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Lactic Acid Bacteria against *Listeria monocytogenes*

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ABSTRACT

***Background*:** *Listeria monocytogenes* is a pathogenic bacterium that can contaminate food and cause public health problems due its ability to form biofilms and resistance to sanitizers, it is responsible for sanitary and economic losses in food producing establishments. The difficulties in controlling biofilms and increasing resistance to traditional antibacterial agents is motivating studies of alternative potential biological agents for the control of pathogenic biofilms, among which lactic acid bacteria (LABs) are included. The objective of this work was to evaluate the activity of LABs against *Listeria monocytogenes* biofilm formation on polystyrene plates, a surface commonly used in the food industry.

***Materials, Methods & Results*:** Lyophilized commercial strains of *Bifidobacterium animalis*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus salivaris* and *Lactobacillus acidophilus* were used. The strain of *Listeria monocytogenes* (L4) was isolated from polystyrene mats from a poultry slaughterhouse cutting room and demonstrated the ability to attach to microplates and resistance to sanitizers (sodium hypochlorite and hydrogen peroxide) at all times, temperatures and tested surfaces. The antimicrobial activity of LABs was evaluated by the agar diffusion method. The LABs that presented action on *Listeria monocytogenes* were selected for the inhibition and/or removal of biofilms in microplates, and all experiments were carried out in triplicate. Only *Bifidobacterium animalis* and *Lactobacillus plantarum* demonstrated action against *Listeria monocytogenes* in the agar diffusion assays and were selected for inhibition and competition assays. Furthermore, competition of LABs against *Listeria monocytogenes* adhesion was evaluated. There was no significant difference between LABs and *L. monocytogenes*, alone or in combination, at temperatures of 30ºC and 37ºC in the *Listeria monocytogenes* inhibition assays on polystyrene surface. The lactic acid bacteria evaluated did not demonstrate inhibition of *L. monocytogenes* adhesin testes with optical density visualization, however, it was possible to identify a reduction in *L. monocytogenes* counts with the application of *Bifidobacterium animals* and *Lactobacillus plantarum* in the testes of competition against biofilm formation. In competition tests *Bifidobacterium animalis* and *Lactobacillus plantarum* have an injunction in *Listeria monocytogenes*, indicating that these lactic acid bacteria can retard *Listeria* biofilm formation on polystyrene surfaces and thus help control the pathogen in the food industry.

***Discussion*:** A potential mechanism to control biofilm adhesion and formation of pathogens for nutrients and fixation on surfaces, multiplication factors and surfaces are a challenge in controlling biofilms of pathogenic microorganisms, alternative measures to traditional methods for inactivating pathogens and biofilm formers bacteria are necessary. In this sense, lactic acid bacteria generate high levels of bacteriocin and are effective in inhibiting the biofilm of pathogenic bacteria, however, our study did not reveal this. We verified that *Bifidobacterium animalis* and *Lactobacillus plantarum* have an inhibitory action on *Listeria monocytogenes*, indicating that these lactic acid bacteria can be used to delay the formation of biofilms by *Listeria* on polystyrene surfaces, helping to control this pathogen in food industry.

Keywords: control of biofilm, pathogenic bacteria, food industry, polystyrene surface, FTDs.

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INTRODUCTION

Listeria monocytogenes is a pathogenic bacterium causing listeriosis, a food-transmitted disease (FTDs) that can cause septicemia, meningitis, and even death in the most severe cases. If contracted during pregnancy, *L. monocytogenes* can result in miscarriage, premature birth, a severe infection of the newborn, or even stillbirth [11,42]. Most outbreaks are associated with the ingestion of ready-to-eat foods based on meat and dairy products that do not undergo cooking or microbial inactivation for consumption [5,21,37].

Other characteristics of the agent are multiplication under refrigeration temperatures, high tolerance to disinfectants, and the ability to form biofilms [9,16]. Biofilms are a set of living microorganisms surrounded by an extracellular polymer matrix (EPS) that can become fixed to and multiply on any surface, being difficult to eliminate due to the protection conferred by the EPS and varying levels of resistance to sanitizers [11,12,33].

The control of *Listeria* biofilms is a challenge for the food industry, using traditional methods, an alternative to these methods would be the application of lactic acid bacteria (LABs) [34]. LABs care a group of microorganisms capable of fermenting with other products, controlling the multiplication of microorganisms by reducing the pH and producing inhibitory compounds [30].

