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Communication

[*Comunicação*]

Standardization of the Spot-on-the-Lawn antagonism test in the inhibition of *Salmonella* Heidelberg by *Lactobacillus salivarius*

[*Padronização do teste de antagonismo* Spot-on-the-Lawn *na inibição de* Salmonella Heidelberg *pelo* Lactobacillus salivarius*]*

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Salmonellosis is a serious public health issue, especially in food safety, due to its zoonotic nature and oral-fecal transmission (Freitas Neto *et al*., 2020). It also poses an economic impact mainly on the poultry export chain due to the imposition of phytosanitary barriers (Gambirage *et al*., 2018). To mitigate the burden of salmonellosis in large-scale animal production, sub-therapeutical dosages of antibiotics are administered as growth promoters, decreasing the salmonellosis challenge and improving zootechnical performance (Choi *et al*., 2023; Gadde *et al*., 2017).

On the other hand, the use of these promoters could lead to induced multi-resistance, not only in *Salmonella* spp. but also in other pathogenic bacteria (Ma *et al*., 2021). In addition, the misuse and improper disposal of antibiotics lead to residuals in animal products and environmental pollution, posing a great risk to public health (Ewbank *et al*., 2021).

To continue exporting poultry products, adapting to the new requirements of import markets was necessary. This demand intensified the search for alternative methods to the use of antibiotics as growth promoters, which resulted in the development of probiotic products (Raposo *et al*., 2019). This class of beneficial microorganisms are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Hill *et al*., 2014). This includes the *Lactobacillus* spp., one of the most common genera in the intestinal core microbiota of domestic birds (Clavijo and Flórez, 2018).

These microorganisms play an important role in the health of the host's intestinal microbiota, not only for its direct antagonism but also for the production of bactericidal substances (Li *et al*., 2022), an important aid in salmonella prevention and control.

The popularity of probiotic products in poultry has been growing, and it is expected to grow even more with the abolishment of products like growth promoters (Andreatti Filho *et al*., 2020). As a relatively new market, it is common for divergences to exist between the evaluation methods of these products. Because of this obstacle, a systematic *in vitro* approach was developed by FAO/WHO (Guidelines…, 2002), which allows a concise evaluation of the quality and viability of these products. Laboratory assays are recommended to assess certain characteristics, such as resistance to gastric and bile acids, adhesion to the mucus or the gut epithelial tissue, hydrolysis of bile salts, and, precisely, antagonistic activity to pathogenic bacteria.

The antagonistic activity of *L. salivarius* as a probiotic acting against pathogens like *Salmonella* is scientifically documented (Miyamoto *et al*., 2000; Thomas *et al*., 2019). Nevertheless, the evaluation of this competition through plate antagonism tests is still not well standardized, as factors such as bacterial concentration, growth, and media volumes may drastically change among procedures, making it a divergence factor in the bioprospecting of these microorganisms.

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The *in vitro* assays that measure bacterial antagonism may be divided into two categories, with the probiotic strain exerting direct and indirect antagonism towards the pathogenic one. Among the most indirect common assays, there are the flip streak and the spot-on-the-lawn (**SOTL**). However, the **SOTL** method is the most efficient, which quantifies through inhibition halos, the sensitivity, and antagonism between bacteria (Barros *et al*., 2009).

The SOTL method was first described in Gratia (1946), and since then, has been widely used and adapted in several studies (Silva *et al*., 2020). Nonetheless, variable factors like bacterial concentration, inoculum, and media volume are prone to vary among assays, as the literature describes a plethora of different methods. Unfortunately, such variations may easy purposeful manipulation, aiming at over or underestimating of results, leading to biased conclusions. In this context, the scope of this study was to investigate such divergent factors of the SOTL method and to propose a standardization model, ensuring greater reliability among studies.

