an *a posteriori* Tukey's test were used for normal data and the Kruskal-Wallis test was used for non-normal data using the program SigmaPlot 11.0. In both cases, values of $P<0.05$ were considered significant (Zar, 1999).

3. Results

3.1. Isolation and identification of potential probiotic bacteria

Seventy-nine bacterial isolates were obtained from all samples. Twenty-three were isolated from the water, 29 from the pond's sediment and 27 from the gut tract of tilapias. Only nine strains presented positive results in the *in vitro* tests (Table 2). Two strains that showed the best performance inhibiting the growth of *A. hydrophila*, *E. tarda*, *P. fluorescens* and *P. putida*, all gram-negative species (Table 2). These two strains were identified as *Bacillus* sp. and *Enterococcus* sp. Both of the identities of these strains had 99% similarity of 16S rRNA gene sequence compared to the bacteria in GenBank (Table 3). The *Bacillus* sp. (C5I18) strain was isolated from the intestine of tilapia and *Enterococcus* sp. (C5S19) was isolated from the pond's sediment.

Table 2
In vitro double-layer test results of probiotic bacteria candidates which showed inhibition against pathogenic bacteria used in this research (AH = *Aeromonas hidrophyla*; EF = *Enterococcus faecalis*; ET = *Edwardsiella tarda*; PF = *Pseudomonas fluorescens*; PP = *Pseudomonas putida*).

Isolated	Origin	AH	EF	ET	PF	PP
C5S17	Sediment					x
C5S20	Sediment		x			
C5S19	Sediment	x		x	X	x
C1A2	Water					x
C5A25	Water					x
C5A13	Water		x			
C1I3	Gut		x			x
C5I18	Gut	x		x	X	x
C1I6	Gut		x			X

3.2. In vivo tests

The total bacterial abundance in the intestines of fish was significantly higher in the treatments where potential probiotic single or mixed were added compared to the Control (Bacil.: $1.46 \pm 0.15 \cdot 10^7$ cells g^{-1}; Enter.: $1.65 \pm 0.23 \cdot 10^7$ cells g^{-1}; Bacil. + Enter.: $1.30 \pm 0.29 \cdot 10^7$ cells g^{-1}; and Control: $1.17 \pm 0.19 \cdot 10^7$ cells g^{-1}) (Fig. 1).

There were also differences in the intestinal microbiota composition of juvenile tilapias among the treatments. The abundance of *Aeromonas* ($0.21 \pm 0.13 \cdot 10^6$ cells g^{-1}) and *P. fluorescens* ($0.28 \pm 0.15 \cdot 10^6$ cells g^{-1}) was significantly lower in the Bacil. treatment compared to the Control ($0.35 \pm 0.17 \cdot 10^6$ cells g^{-1} e $0.51 \pm 0.27 \cdot 10^6$ cells g^{-1}, respectively). Likewise, the abundance of *Pseudomonas fluorescens* ($0.34 \pm 0.15 \cdot 10^6$ cells g^{-1}) was lower in the Enter. treatment compared to the Control. *Bacillus* abundance was higher in both treatments where this bacteria strain was added (Bacil.: $1.0 \pm 0.47 \cdot 10^6$ cells g^{-1}; and Bacil. + Enter.: $0.63 \pm 0.18 \cdot 10^6$ cells g^{-1}) compared to the Control ($0.49 \pm 0.13 \cdot 10^6$ cells g^{-1}). *Enterococcus* abundance ($0.42 \pm 0.15 \cdot 10^6$ cells g^{-1}) was higher in the treatment where only this bacteria strain was added in comparison with the Control ($0.28 \pm 0.16 \cdot 10^6$ cells g^{-1}). The abundance of *Aeromonas* in Bacil. treatment was also significantly lower than in Enter. treatment ($0.30 \pm 0.08 \cdot 10^6$ cells g^{-1}) and in Bacil. + Enter. treatment ($0.34 \pm 0.12 \cdot 10^6$ cells g^{-1}) (Fig. 2).

4. Discussion

We cannot deny the success of commercial probiotics used in aquaculture. However, allochthonous probiotics often have not presented great viability, since the survival rates of these microorganisms are often low (Gatesoupe, 2008). There is a consensus that endemic probiotics are more likely to settle in the cultivated animals, probably due to their ability to easier adapt to the environment being, therefore, a preferential organism to be searched and isolated

Table 1
rRNA-targeted oligonucleotide probes of different bacterial species used in this research. All probes were labeled with fluorochrome Cy3.

