Ciência Animal Brasileira
Brazilian Animal Science

DOI: 10.1590/1809-6891v25e-76376E

e-ISSN 1809-6891
Veterinary Medicine | Research article

Lactic acid bacteria inhibit *Salmonella* Heidelberg biofilm formation on polystyrene surfaces

Bactérias ácido lática para inibir a produção de biofilme de *Salmonella* Heidelberg em superfícies de polisestireno

Luciane Manto[1], Bruna Webber[1], Enzo Mistura[1], Karen Apellanis Borges[2], Thales Quedi Furian[2], Jucilene Sena dos Santos[1], Luciana Ruschel dos Santos[1]

[1] Universidade de Passo Fundo (UPF), Passo Fundo, Rio Grande do Sul, Brasil
[2] Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Rio Grande do Sul, Brasil
*Corresponding author: luruschel@upf.br

Abstract: *Salmonella* spp. is one of the leading causes of gastroenteritis worldwide. *Salmonella* Heidelberg is an emergent pathogen associated with multidrug-resistant outbreaks linked to poultry products. Their high persistence in the environment may be associated with their ability to adhere to different surfaces and form biofilms. Owing to increased antimicrobial resistance worldwide, researchers have investigated the use of lactic acid bacteria (LAB) as a biological control against pathogenic microorganisms. This study aimed to evaluate the ability of LAB to control the formation of *S.* Heidelberg biofilms on polystyrene surfaces. The antibiofilm activity of nine LAB strains, all belonging to *Lactobacillus* genera, related to the inhibition of biofilms produced by *S.* Heidelberg was evaluated *in vitro*. All treatments, except LAB1 (*Lactobacillus salivaris*), showed antibiofilm activity. However, LAB did not reduce bacterial counts. Our results show that LAB can avoid or delay biofilm formation by *S.* Heidelberg on polystyrene surfaces and may be used for *in vivo* studies as a potential alternative to help control this pathogen in food industries.

Keywords: biofilm prevention, adhesion, lactic acid bacteria, *Salmonella* Heidelberg

Resumo: *Salmonella* spp. é uma das principais causas de gastroenterite em todo o mundo. *Salmonella* Heidelberg é um patógeno emergente associado com surtos com multirresistência antimicrobiana vinculados aos produtos avícolas. A sua alta persistência no ambiente pode estar associada com sua habilidade de aderir a diferentes superfícies e formar biofilms. Devido ao aumento da resistência antimicrobiana em todo o mundo, os pesquisadores têm investigado o uso de bactérias acido láticas (BAL) como um controle biológico e de microrganismos patogênicos. O objetivo deste estudo foi avaliar a habilidade de BAL no controle de biofilmes produzidos por *S.* Heidelberg em placas de poliestireno. Foi avaliada a atividade antimicrobiana *in vitro* de nove BAL, todas pertencentes ao gênero *Lactobacillus*, na inibição e na remoção de biofilmes produzidos por *S.* Heidelberg. A formação de biofilme só ocorreu quando a BAL1 (*Lactobacillus salivaris*) foi utilizada. Todos os outros tratamentos demonstraram atividade antimicrobiana. Entretanto, a BAL não foi capaz de reduzir a contagem bacteriana. Os resultados obtidos demonstram que BAL são capazes de previniar ou retardar a formação de biofilme por *S.* Heidelberg em superfícies de

Received: June 06, 2023. Accepted: April 17, 2024. Published: July 04, 2024.

poliestireno e podem ser utilizadas em estudos *in vivo* para determinar o seu potencial alternativo no controle deste patógeno na indústria de alimentos.

Palavras-chave: prevenção de biofilmes, adesão, bactérias ácido láticas, *Salmonella* Heidelberg.

1. Introduction

Foodborne diseases remain a major threat to global health, and *Salmonella* spp. is one of the leading causes of gastroenteritis worldwide[1]. Salmonellosis outbreaks are often associated with the consumption of poultry products[2]. Despite the wide variety of *Salmonella* serotypes, *Salmonella* Heidelberg has recently emerged. The emergence of *S.* Heidelberg, an important pathogen associated with multidrug-resistant outbreaks linked to poultry products, has been observed in North and South America, particularly in Canada, the USA, and Brazil[3, 4, 5]. In addition to multidrug resistance, *S.* Heidelberg can adhere to different surfaces and form biofilms, making it difficult to control[6, 7, 8]. Its high persistence in food processing plants may be associated with outbreaks, which have led to increased concern among such industries[9, 10].

Biofilms are defined as microbial populations that adhere to each other and to an inert or living substrate protected by extracellular polymeric substances (EPS)[11, 12]. These structures make bacterial cells more resistant to disinfection and sanitization processes and play a crucial role in the survival of *Salmonella* in unfavorable environmental conditions, such as poultry slaughterhouses[12, 13]. Due to the increased resistance of *Salmonella* biofilms to disinfectants and antimicrobials, it is important to develop alternative and effective strategies to prevent their formation in food environments[12].

