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Full Length Research Paper

The probiotic potential of lactobacilli isolated from Nile tilapia (*Oreochromis niloticus*)'s intestine

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The potential probiotic acid lactic bacteria isolated from Nile tilapia (*Oreochromis niloticus*)'s intestine was tested for fish farming. In our collection, 10 *Lactobacillus* strains were targeted to confront a series of antibiotics in order to draw their resistance profile, and to test their degree of inhibitory to four pathogenic bacteria, *Staphylococcus aureus, Escherichia coli, Streptococcus* sp. and *Pseudomonas* sp. The power of acidification and tolerance was tested. Overall, our results show that strains BLT31 and BLT21 are fully susceptible and resistance to the tested antibiotics. Strains BLT3, BLT20, BLT21 and BLT23 have a good antagonistic effect against pathogenic bacteria that cause the highest damage in aquaculture. For acid lactic production, strains BLT3, BLT26, BLT27, BLT28, and BLT31 are considered fast since Δ pH ≥ 4U in less than three hours. As for the resistance to pH and bile salts, two strains BLT3 and BLT31 showed significant power which gives them acceptable probiotic potential.

Key words: Probiotics, antibiotics, aquaculture, the Nile Tilapia, inhibitory activities, lactobacilli.

INTRODUCTION

The rapid development of aquaculture, its intensification, and the occurrence of health problems on farms encourage researchers to develop alternatives methods for controlling the microbial environment. One of the methods gaining recognition for controlling pathogens within the aquaculture industry is the use of beneficial or probiotic bacteria (Ringo and Gatesoupe, 1998; Gatesoupe 1999; Verschuere et al., 2000; Irianto and Austin, 2002a, b). In this context, the use of probiotics, which originally involves humans and livestock land, has been expanded to aquatic animals, in the early 1980s. Yasuda and Taga (1980) were the first to suggest the beneficial effect of probiotics on fish. However, initial studies have been published at the end of the 80s (Kosaza, 1986; Gatesoupe et al., 1989).

FAO (2002), and WHO (FAO/OMS, 2002) have developed guidelines for the use of the term probiotics in food and make the following definition: living microorganisms, which when administered in adequate amounts, exert a beneficial effect on the health of the host that ingests them. A variety of probiotic bacteria including yeasts have been targeted as potential probiotics agents. Examples include lactic acid bacteria (Collins et al., 1998; Carr et al., 2002; Carnevali et al., 2004), Bifidobacteria (Picard et al., 2005), Saccharomyces (Czerucka et al., 2007), enteric (Sartor, 2003), and streptococci (Meurman and Stamatova, 2007). However, to be used as probiotics, all of them must be non-pathogenic and non-toxic. In addition, probiotic bacteria must survive the transition niche target and then persist, serving to protect the host against infections caused by pathogenic microorganisms. They will produce metabolites that inhibit the colonization or growth of other microorganisms or by competing with them for resources such as nutrients or space (Ouwehand et al., 1999a, 2001; Forestier et al., 2001; Pinchuk et al., 2001; Fiorillo et al., 2002; Mukai et al., 2002; Servin and Coconnier, 2003; Vine et al., 2004a, b).

In aquaculture, many tests were conducted on several species including Japanese eel (*Anguilla japonica*), red tilapia and shrimp (*Litopenaeus stylirostris*) (Castex et al., 2006). The results showed that a diet supplemented with acid lactic bacteria *Pediococcus acidilactici* MA18/5M allowed these species to improve biologics parameters

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such as mortality, growth rates and feed conversion. Gatesoupe (2002) had also shown an improvement in *Pollachius pollachius* growth as larvae stage, when Artemia received these bacteria for their diet. Maurilio et al. (2002) have shown that the use of bacteria *Streptococcus faecium*, *Lactobacillus acidophilus* and yeast *Saccharomyces cerevisiae* as probiotics in tilapia fry diets improves animals' growth, and mitigates the effects of stress factors.

