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RESEARCH ARTICLE

In vitro study of *Lactobacillus plantarum* properties as a potential probiotic strain and an alternative method to antibiotic treatment of fish

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Abstract. The presence of lactic acid bacteria (LAB) favors the stabilization of intestinal flora, facilitates digestion, improves the assimilability of fodder, and has an immunomodulatory effect on the immune system. According to current research, the application of LAB following antibiotic treatment prevents the development of opportunistic bacteria inhabiting the digestive tract. In the study the potential probiotic properties of *Lactobacillus plantarum* strains, which can be administered as an alternative to antibiotic treatment in aquaculture, were investigated under *in vitro* conditions. The strains of *L. plantarum* were characterized for important properties such as the ability to grow in the presence of 10% fish bile, a tolerance of low pH, and antagonism to pathogens dangerous for fish such as *Aeromonas salmonicida* and *Pseudomonas fluorescens*; therefore, they meeting the criteria for strains with probiotic properties. In view of currently increasing resistance to antibiotics and a decrease of their efficiency, probiotic bacteria can serve to support immunity to infections in the future.

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Introduction

The digestive tracts of healthy fishes exhibit a state of balance among endogenous bacterial strains. Microbes naturally inhabiting the intestine develop a biofilm, the role of which is to inhibit the growth of pathogenic bacteria. Antibiotic therapy, stress, or improper diet can cause disturbances in the balance of the intestinal microflora, resulting in hampered digestion, and, consequently, the reduced assimilation of nutrients and the intensity of foraging, growth rates, and the development of fish.

Pursuant to the definition from 2002 provided by FAO/WHO, probiotics are defined as "living microorganisms that, when administered in adequate amounts, confer a health benefit to the host." Since its publication, this definition has become binding. In light of the appearance of new published scientific evidence, progress regarding probiotic preparations is dynamic. New studies appear to confirm the effect of such preparations on human and animal health, including fishes (Nikoskelainen et al. 2001a, Hoseinifar et al. 2015). Among probiotic bacteria,

lactic acid bacteria (LAB) constitute the most thoroughly investigated group. This group of bacteria includes Gram-positive cocci from the genera *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Enterococcus*, *Oenococcus*, and *Pediococcus*, and Gram-positive bacilli from the genera *Lactobacillus* and *Carnobacterium*. Strains exhibiting probiotic potential include those from the species B*ifidobacterium adolescentis, Bifidobacterium animalis, Bifidobacterium bifidum, Bifidobacterium breve,* and *Bifidobacterium longum*, as well as the species *Lactobacillus acidophilus*, *Lactobacillus casei, Lactobacillus fermentum, Lactobacillus gasseri, Lactobacillus johnsonii, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus rhamnosus*, and *Lactobacillus salivarius* (Hill et al. 2014).

In order for bacteria to be classified as probiotic, they must meet a number of criteria, including the ability to survive in the environment of the digestive tract, antagonistic activity toward pathogens that are dangerous for the organism or have the potential to inhibit their growth, and the ability to produce hydrogen peroxide and compounds with antimicrobial properties. An important property is the ability of the strains to adhere to the gastrointestinal tract mucous membrane, which facilitates the persistence of probiotic strains in the environment over long periods of time (Reid et al. 2011).

The presence of LAB favors the stabilization of intestinal flora, facilitates digestion, improves the assimilability of fodder, and has an immunomodulatory effect on the immune system. Research conducted so far shows that their application following antibiotic treatment prevents the growth of the opportunistic bacteria inhabiting the digestive tract (Son et al. 2009, Andani et al. 2012).