Several studies have investigated the use of lactic acid bacteria (LAB) and probiotic bacteria as biological controls for pathogenic microorganisms[14, 15, 16]. LAB have the main characteristics of producing lactic acid as the main final catabolic product from glucose. They are included in the group of probiotics, which are live microorganisms that confer a health benefit on the host when administered in adequate amounts[12, 17]. LAB have an inhibitory or reducing effect on the microbial consortia of gram-negative and gram-positive bacteria. Competition between pathogenic bacteria and LAB for adhesion sites and nutrients reduces biofilm formation by pathogens[15, 18, 19]. Thus, the application of LAB as a biological control tool is a promising strategy for preventing contamination by pathogenic bacteria in food production facilities.

The aim of this study was to evaluate the ability of LAB to control the formation of *S.* Heidelberg biofilms on polystyrene surfaces.

2. Materials and Methods

Lactic acid bacteria

A total of nine LAB strains were selected for this study: *Lactobacillus salivaris* (LAB1), *Lactobacillus plantarum* (LAB2), *Lactobacillus curvatus* (LAB3), *Lactobacillus reuteri* (LAB4),

Lactobacillus paracasei (LAB5), *Lactobacillus fermentum* (LAB6), *Lactobacillus bulgaricus* (LAB7), *Lactobacillus acidophilus* (LAB8), *Lactobacillus delbrueckii* subsp. *bulgaricus* (LAB9). Freeze-dried commercial strains were acquired from four laboratories: Lemma Supply Solutions (São Paulo, Brazil): LAB1, LAB2, LAB6, and LAB9; Pharma Nostra (Rio de Janeiro, Brazil): LAB4, LAB7, and LAB8; Fagron (São Paulo, Brazil): LAB5; and T.H.T. SA. (Gembloux, Belgium): LAB3.

The strains were reactivated in De Man, Rogosa and Sharpe broth (MRS; Merck, Darmstadt, Germany) at 37 °C for 24 h and seeded onto MRS agar under the same conditions. Strains were identified and selected based on their morphological and biochemical characteristics[20] and kept at −80 °C in MRS broth supplemented with 30% (v/v) sterile glycerol (Sigma-Aldrich, St. Louis, MO, USA).

Salmonella Heidelberg

A strain of *Salmonella* Heidelberg (SH212) originally isolated from the final product of a poultry slaughterhouse was kindly provided by the Food Science and Technology Institute (ICTA) of the Federal University of Rio Grande do Sul (UFRGS). This strain was selected based on its multidrug resistance and biofilm production profiles (Borges et al. 2017). In addition, it contains several virulence-associated genes[8]. The strain was previously serotyped by the Osvaldo Cruz Institute (Fiocruz, Rio de Janeiro, Brazil) and was stored at −20 ºC in brain heart infusion broth (BHI; Oxoid, Basigstoke, UK) supplemented with 20% (v/v) of glycerol. To reactivate the strain, an aliquot was inoculated into BHI medium for 24 h at 37 ºC and then seeded on xylose lysine deoxycholate agar (XLD; Merck, Darmstadt, Germany).

Inoculum preparation

The LAB strains and *S.* Heidelberg isolate were retrieved from frozen culture stocks and cultured overnight at 37 °C in MRS broth and tryptone soy broth (TSB, Oxoid), without glucose, respectively. To prepare the inoculum, McFarland Standard No. 1 (Probac do Brasil, São Paulo, Brazil) was used as a reference to adjust the turbidity of the bacterial suspension to 3×10^8 colony forming units (CFU)/mL.

Competition and adhesion inhibition of *Salmonella* Heidelberg by lactic acid bacteria

The technique was adapted from the methodology proposed by Gong & Jiang[21]. Eleven treatments were evaluated: individual evaluations of each LAB strain (T1–T9),and T10 and T11 corresponded to the two pools of equal proportions of each of the nine LAB strains. Sterile 96-well flat-bottomed polystyrene plates (Kasvi, São José dos Pinhais, Brazil) were used for competition and inhibition assays. The experiments were repeated twice.

For the competition assay, 150 µL of *S.* Heidelberg inoculum and 150 µL of each treatment were inoculated in each well. Each treatment was repeated in nine wells. For positive control, 300 µL of *S.* Heidelberg inoculum was added without the addition of LAB. For negative control, 300 µL of sterile MRS was added. LAB were inoculated individually and in pools as treatment controls. Microplates were incubated at 37 °C for 48 h.