Probe	Specificity	Sequence (5′ - 3′)	Target site (rRNA positions)	% FA*	Reference
NON	Negative Control	TAGTGACGCCGTCGA	–	30	Yokokawa and Nagata, 2005
Bacil 1	*Bacillus*	GCCGCCTTTCAATTTCGAAC	195–209	35	Ichijo et al., 2010
Enter 2	*Enterococcus*	TCCATCAGCGACACCCGAAA	202–221	35	Demanèche et al., 2008
Aero 2	*Aeromonas*	GTAACGTCACAGCCAGCAGA	468–487	35	Kyselková et al., 2009
PsAg1	*Pseudomonas fluorescens*	GATAACTCGTCATCAGCTC	1520–1538	30	Boye et al., 1995

* Percentage of formamide (FA) in *in situ* hybridization buffer.

Table 3
Characteristics of the colonies, cells morphology, and comparison with samples available in GenBank of bacterial isolates sequenced and used in *in vivo* tests.

Isolated	Morfology of bacterial cells	Characteristics of bacterial colonies	Number of base pairs (bp)	Bacterial taxa more approximate in GenBank	Similarity (%)
C5S19	Gram positive cocci	White, bright and with regular edge	302	*Enterococcus* sp.	99
C5I18	Gram positive rod	Slightly yellowish, opaque and with irregular edge	300	*Bacillus* sp.	99

for further applications as probiotics (Balcázar et al., 2007; Carnevali et al., 2004).

The procedures to obtain probiotic bacteria are quite strict regarding various aspects to provide security to the final consumers. Isolating bacteria, testing *in vitro* and *in vivo* to verify the action of these isolates, and testing the pathogenicity in the target organisms and in others organisms involved in the food chain are just some of the steps that must be followed to obtain a commercial probiotic (Merrifield et al., 2010; Verschuere et al., 2000b). These authors suggest that monitoring the microbiota before and after the probiotic addition is also important to determine the efficiency and the changes that occur in the bacterial community by the administration of probiotic bacteria.

Some researchers have evaluated the efficiency of potential probiotic bacteria by utilizing cultivation-dependent techniques for counting probiotic and pathogenic bacteria that were introduced (Avella et al., 2010, 2011; Balcázar et al., 2007; Gopalakannan and Arul, 2011; Merrifield et al., 2009; Nayak and Mukherjee, 2011). Other studies have evaluated the efficiency of probiotic bacteria through indirect indicators, such as hematological parameters and growth performance of raised animals (Al-Dohail et al., 2009; Avella et al., 2010; Balcázar et al., 2007; Brunt and Austin, 2005; El-Dakar et al., 2007; Merrifield et al., 2009; Nayak and Mukherjee, 2011). Nevertheless, there is still little information on the effective colonization of administered probiotics and their interaction with pathogens (Merrifield et al., 2010).

Molecular biology techniques are important tools for performing more accurate monitoring of the added bacteria and also the control of pathogenic bacteria (Merrifield et al., 2010; Verschuere et al., 2000b). Sun et al. (2011) showed no significant changes in the bacterial community using the technique of Denaturing Gradient Gel Electrophoresis (DGGE) after the addition of the probiotic. One possible explanation for this is that this technique allows identifying the presence of microorganisms in any amount due to the amplification of DNA. The results of the DGGE are visualized through the bands of amplified nucleic acid in the gel and it is proportional to the amount of individuals. However, variations in the intensity and size of bands

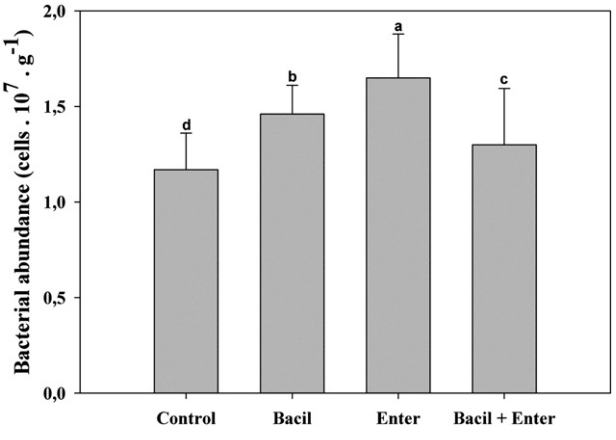

Fig. 1. Total bacterial abundance (cells·10^7 g^{-1}) in fish gut at Control, Bacil., Enter. and Bacil. + Enter. treatments. Different letters indicate statistical differences ($P<0.05$).