LAB have the ability to produce protein substances with antibacterial properties called bacteriocins, such as plantaricin, acidocin, lactocin, lacticin, and nisin (Son et al. 2009, Giri et al. 2013, 2014). Bacteriocins are usually synthesized at the preliminary stages of bacterial growth and are secreted outside of the cell. The mechanism of their functioning involves the development of pores in the cell wall of a susceptible bacterium through which electrolytes contained in the cytoplasm leak out leading to its death (Savadogo et al. 2006). Based on the type of sugar metabolism, LAB are divided into homofermentative (lactic acid is the primary end product of fermentation) and heterofermentative (producing lactic acid as well as acetic acid, formic acid, ethanol, carbon dioxide, diacetyl, acetaldehyde, benzoic acid, etc.). Lactic acid bacteria are able to grow in an acidic environment thanks to complex mechanisms that protect the cell against the passive penetration of the organic acids produced into the microorganism that prevents the acidification of the cytoplasm.

Bacteria from genus *Lactobacillus* have high nutritional requirements. Therefore, they usually occur in nutrient-rich environments (carbohydrates, amino acids). They inhabit the human and animal digestive tracts and the surfaces of plants and are contained in food products (cheeses, yogurts, fermented beverages). Their common property is the ability to anaerobically breakdown carbohydrates by lactic acid fermentation through different metabolic pathways.

L. plantarum strains are Gram-positive bacilli commonly occurring on plants, in fermented foods, and in the digestive tracts of humans and animals, including fish. They are characterized by long-term persistence in the digestive tract. Because of this, they are used deliver vaccines and medical preparations to the host (Rigaux et al. 2009, Bahey-El-Din 2012). Strains of *L. plantarum* are recognized as safe thanks to their staus as GRAS (Generally Recognized As Safe). They are non-pathogenic and do not produce substances that negatively affect human or animal health. Therefore, it is possible to apply these bacteria in the food and pharmaceutical industries, and as probiotic strains. Earlier research on *L. plantarum* strains confirmed their ability to produce antimicrobial substances, such as plantaricin, which is active against specified pathogens and their ability to inhibit their growth (Cebeci and Gurakan 2003).

The objective of this work was to assess under *in vitro* conditions the potential probiotic properties of *L. plantarum*, which can be administered as an alternative to antibiotic treatment in aquaculture.

Material and methods

The experiments involved five strains of *L. plantarum* (225/1, 155/1, 211/1B, 226/1, 274/1) obtained from the collection of strains of the Department of Microbial Biochemistry of the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw, Poland.

The strains were isolated from a sample of raw milk originating from the region of Warmia and Mazury, Poland. The phenotypic and genetic characteristics of the analysed strains of *L. plantarum* were determined. The ability of the strains to use 49 different sources of carbon was examined with the API 50CHL test (Biomerieux) and analyzed with Apiweb software. The enzymatic profile and ability of the strains to produce 19 enzymes was also determined with the API Zym test (bioMerieux). *L. plantarum* cells were cultured on MRS substrate in anaerobic conditions in GENbox jars using atmospheric generators (bioMerieux).

The preliminary research involved determining the antagonistic activity of *L. plantarum* to field bacteria pathogenic for common carp, *Cyprinus carpio* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum), i.e., *Aeromonas hydrophila, Aeromonas salmonicida*, *Pseudomonas fluorescens,* and *Acinetobacter junii* obtained from fish showing symptoms of disease, isolated from skin lesions (ulcers, hemorrhages, hyperemia) and internal organs. The analysis of the antagonism of lactic acid bacteria was performed with the agar slab method described by Strus (1998), where the suspension of *L. plantarum* at density of 2 on the McFarland scale was transferred onto MRS (BTL) medium and incubated at 26°C for 48 h in the presence of 6% CO2. After incubation, 11-mm slabs were cut out from the MRS agar and then transferred onto plates prepared with TSA (BTL) medium inoculated with strains of the field bacteria *A. hydrophila, A. salmonicida*, *P. fluorescens*, and *A. junii*, respectively. The plates were incubated for 24 h at 18°C and 26°C, after which the diameter of the zone of inhibition of growth of the indicator bacteria was measured.