For the adhesion inhibition assay, the microplates were pre-treated with 150 µL of each treatment per well, with nine wells for each treatment. Microplates were then incubated at 37 °C for 48 h. After incubation, 150 µL of *S.* Heidelberg inoculum was added to each well, followed by incubation at 37 °C for 24 h. For the positive control, 300 µL of suspension of each treatment with LAB and *S.* Heidelberg was inoculated. For the negative control, 300 µL of sterile MRS broth was inoculated.

After incubation, the contents of the microtiter plate were poured off, and the wells were washed three times with 300 µL of sterile 0.9% saline solution (Synth, Diadema, Brazil). The attached bacteria were then fixed with 300 µL of methanol (Neon, Suzano, Brazil) per well for 15 min, after which the plates were emptied and dried at room temperature (23 °C). Then, the plates were stained with 300 µL per well of 2% Hucker crystal violet for 5 min. The stain was removed and the plate was gently washed under running tap water. The plates were dried in air. The biofilm was resuspended in 300 µL per well of 33% glacial acetic acid (Nuclear, Diadema, Brazil). The optical density (OD) of each well was measured at 550 nm using Biochrom absorbance reader (Anthos 2010, Cambridge, UK). The OD of each treatment (ODT) was obtained from the arithmetic mean of the respective wells. The cut-off OD (ODC) for the microtiter plate test was defined as three standard deviations above the mean OD of the negative control (sterile MRS). The strains were classified as no biofilm producer (ODT ≤ ODC) or biofilm producer (ODC > ODT)[22].

To evaluate the viable number of microorganisms, the wells of the plates were washed twice with 0.1% buffered peptone water (BPW; Kasvi) and scraped using a platinum handle. The suspensions obtained were homogenized for 30 s in a vortex mixer. The contents were transferred to sterile tubes, and dilutions were performed in 0.1% BPW, followed by seeding on XLD and plate count agar (PCA; Kasvi) for SH212 and LAB, respectively. Bacterial counts were performed by the plate drop method[23]. The plates were incubated at 37 °C for 24 h, and the bacterial counts were expressed as CFU/mL and then transformed into $\log_{10}$ CFU/mL.

Statistical analysis

The results obtained were analyzed using descriptive statistical analysis and grouped according to relative and absolute frequencies. Bacterial counts were analyzed using the analysis of variance (ANOVA) test, and in the significant models, means were compared by the Tukey test ($p<0.05$) using the PASW Statistics program.

3. Results and discussion

Pathogenic and spoilage bacteria can attach to most surfaces in food production plants and produce biofilms. These structures increase the resistance to harsh environmental conditions and antimicrobial compounds[24, 25]. *S.* Heidelberg, an emergent serotype, is highly persistent in slaughterhouse environment and a global threat owing to its increased antimicrobial resistance[3, 10]. Thus, it is important to identify alternative methods for removing

or preventing the formation of bacterial biofilms. LAB can form protective biofilms on surfaces used in food processing plants, and its use as a natural alternative to traditional disinfectants to control the colonization of pathogenic microorganisms has been studied[26, 27].

Previous *in vitro* studies have demonstrated the bioprotective action of LAB and probiotic bacteria on several surfaces against several pathogens[12, 18, 19, 26, 28]. Thus, LAB have attracted the interest of researchers because of their ability to control the formation of *S.* Heidelberg biofilms on polystyrene surfaces widely used in food production plants. Commonly used LAB include several species of *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, and *Pediococcus*[25]. For this study, we selected species of *Lactobacillus* genus, one of the most important genera of LAB. *Lactobacillus* isolates are gram-positive, non-spore-forming, and non-motile bacilli[29].

The results obtained for the bacterial counts of SH212 during the adhesion inhibition and competition by LAB are presented in Table 1.

Table 1 Bacterial counts of *Salmonella* Heidelberg (SH212) for adhesion inhibition and competition assays using lactic acid bacteria (LAB), individually and in pools:

Treatment	Bacterial counts (log_{10} CFU/mL) - mean ± standard deviation	
	Inhibition	Competition
Positive control (SH212)	12.03 ± 0.18[a]	12.32 ± 0.24[a]
LAB1 + SH212	11.68 ± 0.62[a]	12.11 ± 0.28[a]
LAB2 + SH212	11.88 ± 0.24[a]	12.08 ± 0.18[a]
LAB3 + SH212	12.00 ± 0.24[a]	12.29 ± 0.35[a]
LAB4 + SH212	11.92 ± 0.30[a]	12.08 ± 0.25[a]
LAB5 + SH212	11.84 ± 0.28[a]	12.03 ± 0.56[a]
LAB6 + SH212	11.91 ± 0.24[a]	12.18 ± 0.33[a]
LAB7 + SH212	12.03 ± 0.11[a]	12.08 ± 0.35[a]
LAB8 + SH212	11.85 ± 0.50[a]	12.13 ± 0.44[a]
LAB9 + SH212	12.08 ± 0.45[a]	12.31 ± 0.24[a]
pool LAB 1	11.86 ± 0.47[a]	12.12 ± 0.22[a]
pool LAB 2	11.99 ± 0.35[a]	12.11 ± 0.28[a]

Legend: Lactobacillus salivaris (LAB1), L. plantarum (LAB2), L. curvatus (LAB3), L. reuteri (LAB4), L. paracasei (LAB5), L. fermentum (LAB6), L. bulgaricus (LAB7), L. acidophilus (LAB8), L. delbrueckii subsp. bulgaricus (LAB9). Different letters in a column indicate significant differences according to Tukey's test (p<0.05).