This study was conducted in the Ornitopathology Laboratory of the School of Veterinary Medicine and Animal Sciences (FMVZ) at the State University of São Paulo (UNESP), in Botucatu, SP, Brazil. It was approved by the Ethics Committee of the same institution (0032/2019).
The *Salmonella* Heidelberg sample was isolated from a broiler breeder with clinical signs of salmonellosis. The *Lactobacillus salivarius* ATCC 11742 was acquired from the bacterial collection of the Oswaldo Cruz Foundation (FIOCRUZ).

The bacterial samples of *Lactobacillus salivarius* ATCC 11742 and *Salmonella* Heidelberg were submitted to the SOTL antagonism test with modifications [55 – 56]. Three different concentrations of both bacteria were manipulated: concentrated (L1 and S1), intermediate (L2 and S2), and diluted (L3 and S3). Besides, three different volumes of Brain Heart Infusion (BHI) media were inoculated: low (10mL), intermediate (15 mL), and high (20mL). Using three *L. salivarius* ATCC 11742 concentrations (L1, L2, and L3), three *S.* Heidelberg concentrations (S1, S2, and S3), and three media volumes (10, 15, and 20mL), 27 different experimental units were elaborated, with two repetitions each, totaling six inhibition halos measured per experimental unit (Figure 1).

To prepare all three *L. salivarius* ATCC 11742 concentrations, 5 CFU of this bacterium were inoculated in 5mL of DeMan, Rogosa, and Sharpe (MRS) broth, followed by incubation at 38°C for 24 hours. After the incubation process, the culture was poured into another flask containing 400mL of sterile MRS broth, totaling 405mL, proceeding to the same incubation patterns.

From this initial culture (405mL), three different concentrations were elaborated. For the concentrated inoculum (L1), 300mL of the initial culture were separated and centrifuged (3000 x g, 3min, 4°C), followed by disposal of supernatant and resuspension of the pellet, totaling 3mL. The intermediate concentration (L2) was processed from the same initial culture and did not suffer any bioprospection. The lowest concentration (L3) also came from the same initial culture, in which 10 mL were diluted in 990 mL of sterile phosphate-buffered saline (PBS), at a 1:99 ratio.

Subsequently, 10µL of each *L. salivarius* ATCC 11742 concentration was seeded as dots and triplicates, symmetrically arranged in Petri dishes (90x15mm), which contained 15mL of MRS agar. Nine plates per concentration were elaborated, totaling 27 plates. After complete drying, the same plates were incubated at 38°C for 18 hours.

After the incubation process of the plates, three concentrations of *S.* Heidelberg were elaborated (S1, S2, and S3), in a similar way to that previously described for *L. salivarius* ATCC 11742, changing the culture media to Brain Heart Infusion (BHI). Then, 9 sterile tubes containing 10, 15, and 20 mL of BHI at 0.65% of agar were prepared, totaling 27 tubes. Three tubes of each volume were inoculated with 100 µL of each *S.* Heidelberg concentration (S1, S2, and S3), and poured into the incubated plates. All variations among bacteria concentrations and media volume were considered, resulting in 27 different plates, as already described in the experimental design section (Figure 1).