In this research we have choose Tilapia which is a robust fish of the Cichlidae family. Native of warm waters of Africa, living exclusively in fresh water, its biological peculiarity is the practice of mouth brooding eggs and is very undemanding regarding their food and their living conditions. Its holding,amazingly easy, requires no special skill (Trewavas, 1983). Tilapiais the second largest in the world, after carp, for the importance of aquaculture activities. Among these three species recognized the potential of aquaculture, the Nile tilapia, O.niloticus, is by far the one most used in aquaculture worldwide (F.A.O, 2002).

We selected bacteria with probiotic potential from ten strains of lactobacilli isolated from tilapia and identified on the basis of physiological and biochemical analysis results, in a previous study to assess the resistance of these strains to several antibiotics. They are subjected to several tests (criteria) selection of probiotics such as inhibitory activities, resistance to law pH and the tolerance to bile salts.

MATERIALS AND METHODS

Strains and media used

Ten strains of lactobacilli have been isolated and identified from the intestine of Nile tilapia specimens (*Oreochromis niloticus*); they were sampled from the fish farm of Wad El Djemaa (Relizane Province). These strains were kept in a MRS diluted by half with glycerol at a temperature of -18°C. Before use, they were seeded twice in MRS broth and incubated at 30°C for 24 h to 48h for regeneration. These strains are identified by physiological and biochemical tests as *Lactobacillus plantarum* (BLT3, BLT 20, BLT 23, BLT 29, and BLT30) and *Lactobacillus casei* (BLT 21, BLT 26, BLT 27, BLT 28, and BLT 31).

Antibiotic resistance

The antibiotic resistance was determined by the method of dissemination of antimicrobial disks and measurements of diameters inhibition (Kirby Bauer method). After adjustment of inoculums density (10^6 UFC) then the standard 0.5 Mac Farland, Mueller Hinton medium was inoculated, let dry. Eleven different antibiotic disks: Penicillin G, Ampicillin, Cefoxitin, Oxacillin, Vancomycin, Chloramphenicol, Clindamycin, Rifampicine, Tetracycline, Kanamycin, Ciprofloxacin ((bioMérieux, Marcy-l'Etoile, France) are deposited. The average results of three readings are expressed in Sensitive (S) or resistant (R) according to the standards of the Committee of the antibiogram of French Society of Microbiology (1996). A strain with known antibiotic resistances (*Staphylococcus aureus* ATCC 25923) was used as the control strain.

Inhibitory activities

The antimicrobial activity is thought to be an important means for lactic acid bacteria (LAB) to competitively exclude or inhibit invading bacteria (Carr et al., 2002; Roos and Holm, 2002). Some of them act by secreting non-specific antimicrobial substances such as short-chain fatty acids (Carr et al., 2002) or hydrogen peroxide (Eschenbach et al., 1989), while others produce toxins with very narrow ranges of killing, such as bacteriocins, and bacteriophages (Smith et al., 2007; Tagg and Dierksen, 2003). The antimicrobial activity was detected by the diffusion method of Schillinger and Lucke (1989). The medium used was Mueller-Hinton. The pathogenic bacteria which were targeted are *Staphylococcus aureus* ATCC 25923, *Escherichia coli ATCC 25921*, *Streptococcus sp.* and *Pseudomonas sp*. Two to three wells of 5 mm diameter were performed in Mueller- Hinton agar. Petri dish was flooded by 0.1 ml of the pathogenic strain, then the wells were filled with 60 µl of filtered supernatant which was obtained after centrifugation at 10 000 t/min in 20 ml of medium cultivated MRS with lactic strain. After 24 h of incubation at 30°C, the diameters of inhibition zones appearing around the wells were measured.

Measure the acidity produced by bacteria

The acidifying power strains on MRS (De Man Rogosa Sharpe) medium liquid was estimated by titration with sodium hydroxide using phenolphthalein as an indicator. Each strain was inoculated into 10 ml of sterile skim milk (10% w/v). Pre-cultures were prepared by incubation at 30°C until coagulation. 3% of the pre-culture was transferred aseptically in 100 ml of skim milk. The mixture was distributed in sterile tubes at 10 ml/tube.