The application of LABs to neutralize the proliferation of other bacteria on contact surfaces is based on the principle of competitive exclusion, where LABs with biofilm formation capacity can limit the multiplication of pathogens through competition for nutrients, production of antimicrobial substances, and physicochemical changes of surfaces [10,17,19,24,25,37].

MATERIALS AND METHODS

Lactic Acid Bacteria (LABs) and Listeria monocytogenes

Lyophilized commercial strains of *Bifidobacterium animalis*, *Lactobacillus fermentum*, *L. plantarum*, *L. salivaris* and *L. acidophilus* were used. The strains were reactivated in MRS Broth[1] at 30°C for 18 h, and sown in MRS Agar[1] under the same conditions. The isolates were kept at -80°C in MRS broth supplemented with 20% (v/v) of sterile glycerol.

The strain of *Listeria monocytogenes* (L4) was isolated from polystyrene mats from a poultry slaughterhouse cutting room [28] and demonstrated the ability to attach to microplates and resistance to sanitizers (sodium hypochlorite and hydrogen peroxide) at all times (0, 4, 12, and 24 h), temperatures (42 ± 1ºC, 36 ± 1ºC, 25 ± 1ºC, 9 ± 1ºC, and 3 ± 1ºC) and tested surfaces (stainless steel, polyethylene, and polystyrene) (unpublished data). *L. monocytogenes* ATCC7644 was used as a control. Both isolates were stored at -20°C in Broth Brain Heart Infusion (BHI)2 with 20% glycerol and were reactivated in BHI broth for 18 h to 37°C with confirmation in Triple Sugar Iron (TSI), Lysine Iron Agar (LIA), indole, urea, citrate, nitrate, methyl red, ramanose, and mannitol[2].

Preparation of the LABs free-cells supernatants

For the preparation of the free-cells supernatants, the LABs were incubated in MRS Broth at 37°C for 24 h. Next, the supernatant of each culture was obtained by centrifugation at 16128 *g* for 15 min, neutralized with 1 M NaOH pH 6.5, filtered in a 0.22 µm filter and partially purified [14]. The cell suspension of the LABs was concentrated in a centrifuge at 448 *g* for 6 min. The supernatant and bacteria suspended in MRS broth were removed.

Inhibition assays of Listeria monocytogenes *by LABs in the agar diffusion method*

The antimicrobial activity of LABs was evaluated by the agar diffusion method [3]. *L. monocytogenes* suspensions equivalent to 0.5 of the MacFarland scale were sown in BHI agar and after 5 min, 8 mm orifices were punctured with a sterile punch and a volume of 80 µL of the LABs supernatant (106 CFU/µL) was inoculated. Tetracycline discs (30 µg)[3], were used as a positive control. The plates were incubated at 37ºC and after 24 h the diameters were measured in millimeters. The LABs that presented action on *L. monocytogenes* were selected for the inhibition and/or removal of biofilms in microplates. All experiments were carried out in triplicate.

Polystyrene microplate assays

Only *Bifidobacterium animalis* and *Lactobacillus plantarum* demonstrated action against *Listeria monocytogenes* in the agar diffusion assays and were selected for inhibition and competition assays [35]. To evaluate the inhibition of *Listeria* on the polystyrene surface, 100 µL of the suspension of LABs was incubated individually at 30°C and 37°C for 48

h. After 24 h, 200 µL of LABs were added individually, and after 48 h, 100 µL of *Listeria* cultures. The plates were incubated for 24 h at 30°C and 24 h at 37°C, totaling 48 h of incubation for *Listeria*. LABs were grown individually in MRS broth for 18-24 h at 37°C ± 1°C and adjusted to Mac Farland scale 1 (3.0×10^8 CFU/µL).

To evaluate the competition of LABs in the face of the adhering of *L. monocytogenes*, 200 µL of bacteria suspension were inoculated in each well and 200 µL of the suspension of each LABs, individually. As a positive control, 200 µL of the suspension of LABs was added and as a negative control 200 µL of sterile MRS. The plates were incubated at 30°C and 37°C for 48 h, the wells rinsed 3 times with 200 µL of sterile NaCL 0.85% and dried at room temperature. Next, the bacteria were fixed with 250 µL of methanol p.a., which was removed after 15 min and the plates dried at room temperature. After being flushed with 200 µL of 2% Hucker violet crystal for 5 min, washed in running water and dried at room temperature, the absorbance reading was performed before and after the addition of 250 µL of glacial acetic acid 33%, in ELISA reader[4] at 550 and 595 nm.