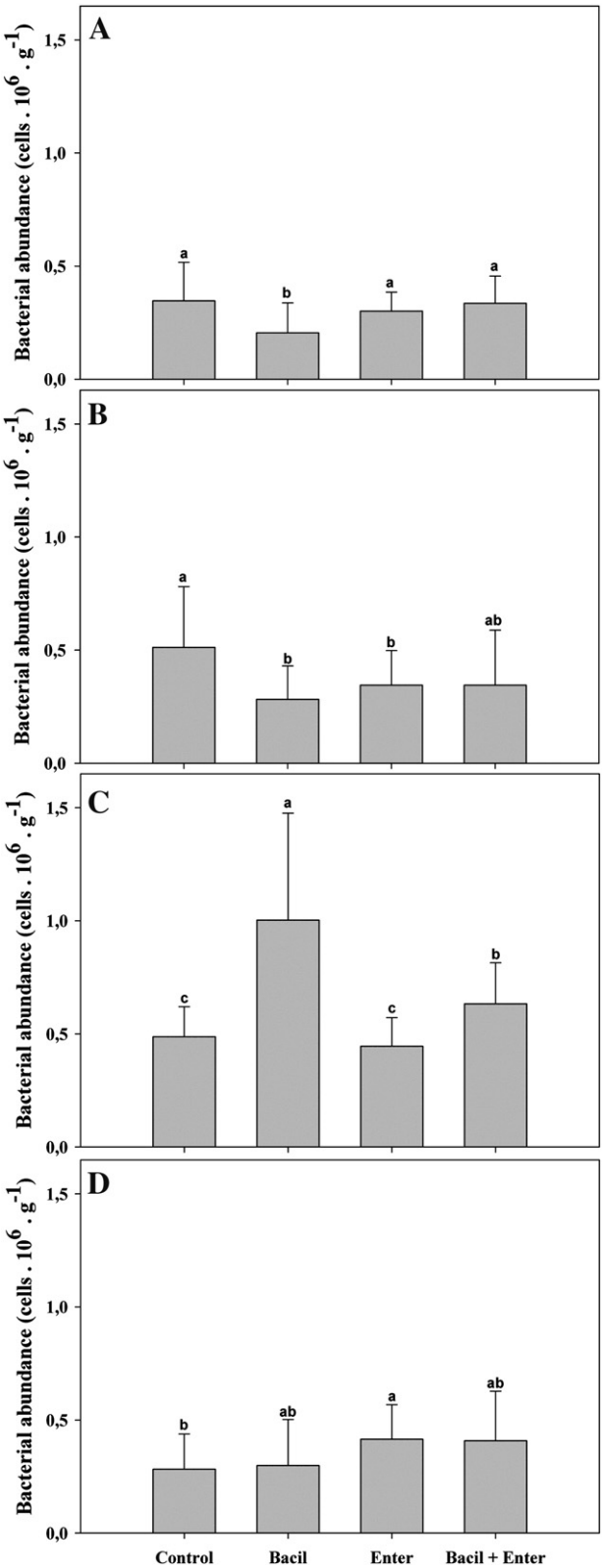

Fig. 2. Specific bacterial abundance (cells·10^6 g^{-1}) of *Aeromonas* (A), *Pseudomonas fluorescens* (B), *Bacillus* (C) and *Enterococcus* (D) in fish gut at Control, Bacil., Enter. and Bacil. + Enter. treatments. Different letters indicate statistical differences ($P<0.05$).

can occur and do not permit precise quantification of the number of individuals in the sample. Therefore, in our work, we proposed to use another molecular biology technique, the Fluorescence *In Situ* Hybridization (FISH) technique. FISH is a culture-independent molecular technique that allows visualization and direct counting of bacterial cells specifically labeled. It is based on the use of fluorescent probes that are specific for bacterial groups, genera or species (Zwirglmaier, 2005). Through the FISH technique, we can quantify and follow changes in the number of probiotics and pathogens microorganisms. Thus, the microbial community structure (taxa and number of each taxa of bacteria) allows us to verify the probiotic efficiency.