The ability of *L. plantarum* to pass through the intestine was analysed by examining lactobacilli growth in the presence of bile. Bile was sampled from rainbow trout through punctures of the gallbladder, and then it was stored at a temperature of -26°C (Nikoskelainen et al. 2001b). For the assay, bacterial suspensions were prepared in PBS at a density of 10^7-10^8 CFU ml^{-1}. Then, 500 µl of bacterial suspension was centrifuged and resuspended in sterile PBS or in sterile PBS with the addition of 10% fish bile. The samples were incubated for 1.5 h at 26°C and then they were serially diluted in sterile PBS and viable counts were determined by plate count using the MRS agar medium. The plates were incubated for 48 h at 26°C. The ability of the analysed bacterial strains to survive in the presence of bile salt was determined based on the number of colonies grown. Survival was calculated as the logarithm of the numbers of colony forming units after 1.5 h incubation in the presence of bile compared to the logarithm of the number of colony forming units developed after 1.5 h of incubation with no addition of bile to the medium.

The resistance of the analysed strains of *L. plantarum* to low pH was examined by adding bacterial suspension at density of 10^7-10^8 CFU ml^{-1} to a MRS bullion previously adjusted by addition of HCL 1 N to pH 2, 3, 4, or 5 and incubating it for 1.5 h at 26°C (Prasad et al. 1998). After this period, the bacteria were plated onto MRS agar medium and incubated for 48 h at 26°C. A bacterial culture grown on medium with pH 6.4 served as the control sample. The ability of the analysed bacterial strains to survive in an environment with a low pH was determined based on the number of colonies obtained. Survival was calculated as the logarithm of the numbers of colony forming units after 1.5 h incubation with the addition of hydrochloric acid compared to the logarithm of the number of colony forming units developed after 1.5 h of incubation with no addition of hydrochloric acid.

The ability of *Lactobacillus* to produce hydrogen peroxide (H_2O_2) was determined by growing the strains on MRS agar medium with the addition of 2.2'-azino-bis (3-ethylobenzotiazolino-6-sulfonic) acid (ABS) and peroxidase (Sigma Aldrich) for 48 h

Table 1
Antagonistic activity of bacteria from the genus *Lactobacillus* to field strains of bacteria pathogenic for common carp, at 26°C (mean ± SD, n=3)

	Growth inhibition zone (mm)				
Pathogenic bacteria	*L. plantarum* 225/1	*L. plantarum* 155/1	*L. plantarum* 211/1B	*L. plantarum* 226/1	*L. plantarum* 274/1
P. fluorescens strain 1	20.67±0.58	21.33±1.53	20.67±1.15	19.67±1.53	19.00±1.73
P. fluorescens strain 2	20.33±0.58	18.00±2.65	21.00±1.00	19.67±0.58	19.33±1.53
A. hydrophila strain 1	18.00±1.73	17.67±2.08	18.33±2.08	17.00±1.73	17.33±2.08
A. hydrophila strain 2	18.67±1.53	17.67±2.08	18.33±2.08	17.00±2.65	18.33±2.08
A. hydrophila strain 3	16.33±2.31	16.33±1.53	15.67±0.58	17.00±2.00	15.33±2.52
A. salmonicida	16.33±1.53	16.00±1.00	16.00±1.73	16.33±1.53	15.00±2.65
A. junii	15.33±1.15	14.33±1.15	15.00±1.73	13.67±1.53	16.33±2.52

at 26°C. The plates were then left at room temperature for another period of 5-8 h (Berthier 1991). After this time, changes in the color of the substrate to violet should have occurred around the bacteria obtained, which expressed the ability of the lactic acid bacteria to produce hydrogen peroxide.

Determinations of the surface properties of *L. plantarum* such as the ability to produce mucus and autoaggregation ability were performed as follows. The first experiment was done according to Hogt et al. (1983), where bacteria were cultured in glass test tubes for 24 h at 26°C. After this time, the tubes were emptied, and 0.1% alcohol solution of safranin was poured against their walls. Mucus with pinkish colour indicated positive reactions. The second experiment was the assessment by naked eye of the flocculation of the suspension of the analysed strains in a NaCl solution (Ahmed et al. 1992).