To evaluate the viable number of microorganisms, the wells of the plates were washed 3 times and scraped with a platinum handle. The suspensions obtained were transferred to sterile tubes, and dilutions were performed in 0.85% saline solution (w/v), followed by sowing in BHI Agar and Palcam Agar[3] to *L. monocytogenes* by plate drop method (inoculation of 5 drops of 10 µL of each dilution). The plates were incubated at 37°C for 24-48 h, the bacterial counts performed and the percentage of inhibition and competition was calculated [13] as follows:

$$\text{Biofilm Reduction}\% = (OD_{Control} - OD_{Treatment}) \div OD_{Control} \times 100\%$$

Statistical analysis

The mean of the data in triplicate of the experiments was analyzed by ANOVA, and the difference between the means was evaluated by the Tukey test with a 95% confidence interval ($P < 0.05$) in Statistic 7.

RESULTS

The results of action of LABs are shown in Figure 1. Although the LABs evaluated in our study did not show inhibition of *Listeria* adhesin trials with measurement of optic density, it was possible to identify a reduction in *L. monocytogenes* count with the application of *Bifidobacterium animals* and *Lactobacillus plantarum* in the competition trials against the formation of biofilms.

In our work, the results of the competition trials showed that *Bifidobacterium animalis* and *Lactobacillus plantarum* have an injunction on *Listeria monocytogenes*, indicating that these lactic acid bacteria can be used to delay the formation of biofilms by bacteria on polystyrene surfaces and, thus, assist in the control of this pathogen in the food industry.

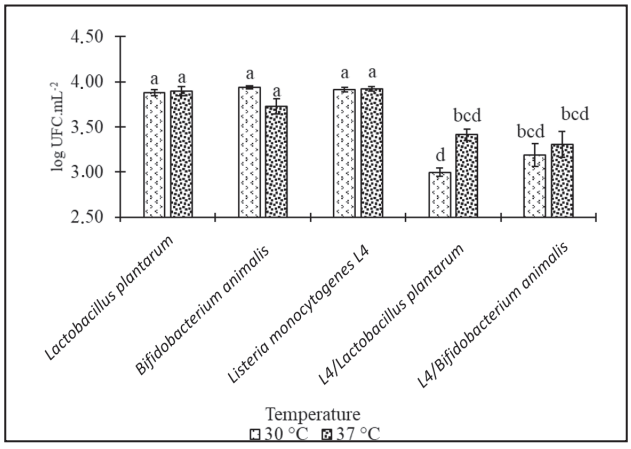

Figure 1. Quantification of biofilm in competition trials between acid lactic bacteria and *Listeria monocytogenes* in polystyrene plates. Means followed by the same letters do not differ from each other by the Tukey test ($P < 0.05$).

DISCUSSION

There was no significant difference between LABs and *L. monocytogenes*, alone or in combination, at temperatures of 30 and 37ºC ($P < 0.05$) in the *Listeria* adept inhibition assays on polystyrene surface. These results differ from *Lactobacillus plantarum* generate high concentrations of bacteriocin and was effective at inhibiting biofilm of *Listeria monocytogenes* [34] as well as *Lactobacillus plantarum* and *L. fermentum* as capable of adhering in polystyrene and inhibiting biofilms of other pathogens (*Pseudomonas aeruginosa* and *Salmonella* Typhimurium) [2].

Other studies evaluating the effects of LABs against *Listeria monocytogenes* biofilms on different surfaces also contradict our results, when testing stainless steel surfaces (24 h at 30°C) but reporting inhibition by the superior by *Lactobacillus curvatus* (2.17 log CFU/cm^2) than by *L. plantarum* (1.45 log CFU/cm^2) [19].