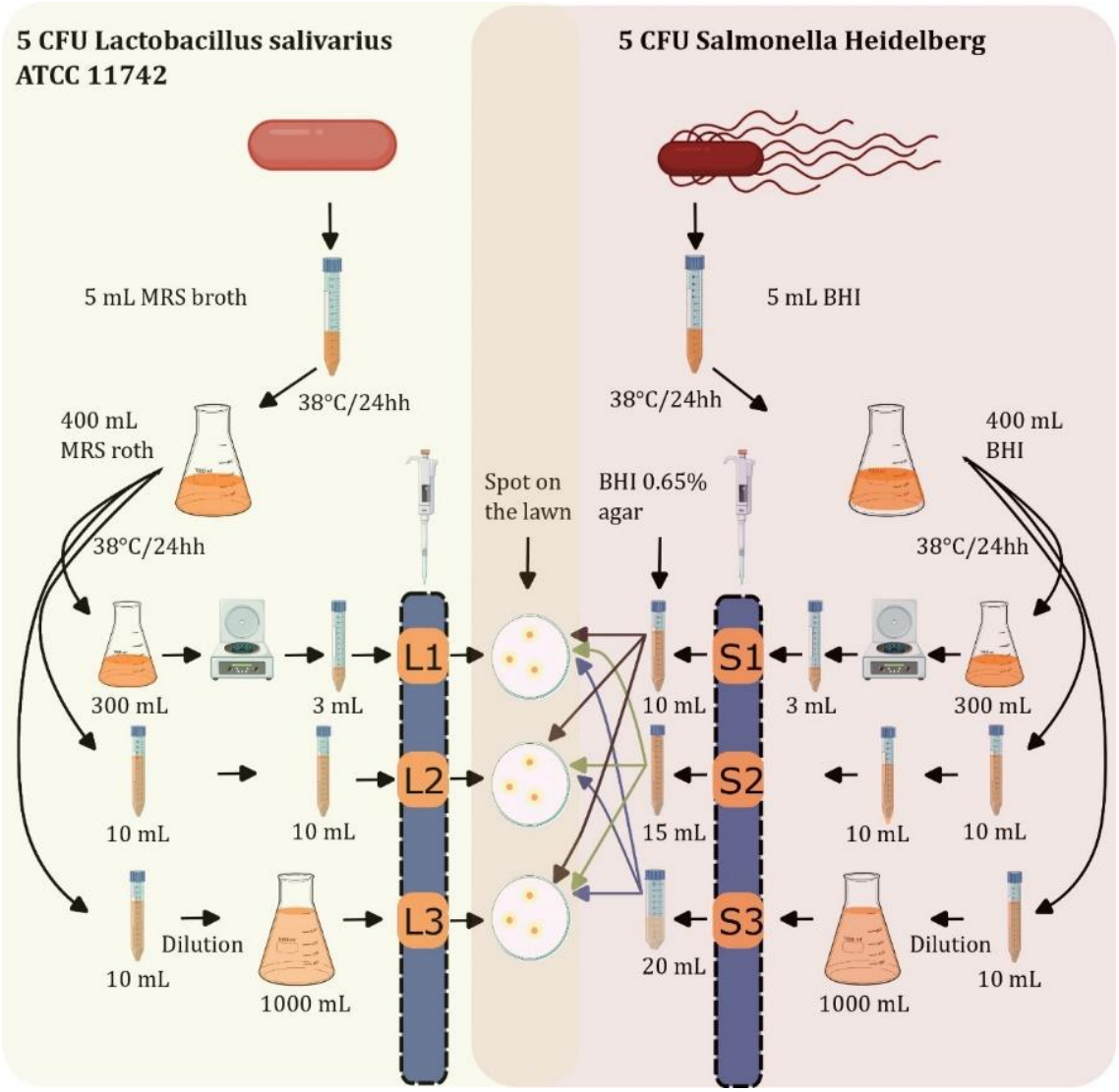

Figure 1. Diagram of the experimental design process

After the complete solidification of the BHI recently poured on, the plates were submitted to incubation at 38 °C for 18 hours. Then, the plates were read according to the inhibition halos formed by the antagonism of *L. salivarius* ATCC against *S.* Heidelberg.

Bacterial quantification was performed through serial decimal count at the manipulation of each microorganism. Thus, an aliquot of 100 µL of each bacteria concentration was separately homogenized in 900 µL of PBS (10^{-1}), followed by inoculation of 100 µL and spreading with a Drigalski spatula, on plates containing MRS agar for *L. salivarius* ATCC 11742 and Brilliant Green Agar (BGA) for *S.* Heidelberg. This process was performed until it reached 10^{8} of dilution, which took seven attempts. Then, all plates were incubated at 38 °C for 24 hours.

The assumptions of normal distribution and homoscedasticity were evaluated through Shapiro-Wilk and Bartlett's tests, respectively. Comparisons between groups were performed through the Kruskal-Wallis test [Median (1° and 3° quartiles)], and multiple comparisons were adjusted with Dunn's test. The analyses were done through the statistical package Graph Pad Prism 8 (8.0.1).