Statistical analyses

The results obtained are shown as the average of three independent experiments, and variation is expressed as standard deviation. Analysis of variance (ANOVA) and the Duncan's multiple range test were used to determine significant differences ($P < 0.05$) among the various *L. plantarum* strains tested. All statistics were performed using Statistica 8.0 (StatSoft) for MS Windows.

Results

The results of the assessment of the antagonistic activity of *L. plantarum* strains against fish pathogenic bacteria at incubation temperatures 18 and 26°C are presented as the average diameter of growth inhibition zone ± standard deviation in Tables 1 and 2. There were significant differences ($P < 0.05$) between *L. plantarum* strains 225/1 and 274/1 against *A. hydrophila* strain 1 and *A. junii* and between *L. plantarum* strains 225/1 and 226/1 against *A. hydrophila* strain 2 at the of temperature 18°C. There were no significant differences ($P < 0.05$) among analysed *L. plantarum* strains at a temperature of 26°C.

The diameter of the growth inhibition zones differed significantly ($P < 0.05$) after incubation at 18°C compared to 26°C for all the *L. plantarum* strains examined and *A. hydrophila* strain 1, *A. hydrophila* strain 2, *A. hydrophila* strain 3, *A. salmonicida*, and *A. junii*, but not in the case of *P. fluorescens*.

An important parameter of bacteria is their resistance to bile, which permits surviving the passage through the digestive tract and reaching and colonizing the target site. All the analysed strains survived incubation in bile salts and were exhibited high resistance (Fig. 1).

The survival of *Lactobacillus* was examined at low pH, i.e., 2, 3, 4, and 5. All of the strains analyzed survived incubation at pH 2. A considerable decrease

Table 2
Antagonistic activity of bacteria from the genus *Lactobacillus* to field strains of bacteria pathogenic for common carp, at 18°C (mean ± SD, n=3)

	Growth inhibition zone (mm)				
Pathogenic bacteria	*L. plantarum* 225/1	*L. plantarum* 155/1	*L. plantarum* 211/1B	*L. plantarum* 226/1	*L. plantarum* 274/1
P. fluorescens strain 1	21.33±1.15	22.33±1.53	22.33±2.08	22.33±2.31	21.67±1.15
P. fluorescens strain 2	21.00±1.73	21.00±2.65	20.67±2.08	21.33±0.58	22.00±1.00
A. hydrophila strain 1	24.67±2.08^{a}	26.00±2.65^{a,b}	26.33±1.53^{a,b}	26.00±1.73^{a,b}	29.67±4.04^{b}
A. hydrophila strain 2	23.33±1.53^{a}	26.33±1.53^{a,b}	26.33±1.15^{a,b}	29.00±3.00^{b}	26.67±2.52^{a,b}
*A. hydrophil*a strain 3	21.33±1.53	21.67±3.06	22.33±3.21	22.00±1.73	22.33±1.53
A. salmonicida	25.00±2.65	27.67±2.08	25.67±3.79	25.33±1.53	28.33±3.21
A. junii	19.67±2.08^{a}	23.00±2.65^{a,b}	22.33±1.15^{a,b}	21.67±2.08^{a,b}	24.33±3.21^{b}