Under magnetic stirring, Dornic soda (N/9) was added drop by drop, until a persistent pink color was formed. We denote the volume of NaOH used. The acidification kinetics was performed with regular intervals of 0, 2, 4, 6, 8, 10, and 24 h. The results were expressed in degrees Dornic. The acidification was also measured with respect to time by the method of Lombardi et al. (2002) and Ayad et al. (2004). The value of the acidification was calculated by the difference in values of pH ($\Delta pH = pH_{\text{at time}} - pH_{\text{at time zero}}$). The cultures were considered as fast, medium and slow when Δ pH = 0.4 (pH unit) after 3, 3 to 5 and > 5 h, respectively.

Tolerance to bile salts

The concentration of intestinal bile is 0.3% (w/v) and the residence time of food through the small intestine is approximately 4 h (Prasad et al., 1998). The experiment was applied to the concentration of bile for 4 h. The strains were inoculated in MRS broth enriched with 0.3% bile salts (Oxoid) and incubated at 30°C. During the 4 h incubation, growth was verified by measuring the optical density (OD_{600}) and by counting on MRS solid every hour.

Resistance to acidic pH

While in the stomach, the pH can be as low as 1, most *in vitro* assays using a pH 3.0. Due to the fact that a significant decrease in the viability of strains was often observed at pH 2 and below, and because the food stayed for 3 h, this period was considered (Prasad et al., 1998). After 18 h of incubation in MRS broth and after centrifugation for 10 min at 5000 rev/min at 4°C, the pellet was washed once with phosphate-buffered saline (PBS) at pH 7.2, and

then resuspended in PBS pH 1, 2 and 3. It was incubated at 30°C (El-Naggar, 2004; Yavuzdurmaz, 2007). During the 3 h incubation, growth was verified by measuring the optical density (OD_{600}) and counting on MRS solid every hour.

RESULTS

Antibiotic resistance

The results showed that eight of the ten strains tested were multiresistant to various antibiotics such as penicillin, oxacillin, chloramphenicol and kanamycin. In contrast, ampicillin, vancomycin, and clindamycin were most active antibiotics against all strains. The strains BLT21 and BLT31 were completely sensitive to all antibiotics tested.

Inhibitory activities

The diameter of inhibition zones (Table 2) showed that most of the isolates had an antibacterial effect on the pathogenic micro-organisms tested.

Measure of the acidity produced by bacteria

For the production of lactic acid, strains BLT3, BLT 26, BLT27, BLT28 and BLT31 were considered fast because Δ pH ≥ 4U in less than three hours and the strains BLT21, BLT26, BLT27 and BLT 30 were considered medium to stem acidifying. The strain BLT23 was the only strain with a slow power of producing lactic acid (Figures 1 and 2).

Tolerance to bile salts

For resistance to bile salts, all strains were able to grow in these conditions and the results are shown in Figure 5.

Resistance to acidic pH

Resistance to low pH values is one of the criteria for selecting probiotic strains (Ouwehand et al., 1999; Çakir, 2003). Resistance to pH 3 is often used *in vitro* assays to determine resistance to the pH of the stomach. In our experiment, all strains survived at pH 3 (Figure 3); with respect to pH 2, only three strains survived during the incubation time for 3 h. The others did not survive after 1 h of incubation (Figure 4). Growth was not detected in pH 1 for all strains tested.

DISCUSSION

The aquatic cultures are continuously exposed to a wide range of microorganisms, some which are pathogenic (Reilly and Kaferstein, 1999). Efforts to prevent and control invasion by disease-causing agents have concentrated on good husbandry techniques and the use of vaccines (Corripio-Miyar et al., 2007) and antibiotics (Smith et al., 2007). The use of vaccines is laborious, costly, and highly stressful to the animals. The use of antibiotics will result in the selection of antibiotic-resistant bacteria and the residues of the drugs remain active long after use, as free unused antibiotic (Matyar, 2007). An alternative approach to disease prevention in aquaculture is to use the probiotics (Vijayan et al., 2006). The strains used in this study were isolated from several specimens of Nile tilapia, a fish with important position in the world of aquaculture.