Medians of the inhibition halos—formed under the high (S1 = 7.8 x 10^{11}) and intermediate (S2 =

4.8 x 10^9) concentrations of *S.* Heidelberg—were higher ($p < 0.05$) when the highest concentration of *L. salivarius* ATCC 11742 (L1 = 4.6 x 10^{11}) was also used under the volumes of 10, 15, and 20mL of BHI (Table 1). These data demonstrate the versatile behavior of *L. salivarius* ATCC 11742, as its antagonism against *S.* Heidelberg intensifies when presented at a higher concentration level. The *Lactobacillus* genera may produce bactericidal substances that act against pathogens like *Salmonella*. Thus, a greater number of *L. salivarius* ATCC 11742 cells may lead to higher concentrations of bactericidal substances diffused in the media, which consequently leads to greater inhibition of *S.* Heidelberg.

These findings are expected to be of considerable significance for forthcoming research involving the use of *L. salivarius* ATCC 11742 as a probiotic culture, with the objective of alleviating *S.* Heidelberg infections. Based on our findings, to achieve statistically distinguishable outcomes when confronting high *S.* Heidelberg concentrations in experimental challenges, it is recommended to employ an equivalently elevated concentration of the probiotic.

Also, the *Lactobacillus* sp. antagonizes *Salmonella* sp. indirectly through the stimulus of the immune system and directly by producing bactericidal substances (Clavijo and Flórez, 2018). To exert this behavior, bacteria may use the quorum sensing mechanism, which is triggered by the identification, production, and secretion of oligopeptides, to regulate the unicellular behavior collectively, inhibiting pathogens such as *Salmonella* sp. (Okamoto *et al*., 2018). It is a consensus that gram-positive bacteria such as *Lactobacillus* trigger this behavior when a minimum bacterial concentration is reached (Hawver *et al*., 2016). This process may be manipulated by increasing the number of cells of these microorganisms in the spot-on-the-lawn antagonism test, resulting in higher medians of inhibition halos (Table 1). With the manipulation of the bacterial concentration upper wise, it is possible that the *quorum sensing* mechanism is activated, stimulating the *L. salivarius* ATCC 11742 cells to inhibit the S. Heidelberg even more.

Under the diluted *S.* Heidelberg concentration (S3 = 2.2 x 10^7), no difference was found ($p>0.05$) between the inoculums of all *L. salivarius* ATCC 11742 concentrations (L1 = 4.6 x 10^{11}, L2 = 2.1 x 10^9, and L3 = 7.4 x 10^7), in all media volumes used (10, 15, and 20 mL [Table 1]). It might suggest that a low concentration of *S.* Heidelberg does not affect its antagonism against *L. salivarius* ATCC 11742 in the **SOTL** test, even with different concentrations of this bacteria (L1, L2, and L3). This data suggests that for a difference to be found among the use of different probiotic concentrations, there must also be a minimum concentration of *S.* Heidelberg as well, in the **SOTL** test.

Thus, when considering the application of this probiotic strain for mitigating *S.* Heidelberg infections in broilers, it may be prudent to initially assess the pathogen's abundance in the chick's ceca before initiating therapeutic treatment. If *S.* Heidelberg is present in low concentrations, the use of this probiotic strain might not yield desired results. It is essential to emphasize that further *in vivo* studies are strongly recommended to validate these hypotheses.

The formation of inhibition halos in the **SOTL** test is liable to changes under the influence of different microorganism concentrations ($p<0.05$; Table 1). One of the mechanisms that enable such action-reaction of *Lactobacillus* is biotic stress induced by different *S.* Heidelberg concentrations. The biotic stress suffered by LAB is a survival mechanism, developed as these microorganisms are submitted to different stress conditions, always adapting to the environmental changes, ensuring their proliferation (Papadimitriou *et al*., 2016). This fact may also explain the reason for no difference to be found among higher, middle, or lower use of *L. salivarius'* concentrations (L1, L2 and L3) against the lower of *S.* Heidelberg (L3), in all media volumes (10, 15 and 20mL).