^{a,b,c} – significant differences between marked values at $P < 0.05$

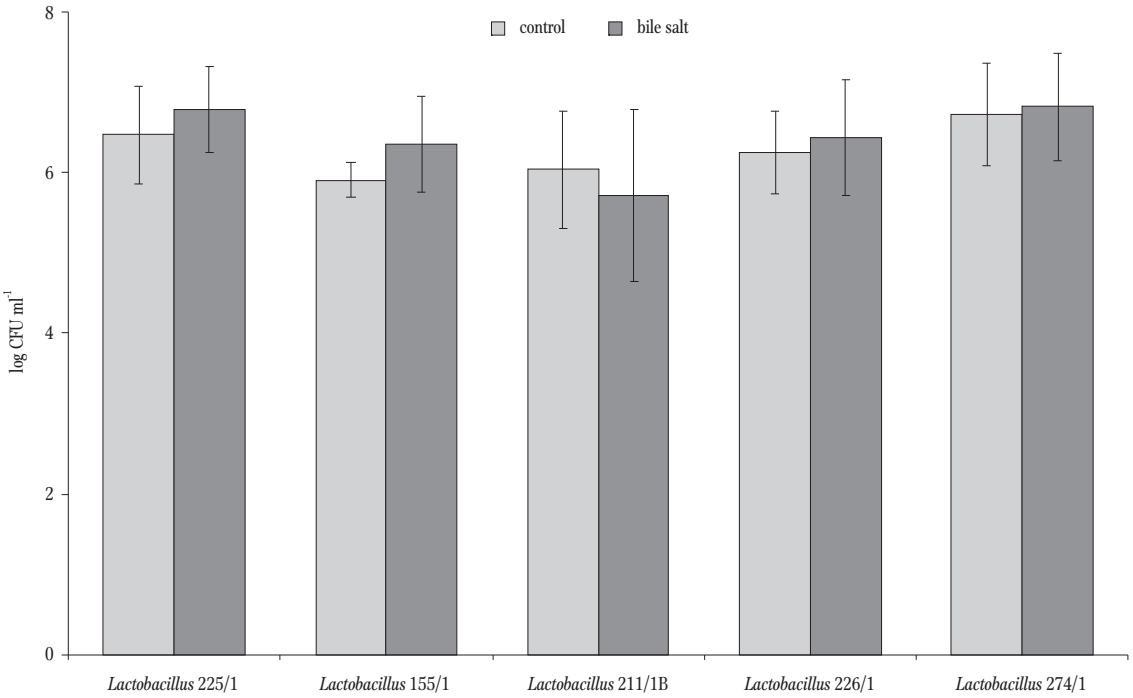

Figure 1. Effect of the concentration of bile salts on the survival of the analysed *Lactobacillus* strains.

in the number of bacterial cells was observed; however, depending on the strain it was lower by three log orders (strain 225/1), four log orders (strain 226/1 and 274/1), or five log orders (strains 155/1 and 211/1B). The bacteria analyzed exhibited good resistance to pH 3. Only in the case of strain 211/1B was a decrease in the number of bacterial cells observed by one log order. No statistically significant differences were recorded among the groups of bacteria incubated in pH 4 and pH 5 (Fig. 2).

In the course of experiments to determine the ability of the *L. plantarum* bacterial strains to produce hydrogen peroxide, no change in the color of the substrate around the strains was observed.

During the investigation of the surface properties of *L. plantarum* strains, a pink biofilm and