The researches on inhibitory bacteria or bacteria producing inhibitory substances against pathogenic bacteria are actual studies. Thus, for our study on testing antimicrobial resistance, the isolates showed multidrug resistance to the several used antibiotics, because lactic acid bacteria are intrinsically resistant to antibiotics (Salminen et al., 1998). These results are consistent with various reports indicating that lactic acid bacteria are usually resistant to principal antibiotics, such as penicillin G, ampicillin, chloramphenicol and ciprofloxacin (Coppola et al., 2005) especially, when penicillin G, amoxicillin, oxacilin, cefoxitin, ceftriaxone, and chloramphenicol are the most commonly used antibiotics in aquaculture.

Studies of Herros et al. (2005) on *L. plantarum* isolated from different sources were resistant to the same antibiotics. Multi-drug resistance of most isolates is also reported by several authors (Bhattacherjee et al., 1988; Pathak et al., 1993; Goni-Urriza et al., 2000; Rhodes et al., 2000) and according to these studies, the increasing use and misuse of antibiotics has created resistant bacteria through the transfer of resistance plasmids between them. For BLT3 and BLT31 strains, they were sensitive to all used antibiotics. Vancomycin is active in all gram-positive bacteria (Reynolds, 1989). This is consistent with our results since all strains were susceptible to this antibiotic (Table 1).

In addition, the results of inhibitory power of the ten strains against pathogenic bacteria that cause the greatest losses in aquaculture showed that our strains had a good antagonistic effect particularly strains BLT3, BLT21, BLT23 and BLT26. This inhibitory activity against *S. aureus*, *E. coli, Streptococcus sp* and *Pseudomonas sp.* by our strains, observed *in vitro* showed us the possibility to exploit this activity for use as a means to exclude pathogenic bacteria through the production of inhibitory compounds, improve water quality, enhance the immune response of host species, and enhance the nutrition of host species through the production of supplemental digestive enzymes (Thompson et al., 1999; Verschuere et al., 2000). Our results agree with those of Nogami and Maeda (1992), Austin et al. (1995), Rengpipat et al. (1998), Gram et al. (1999), and similar to the work of Jayanth et al. (2001) on some marine

Table 1. Isolates of antibiotics resistance results.

Antibiotic	BLT3	BLT20	BLT21	BLT23	BLT26	BLT27	BLT28	BLT29	BLT30	BLT31
Penicillin G (10 µg)	R	S	S	S	R	R	R	R	R	S
Ampicillin (10 µg)	S	R	S	S	S	S	S	R	S	S
Cefoxitin (30 µg)	S	S	S	R	S	S	S	S	R	S
Oxacillin (1 µg)	R	R	S	R	R	R	S	R	R	S
Vancomycin (30 µg)	S	S	S	S	S	S	S	S	S	S
Chloramphenicol (30 µg)	S	R	S	R	R	S	S	R	S	S
Clindamycin (2 µg)	S	S	S	S	S	S	S	S	S	S
Rifampicine (5 µg)	S	R	S	R	S	S	R	S	R	S
Tetracycline (30 µg)	R	R	S	S	R	S	R	S	S	S
Kanamycin (30 µg)	S	S	S	R	R	S	R	R	R	S
Ciprofloxacin (5 µg)	R	S	S	S	R	S	S	S	S	S

(R): Resistant, (S): sensitive.

Table 2. Diameter of inhibition zones (mm).

Strain	Diameter of inhibition zone (mm)			
	***E. coli* ATCC 25921**	***S. aureus* ATCC 25923**	***Streptococcus* sp.**	***Pseudomonas* sp.**
BLT3	22 ± 1.41	20 ± 0	15 ± 2.82	13 ± 4.24
BLT20	25 ± 2.82	18 ± 2.82	17 ± 4.24	21 ± 2.82
BLT21	22 ± 4.24	34 ± 1.41	20 ± 1.41	27 ± 1.41
BLT23	18 ± 1.41	24 ± 0	10 ± 0	-
BLT26	11 ± 4.24	22 ± 4.24	10 ± 4.24	17 ± 2.82
BLT27	9 ± 0	-	14 ± 4.24	19 ± 4.24
BLT28	-	15 ± 2.82	-	17 ± 0
BLT29	-	16 ± 1.41	11 ± 2.82	13 ± 2.82
BLT30	18 ± 5.65	12 ± 0	13 ± 4.24	15 ± 0
BLT31	-	21 ± 1.41	9 ± 5.65	17 ± 1.41