Regarding the three concentrations of both bacteria associated with different volumes of media, the halo medians tend to increase ($p<0.05$) as the volume of media also increases (Table 1). The addition of larger volumes of media in all three *S.* Heidelberg concentrations dilutes these concentrations even more, decreasing the *S.* Heidelberg's challenge. This fact led to higher medians in inhibition halos ($p<0.05$) when compared with volumes of 10 and 20mL (Table 1).

Table 1. Inhibition halos [median (1° quartile – 3° quartile)] of *Salmonella* Heidelberg by *Lactobacillus salivarius* ATCC 11742, according to different bacterial concentrations and media volumes (mL) at 0.65% of agar

Salmonella Heidelberg	*Lactobacillus salivarius* ATCC 11742	Volume		
		10	15	20
S1	**L1**	12 (10.7-12)Ab	14 (12.7-14.2)Bab	22 (21.7-22)Aa
	L2	11.5 (10.7-12)Ab	16 (15.7-16)Aab	23.5 (21.5-24)Aa
	L3	8 (8-9)Bb	13.5 (12.7-14)Bab	18 (17-18)Ba
S2	**L1**	17 (16-20.2)Ab	18 (17.5-20.2)Ab	30.5 (28-32)Aa
	L2	14 (12.7-15)Bb	19 (17.5-20.2)Aab	21.5 (20.7-22)Ba
	L3	13.5 (12-15.2)Bb	16 (15-16)Bab	24 (21.7-26)Aa
S3	**L1**	20 (19.7-22.2)Ab	24.5 (22.5-25.2)Aab	31.5 (29.5-35)Aa
	L2	20.5 (19.5-21)Ab	24 (23.7-25.7)Aab	33 (31.5-33.2)Aa
	L3	18 (17-18.7)Ab	24 (23.5-24.2)Aab	30 (25.5-32)Aa

Bacterial concentrations (colony-forming units per mL) of *Salmonella* Heidelberg: S1 = 7.8 x 10^{11}; S2 = 4.8 x 10^{9}; S3 = 2.2 x 10^{7}. *Lactobacillus salivarius* ATCC 11742: L1= 4.6 x 10^{11}; L2 = 2.1 x 10^{9}; L3 = 7.4 x 10^{7}. Different upper- and lower-case letters indicate significant differences in columns and lines, respectively, according to the Kruskal-Wallis test with Dunn's adjustment ($p<0.05$).

Regarding the concentrations of *S.* Heidelberg, the medians of inhibition halos tend to increase as the lowest concentration of *S.* Heidelberg (S3) is inoculated, in all volumes of media (10, 15, and 20mL) and concentrations of *L. salivarius* L1 (A, B, C, Figure 2), L2 (D, E, F), and L3 (G, H, I). These findings support the results found in Table 1, as the inhibition halos decrease as the *S.* Heidelberg's concentration also decreases.