In aquaculture, FISH technique has been used to characterize the microbiota of water and wastewater (Garcia and Olmos, 2007; Paungfoo et al., 2007; Payne et al., 2007; Pereira et al., 2011), the formation of biofilm (Cytryn et al., 2006), the microbiota of the intestinal tract of fish (Asfie et al., 2003; Balcázar et al., 2010; Huber et al., 2004).

The two strains of potential probiotic bacteria isolated in our study were identified as *Bacillus* sp. and *Enterococcus* sp. Species of these same genera are already used as probiotic in aquaculture (Kumar et al., 2006). However, in our study, the *Bacillus* sp. had a better performance in comparison to the other treatments, always showing abundances in the tilapia intestine tract nearly twice that of *Enterococcus* sp. at the end of the experiment.

Even though results of other *in vitro* (Chau et al., 2011; Shakibazadeh et al., 2012; Sica et al., 2010, 2012; You et al., 2005) and *in vivo* tests (Ravi et al., 2007) showed the potential of probiotic of bacteria isolated from pond's sediment, the better performance of *Bacillus* sp. in this work, may be related to the fact that this strain has been isolated from the gut of tilapia. It probably facilitates the incorporation and colonization of this strain when offered together with commercial feed.

Aeromonas and *P. fluorescens* are normally found in the intestine of tilapia (He et al., 2009), being a major route of infection in fish. The control *Aeromonas* population is of paramount importance, since some species of this genus, such as *A. hydrophila*, are highly pathogenic to fish (Aly et al., 2008a; Li and Cai, 2011). Similarly, *Pseudomonas* species are important pathogens in fish (Zhang et al., 2009), although some species were tested as probiotic (El-Rhman et al., 2009). Therefore, these bacterial species should be monitored and controlled to avoid further opportunistic infections.

The probiotic action of *Bacillus* species has been already demonstrated in several studies with different species of raised aquatic organisms. In studies with tilapia, for example, *Bacillus* increased resistance and survival when exposed to *Aeromonas* and *Pseudomonas* (Aly et al., 2008a, 2008b). The fish presented an increase in the phagocytic activity of leukocytes (Aly et al., 2008c) and better immune response (Ridha and Azad, 2012). Similar effects were observed for several other fish species (Avella et al., 2010; Brunt et al., 2007; Kumar et al., 2006; Newaj-Fyzul et al., 2007; Raida et al., 2003; Sugita et al., 1998) and shrimps (Balcázar and Rojas-Luna, 2007; Rengpipat et al., 2003; Vaseeharan and Ramasamy, 2003). In general, the use of *Bacillus* species as probiotic increases the animal's resistance to bacterial diseases and, consequently, their survival.

In our results, we observed the efficient action of *Bacillus* sp. in the control of *Aeromonas* and *Pseudomonas* populations in both *in vitro* and *in vivo* tests. The gut of tilapias was colonized by *Bacillus* sp. The number of cells of *Bacillus* sp. increased, while there was a reduction of putative pathogens in juveniles of tilapia. However, other subsequent tests must be performed to confirm the probiotic action of these strains, following the suggestions of Verschuere et al. (2000b) to obtain efficient probiotic species.

In summary we can conclude that the Fluorescent *in situ* Hybridization (FISH) technique is an excellent tool for monitoring potential probiotic, putative pathogenic, or any other kind of bacteria present in the fish gut content. This technique can be employed in any research where direct visualization of bacteria is necessary in order to better understand physiological and metabolic processes. In this study the use of FISH allowed to demonstrate that the strain of *Bacillus* sp., an endemic bacteria isolated from the tilapia gut, showed efficient residence in the fish intestine tract and a good control of putative pathogenic bacteria populations (*Aeromonas* and *Pseudomonas fluorescens*) also isolated from the same aquaculture system.

Acknowledgements

We thank the Empresa de Pesquisa Agropecuária of Minas Gerais (EPAMIG), especially Thiago Freato, for the support provided at the Fazenda Experimental de Leopoldina (FELP/EPAMIG); Thiago Nascimento, Juliana Resende and Cláudia Fontes for help in the bacterial antagonism *in vitro* assay; Cintia Coelho and Julliane Medeiros for help in bacterial DNA extraction and analysis; Edmo Rodrigues and Raiza Azevedo for help in processing samples for analysis by FISH. Financial support and grants consent by CNPq (Process 145337/2009-0).

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