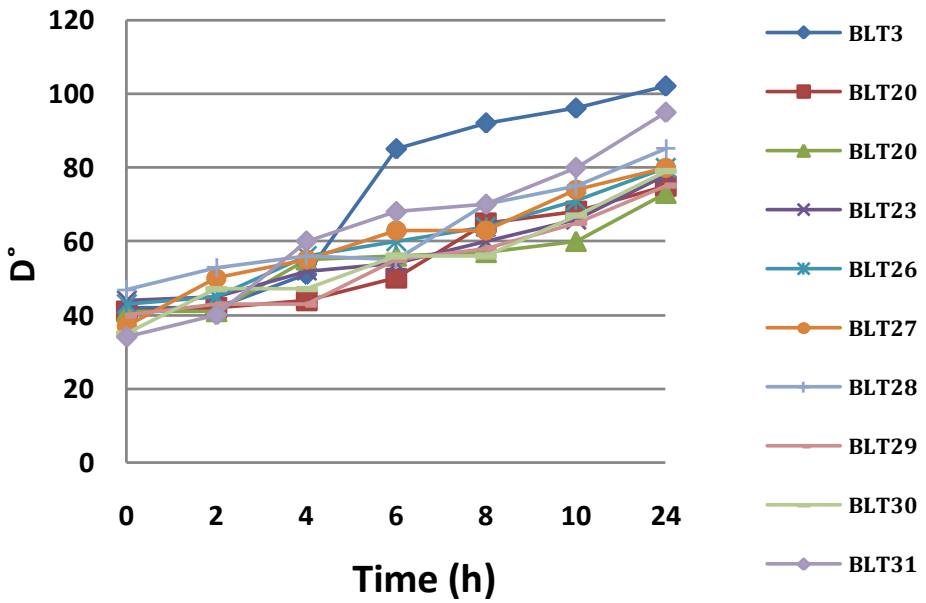

Figure 1. Kinetics of Dornic Acidity evolution for the ten acid lactic bacteria.

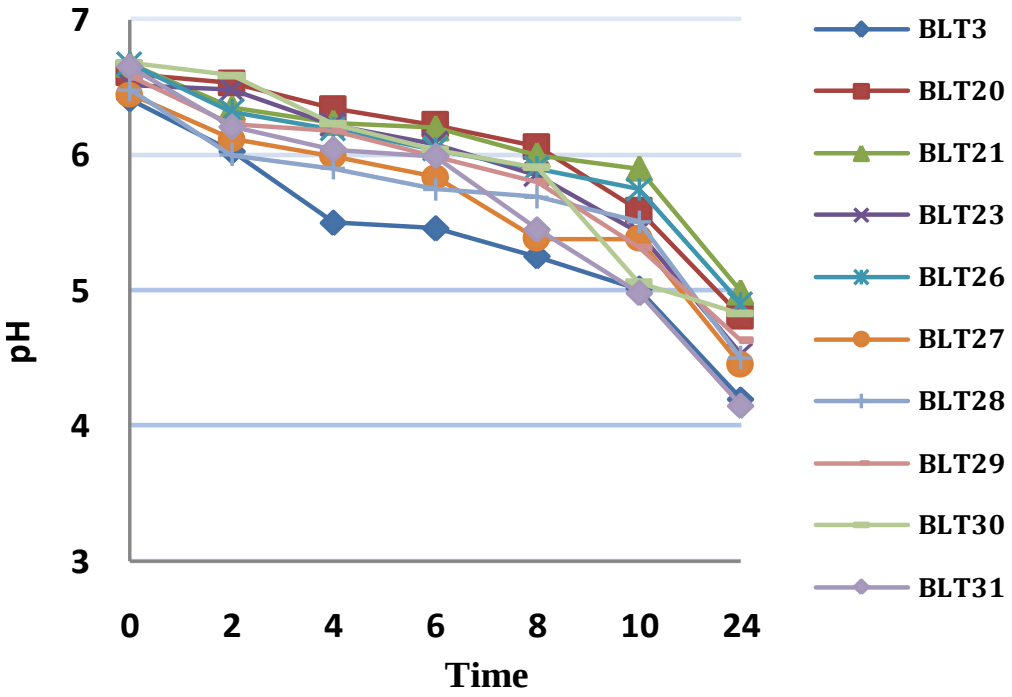

Figure 2. Kinetics of pH variation of the isolates.