Gomaa et al.[30] demonstrated that commercial probiotic strains of *L. acidophilus* and *L. paracasei* inhibit the multiplication of *S.* Heidelberg isolates *in vitro*. According to the authors, pathogen inhibition can be attributed to several factors, including pH reduction caused by probiotic fermentation. Low pH values, approximately 4.4–5.2, reduce *S.* Heidelberg multiplication[31]. Thus, we expected the addition of LAB to reduce the bacterial counts of

SH212. However, this was not observed in this study. No significant differences (p>0.05) were observed in bacterial counts between the positive control and treatments, regardless of the evaluated LAB and test (inhibition or competition).

It is possible that even if LAB did not completely eliminate *S.* Heidelberg, competition for adhesion sites by LAB could prevent SH212 adhesion. Thus, antibiofilm activity was also evaluated. The results of biofilm formation by SH212 in inhibition and competition assays are presented in Table 2.

Table 2 Evaluation of biofilm formation by *Salmonella* Heidelberg (SH212) in inhibition and competition assays.

Treatment	Biofilm production: positive (+) or negative (-)	
	Inhibition	Competition
Positive control (SH212)	+	+
LAB1 + SH212	+	+
LAB2 + SH212	-	-
LAB3 + SH212	-	-
LAB4 + SH212	-	-
LAB5 + SH212	-	-
LAB6 + SH212	-	-
LAB7 + SH212	-	-
LAB8 + SH212	-	-
LAB9 + SH212	-	-
pool LAB 1	-	-
pool LAB 2	-	-

Legend: Lactobacillus salivaris (LAB1), L. plantarum (LAB2), L. curvatus (LAB3), L. reuteri (LAB4), L. paracasei (LAB5), L. fermentum (LAB6), L. bulgaricus (LAB7), L. acidophilus (LAB8), L. delbrueckii subsp. bulgaricus (LAB9).

The positive control (SH212, without treatment) exhibited biofilm formation, demonstrating the ability of this isolate to produce these structures. Of the 11 treatments evaluated, biofilm formation occurred only when LAB1 (*L. salivaris*) was used. All other treatments showed antibiofilm activity against SH212. The presence of LAB prevented biofilm formation in both the tests. This effect can be explained by the ability of LAB to aggregate with potential pathogens, block their adhesion sites, and produce antimicrobial substances such as hydrogen peroxide and biosurfactants that inhibit their multiplication and hinder adhesion[12, 26, 28].

Previous studies have demonstrated antibiofilm activity against several pathogens, including *S.* Gallinarum, *S.* Typhimurium, and *S.* Enteritidis[14, 15, 32, 33]. However, there are few

studies on *S.* Heidelberg, which makes it difficult to compare results and reinforces the need for further analyses to evaluate the action of LAB against this serotype.

4. Conclusion

Our results show that LAB can avoid or delay biofilm formation by *Salmonella* Heidelberg on polystyrene surfaces and may be used for *in vivo* studies as a potential alternative to help control this pathogen in food industries.

Conflict of interest statement

The authors have no competing interests.

Author contributions

Conceptualization: L. Manto and L. R. dos Santos. Data curation: L. Manto and L. R. dos Santos. Formal analysis: L. Manto, K. A. Borges, T. Q. Furian, and L. R. dos Santos. Investigation: L. Manto, B. Webber, E. Mistura, and J. S. dos Santos. Methodology: L. Manto, B. Webber, E. Mistura, and J. S. dos Santos. Project administration: L. Manto and L. R. dos Santos. Supervision: L. R. dos Santos. Validation: K. A. Borges, T. Q. Furian, and L. R. dos Santos. Writing (original draft): L. Manto, K. A. Borges, T. Q. Furian, and L. R. dos Santos. Writing (review and editing): L. Manto, K. A. Borges, T. Q. Furian, and L. R. dos Santos.

Acknowledgment

The authors thank Dr. Eduardo Cesar Tondo and the Institute of Food Science and Technology (ICTA) of the Universidade Federal do Rio do Sul (UFRGS) for kindly providing *Salmonella* Heidelberg strain.

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