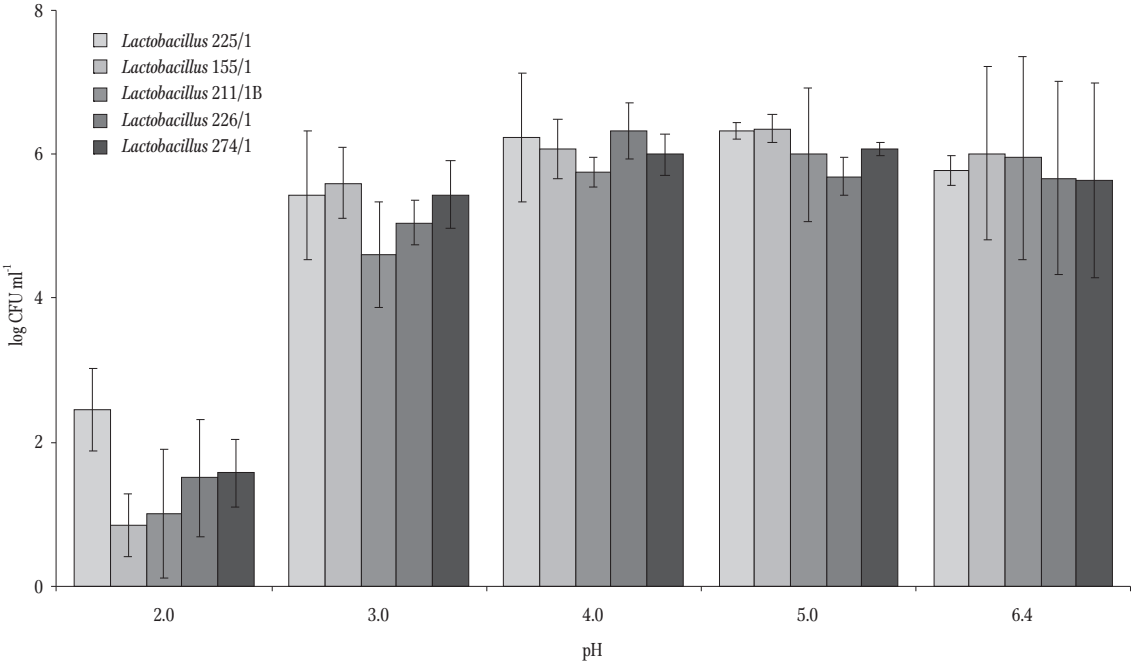

Figure 2. Survival rate of *Lactobacillus plantarum* strains after 1.5 h of incubation in media of different pH.

autoagglutination in the physiological NaCl solution were observed.

Discussion

The key features of probiotic bacteria are their antibacterial properties against pathogens, in this case those of fish. Numerous studies have shown that the antagonistic activity of *L. plantarum* is related to their ability to produce metabolic products and bacteriocins (Ringø and Gatesoupe 1998, Vazquez et al. 2005). The production of antibacterial substances depends on temperature, among other factors, but this does not always correspond with the optimum temperature for bacterial growth. At lower temperatures bacterial growth is slower but the amount of metabolites can increase (Goderska et al. 2012). Our own results correspond with the findings above. At 18°C the antagonistic activity of the *Lactobacillus* strains analyzed against almost all the pathogenic bacteria tested was higher than at 26°C. This is important because in the case of salmonid fish, bacterial diseases usually occur in the temperature range of 16 to 22°C. Psychrophilic bacteria that are a factor of etiological diseases in early spring are also a threat to common carp. Geraylou et al. (2014) provide evidence that certain strains of *Lactococcus lactis* ssp. *lactis* isolated from the digestive tract of Siberian sturgeon, *Acipenser baerii* (Brandt, 1869), inhibit the growth of *A. hydrophila*, *A. salmonicida*, *Yersinia ruckeri*, *Flavobacterium columnare*, and *Vibrio anguilarum*. Another study (Allameh et al. 2013) shows that, in diffusion tests, *L. fermentum* isolated from the stomach of snakehead, *Channa striata* (Bloch), inhibited the growth of the pathogens *A. hydrophila, Pseudomonas aeruginosa*, and *Shewanella putrefaciens*. *In vivo* studies also confirm the effect of probiotic bacteria (*L. plantarum, Lactobacillus delbrueckii*, *Lactobacillus sakei*, *L. rhamnosus*) on pathogens in various fish species, e.g., in rainbow trout, Atlantic salmon, *Salmo salar* L., longtooth grouper, *Epinephelus bruneus* (Bloch), Nile tilapia, *Oreochromis niloticus* (L.), and roho labeo, *Labeo rohita* (Hamilton) (Nikoskelainen et al. 2001a, Harikrishan et al. 2010, Ngamkala et al. 2010, Giri et al. 2013).