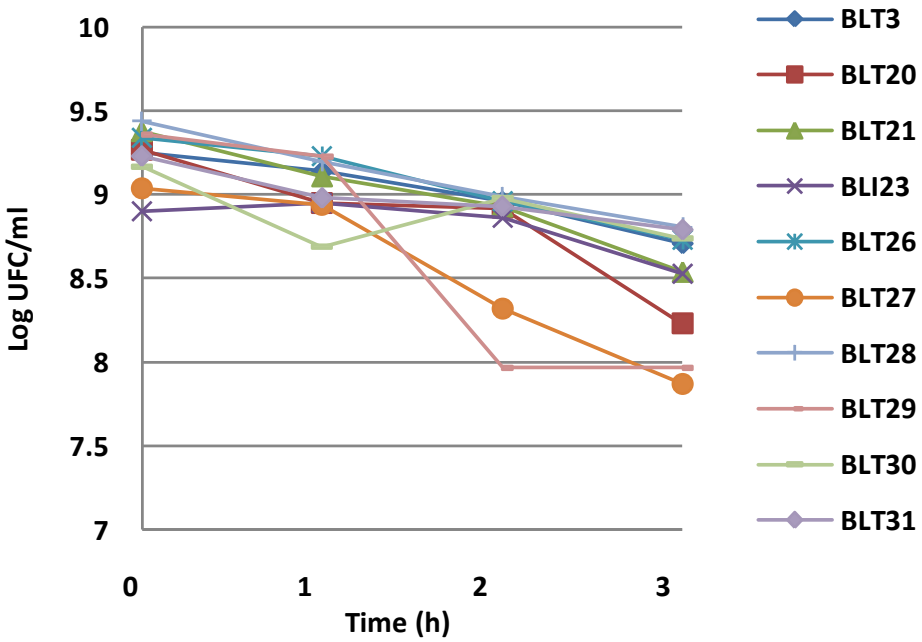

Figure 3. Tolerance of strains to pH 3.

bacteria.

However, Sugita et al. (1996) reported that among 304 strains isolated from the intestinal tract of fish, only 3.2% had an inhibitory capacity to other organisms. Most strains showed a rapid or medium acidification activity which created rapidly a hostile environment for pathogenic bacteria. The tolerance to bile salts and acid pH is considered a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host (Havenaar et al., 1992). It is mentioned that

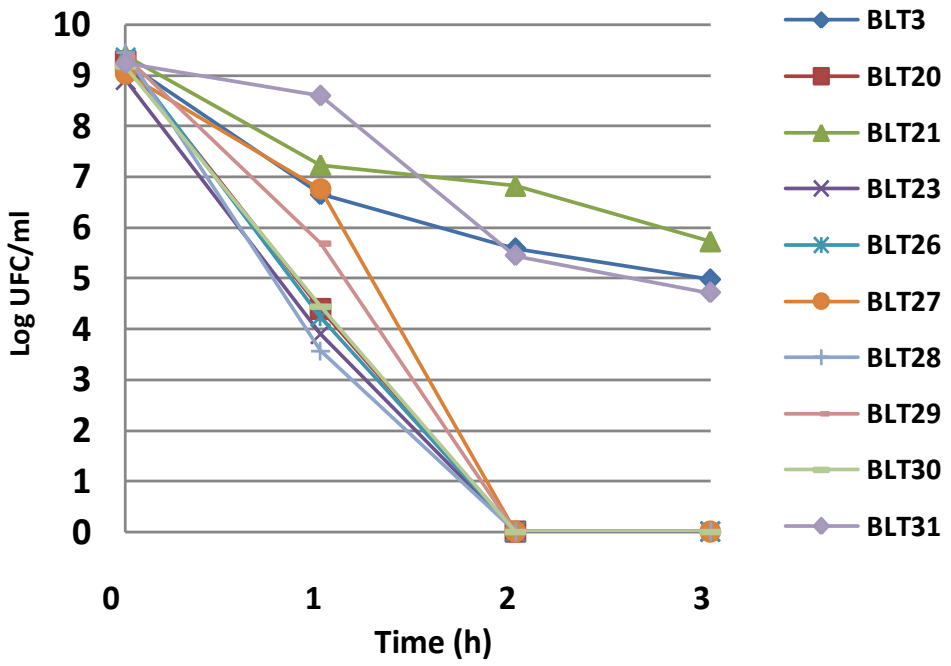

Figure 4. Tolerance of isolates to pH 2.