Listeria monocytogenes can be detected in several environments, including soil, water, vegetation, drains, animal and human feces, among others. It can adhere and form biofilms that are aggregates composed of one or more living microorganisms organized and linked to each other, surrounded by extracellular polymeric substances (EPS) and other substances. These microorganisms after adhesion on surfaces such as stainless steel, glass, plastics, and others, form microcolonies and produce EPS allowing the maturation of the biofilm that over time become permanent reservoirs of pathogenic bacteria whose penetration of sanitizing agents is inhibited by the developed resistance that is superior to planktonic cells. In this way, pathogenic bacteria such us *L. monocytogenes* can persist in industrial processing environments, especially in equipment and food contact surfaces, and cause continuous contamination of food products that are handled after heat treatment [1,4,11,20,22,27].

Strains of the bacterium isolated from food products have shown resistance to common antibiotics such as penicillin, ampicillin, oxacillin, clindamycin, and tetracycline confirming the trend of resistance to a wide range of antimicrobial agents. Antimicrobial resistance of *L. monocytogenes* isolates from food has been described in beef, pork, chicken, fish, and dairy production chains and this fact may have serious consequences for public health where future outbreaks may be more difficult to control [7,15,23,29,40].

In our work, we selected *Lactobacillus plantarum* for the trials with *Listeria* biofilms because this LAB is cited as effective against this pathogen, which was not confirmed in our studies of inhibition of the disease. In this context, *Lactobacillus curvatus*, *L. fermentum* and *L. delbrueckii* inhibited polystyrene listerias biofilms with 24 h of multiplication, which can be attributed to the potential for adhesion according to the type of LAB evaluated [6]. This hypothesis can be corroborated by suitability and formation of biofilms by *Bifidobacterium* and *Lactobacillus* (including *Bifidobacterium animalis* and *Lactobacillus plantarum* used in our study) on stainless steel, glass, and polycarbonate surfaces was superior by LABs *Bifidobacterium infantis* and *Lactobacillus reuteri* and in less time the other surfaces teste [32].

In addition to the characteristic properties of each species of LAB, the surface material is an important factor in the adhesion and biofilm formation of *Listeria monocytogenes* [9] when the bacteria support on surfaces commonly used in food processing areas such as aluminum, rubber, stainless steel, polycarbonate, and polypropylene. The polystyrene surface mimics some of the plastic materials used in food processing plants and presents a good in vitro stimulation [26] such as polyethylene of cutting plates and the polyurethane present in conveyor belts of poultry slaughterhouses.

When evaluating the competition in polystyrene plates, the LABs in the study interfered with the performance of *L. monocytogenes*, with no significant difference in antimicrobial activity between *Lactobacillus plantarum* and *Bifidobacterium animals*. Our results agree with studies that showing the influence of *Lactobacillus sakei* on the adhesion of *Listeria monocytogenes* on stainless steel surfaces [36]. Another competition test used *Lactobacillus paraplantarum* showed a significant reduction in *Listeria monocytogenes* in stainless steel (2.4 log in 24 and 48 h and 1.86 log in 72 h) [38]. The temperature, nutrient availability and variability among strains are important factors in the formation of *L. monocytogenes* biofilm in polystyrene plates [9].

Competition for nutrients and fixation on surfaces is the mechanism with the greatest potential to control the adhesion and formation of biofilms of pathogenic microorganisms [8,14,37]. The multiplication factors and the surfaces are a challenge of controlling biofilms of pathogenic microorganisms [18,31,39]. The practical application of LABs can be evaluated with the reduction of biofilms of *L. monocytogenes*, *Salmonella* spp. and *Escherichia coli* O157:H7 with probiotic characteristics and without risk to the consumer, using strains of Bacteriocin-producing *Lactobacillus* (*L. lactis*, *L. sakei*, and *L. curvatus*) and non-producers (*Lactococcus lactis* subesp. *lactis*, *Lactobacillus helveticus*, *L. casei* and *Weissella viridescens*) [12]. Furthermore, the reduction of *Listeria monocytogenes* in poultry processing plants indicates the potential of the control strategy with lactic acid bacteria [41].

CONCLUSION

In our work, the results of the competition trials showed that *Bifidobacterium animalis* and *Lactobacillus plantarum* have an injunction on *Listeria monocytogenes*, indicating that these lactic acid bacteria can be used to delay the formation of biofilms by bacteria on polystyrene surfaces and, thus, assist in the control of this pathogen in the food industry.

MANUFACTURERS

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***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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