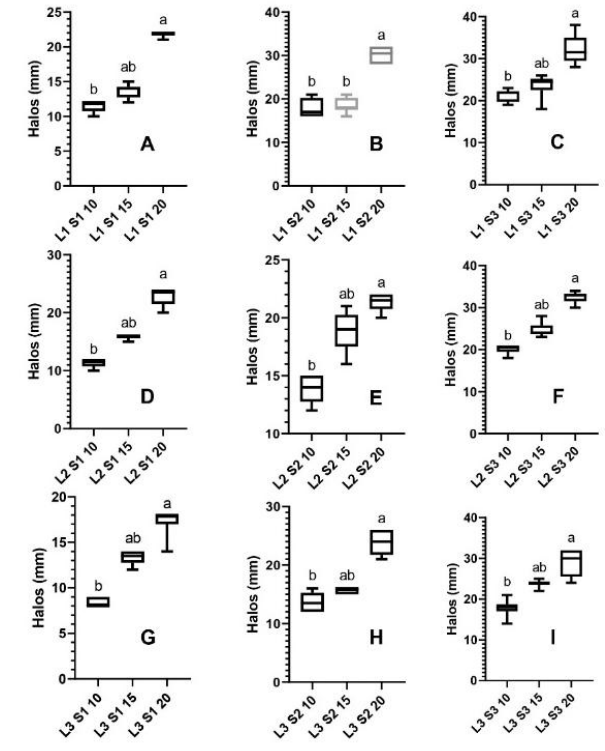

Figure 2. Medians (maximum-minimum) of inhibition halos of *Salmonella* Heidelberg *vs Lactobacillus salivarius* ATCC 11742 in volumes of 10, 15 and 20mL on the Spot-on-the-Lawn test. Bacterial concentrations are expressed in colony-forming units per mL. *L. salivarius as: L1 = 4.6 x 10^{11}; L2 = 2.1 x 10^{9}; L3 = 7.4 x 10^{7}*. *S.* Heidelberg as: S1 = 7.8 x 10^{11}; S2 = 4.8 x 10^{9}; S3 = 2.2 x 10^{7}. Different letters indicate a statistical difference.

The results of the **SOTL** antagonism test are decisive in the classification of probiotic products, making it useful against pathogenic bacteria. The inhibition of halos formed may vary depending on different bacterial concentrations (Table 1), which is justified by the increase in the microbiological challenge when *S.* Heidelberg is at a higher concentration. Furthermore, a decrease in the concentration of *L. salivarius* ATCC 11742 and a subsequent decrease in the production of bactericidal substances reduced (Table 1; $p<0.05$) the inhibition halos of *S.* Heidelberg.

Although this study provides insights into specific strains of probiotics and *Salmonella*, it is likely that other microorganisms from similar genera may respond similarly. However, it is important to exercise caution when extrapolating these findings. One example is the variation in behavior observed among different species of *Salmonella*, such as non-typhoidal and avian-specific strains (*S.* Gallinarum and *S.* Pullorum). Both avian *Salmonella* strains possess highly divergent antigenic structures compared to those found in typhoidal *Salmonella* (Freitas Neto *et al*., 2020). Despite their shared genus classification, it is probable that they may respond differently, even when submitted to the same conditions.

Using extremes concentrations for both bacteria involved in the *Spot-on-the-Lawn* antagonism test is highly contraindicated, as results may vary mor than expected and thus, not be reliable. The present study recommends a standardization model with similar values for the concentration of both bacteria to avoid high variability, and non-replicability as well as to ensure reliable results. Using a nephelometric scale such as McFarland (0.5 tube; 1.5 x 10^8 CFU/mL) is highly advised, which is a turbidity standard pattern used to pre-assess the bacterial concentration in liquid culture media. It is also advised to perform bacterial quantification through serial decimal counts, ensuring similar concentrations of both probiotic and pathogen bacteria.

Keywords: poultry, probiotic, antagonism, *Salmonella*, *Lactobacillus*

RESUMO

Neste estudo, as principais variáveis do teste de antagonismo Spot-on-the-Lawn, como concentrações de Salmonella Heidelberg *e* Lactobacillus salivarius *ATCC 11742 e volumes de meio foram investigadas, sendo ao final proposto um modelo de padronização, visando à diminuição de variações individuais e à replicabilidade do teste. Três concentrações de cada bactéria foram preparadas (concentrada, intermediária e diluída), além de três volumes de caldo Brain Heart Infusion (10, 15 e 20mL). O teste de antagonismo foi realizado, sob todas as variações, entre concentrações bacterianas e volumes de meio, resultando em 27 unidades experimentais diferentes e nove halos de inibição por unidade. As comparações permitem concluir que o uso de valores extremos para as concentrações de ambas as bactérias e os volumes de meio leva à super ou subestimação dos halos de inibição. Assim, o ideal é a utilização de concentrações bacterianas e de volumes de meio similares e intermediárias.*

Palavras chave: avicultura, probiótico, antagonismo, Salmonella, Lactobacillus

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