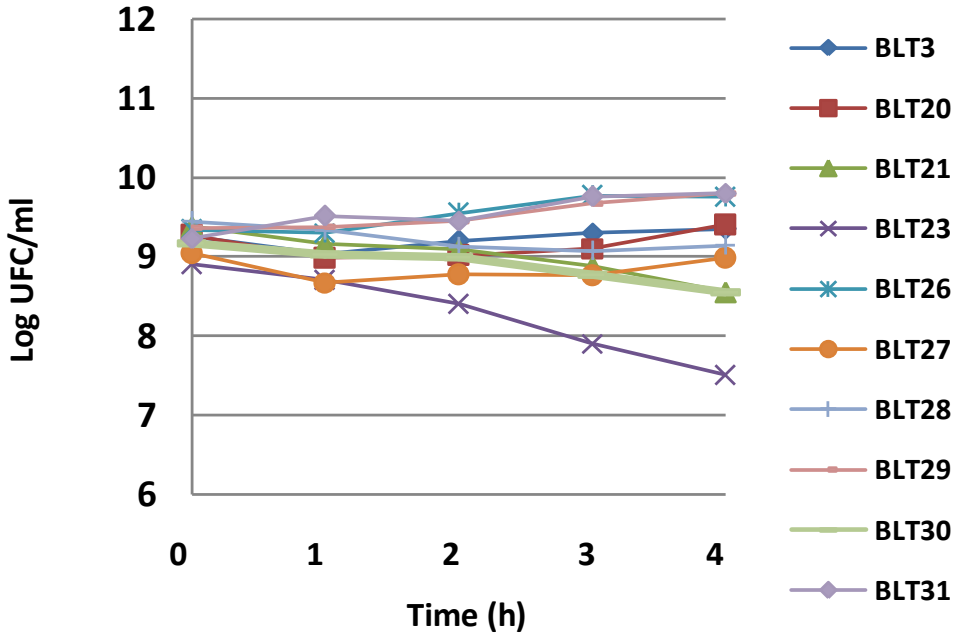

Figure 5. Tolerance of lactobacilli to 0.3% bile salts.

resistance to bile salts varies greatly among the lactic acid bacteria and also between strains of the species themselves (Xanthopoulos, 1997). Before reaching the intestinal tract, probiotic bacteria must first survive transition through the stomach where the pH can be as low as 1.5 to 2 (Dunne et al., 2001).

Maragkoudaki et al. (2005) tested 29 strains of lactobacilli of dairy origin for their probiotic potential. The resistance of these bacteria at a pH between 1 and 3 revealed that all strains are resistant to pH 3 for 3 h, and most have lost their viability in 1 h in a pH 1. In addition, all strains tolerated a concentration of 0.3% bile salt for 4 h. In our study, for pH 3, we obtained the same results. In contrast to pH 1, we had no growth, whereas, for bile

salts, the results were similar to those obtained in this study since it was noted that all strains grew at this concentration.

Conclusion

This study aimed to characterize and determine the properties of some probiotic lactobacilli isolated from the intestine of Nile tilapia (*Oreochromis niloticus*). This potential was investigated by the application of several tests such as resistance to acid pH, bile salts, inhibitory effect and antibiotic resistance. Two of our strains (BLT3 and BLT31) were sensitive to antibiotics that were tested, the most widely used antibiotics in aquaculture areas, had the largest inhibition against pathogenic bacteria and can survive under stressful conditions of our experiments. These results showed positive traits for our strains, which gives them a good probiotic potential. Therefore, some additional studies should be done to know the power of adhesion, the stability of the strain to manufacturing processes and the influence of the incorporation of these bacteria in the diet of fingerlings of Nile tilapia on their growth and animals' performances.

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