All the strains of *L. plantarum* analyzed survived 1.5 h incubation in bile, even though the concentration of bile used in the experiment was relatively high. In fish, bile is produced in the liver from cholesterol and secreted to the intestine where its concentration varies from 0.4 to 1.3% (Balcazar et al. 2008). Its task is to emulsify fats. The activity of bile salts is more destructive for bacteria cells than low pH, because lipid emulsifiers can damage bacterial cell membranes. Nikoskelainen et al. (2001a) recorded no significant differences in survival between the control group and *L. rhamnosus* LC 705, *Bifidobacterium lactis* Bb 12, *Lactobacillus bulgaricus*, *Enterococcus faecium* Tehobak, *L. rhamnosus* ATCC 53103, or *L. johnsonii* La 1 incubated in 10% fish bile. Only in the case of *L. casei* Shirota, did the number of living bacteria decrease by 19.7%. Balcazar et al. (2008) reports that *Lc. lactis*, *L. plantarum*, and *L. fermentum* isolated from the digestive tract of healthy rainbow trout survived 1.5 h incubation in 10% bile. No significant changes were recorded in the number of bacterial cells between the control group and the group incubated with bile. The tolerance of the analysed strains to bile is presumably related to the ability to produce the enzyme bile salt hydrolase (BSH), which protects them against the toxic activity of bile (Schillinger et al. 2005). According to literature data, not all lactic acid bacteria exhibit BSH activity. It usually occurs in bacteria isolated from an environment rich in conjugated bile acids, i.e., the intestinal flora (Ziarno 2005).

In the study we also assessed the survival of *L. plantarum* in acidic environments in a pH range of 2 to 5. The threshold value was determined as pH 2, although such an extremely low value in fish occurs only in an empty stomach, and the analysed bacteria are supposed to be supplied with fodder that buffers acidic reactions. All the bacterial strains analyzed survived 1.5 h incubation in an environment of pH 2 and showed high resistance to higher pH values. A similar tendency was observed by Goderska et al. (2002), who found that in an environment of pH 2 *L. plantarum* T-106 survived an incubation of 24 h in pH for 3 to 11 days and in pH 4 and 5 for 31 days. Haller et al. (2001) report that neither *L. johnsonii* La1 nor *L. plantarum* survived exposure to pH 1.5. Balcazar et al. (2008) found that *Lc. lactis*, *L. plantarum*, and *L. fermentum* survived 1.5 h of incubation at pH from 2.5 to 6.5, but only *L. plantarum* tolerated pH 2.

The bacterial strains analyzed did not produce hydrogen peroxide. However, the lack of this property does not limit their usefulness as potentially probiotic bacteria, as tolerance to low pH and bile salts is the most important criterion in the assessment of strains.

The autoaggregation abilities of the bacteria analyzed and the mucous produced by the microorganisms, which was confirmed by the presence of a pink biofilm appearing on the walls of the test tubes and autoagglutination occurring in physiological solution of NaCl, suggest their non-specific adhesion to mucous membranes, which indicated surface activity that facilitates their adhesion to intestinal cells. Both of these properties favor the colonization of the digestive tract and the development of biofilm which, by limiting the adhesion of pathogenic bacteria to receptors on the surface of the intestinal mucous membrane, protects it against colonization by pathogenic strains. The longer probiotic bacteria are in contact with the digestive tract thanks to their adhesion abilities, the longer they can influence the fish bodies and confer better clinical effects. Bacteria with no adhesion properties can only remain in the intestines until they are removed together with undigested food.

Conclusion

In conclusion, our results demonstrated that the analysed *L. plantarum* strains are characterized by important properties such as the ability to grow in the presence of bile, a tolerance to low pH, and antagonism against pathogens dangerous for fish. Therefore, they meet the most important criteria determined for probiotic strains. This suggests that it is possible to apply them in aquaculture as an alternative to antibiotic treatment.

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