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Research Article
Measurement of *in vitro* Inhibition by *Lactobacillus* spp. Against *Salmonella* Heidelberg

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Abstract

Background and Objective: *Salmonella* Heidelberg is considered to be a microorganism of great concern in the poultry industry, since it causes losses for farmers and is a great risk to public health. Brazil stands out worldwide regarding poultry exports and because of this, researchers have increasingly been trying to reduce the incidence of this pathogen, mainly through taking care of the intestinal health of the birds. A healthy intestinal microbiota comprises many microorganisms and among them, *Lactobacillus* spp. is noteworthy given that studies have already proven its effectiveness in inhibiting several pathogenic microorganisms *in vitro*. The objective of the present study was to correlate the results obtained using two methods for measuring antagonist activity between *Lactobacillus* spp. and *Salmonella* Heidelberg and to observe whether the correlation between the two methods tested was positive or negative. **Methodology:** Ten strains of *Lactobacillus* spp. were used and these showed positive results regarding inhibition of *Salmonella* Heidelberg, both in the Spot on the Lawn test and in the concomitant culture. The two tests were carried out to measure the inhibitory capacity of *Lactobacillus* spp. on *Salmonella* Heidelberg, by correlating the size of the inhibition halo obtained in the Spot on the Lawn test with the *Salmonella* Heidelberg count in the concomitant culture. **Results:** It has been observed that *Lactobacillus* spp. have a great capacity of inhibition against *Salmonella* Heidelberg. However, this capacity varies according to the strain of *Lactobacillus* spp., in addition, other factors may also influence this ability as the temperature, the culture medium and the time of exposure between the samples. **Conclusion:** Although all *Lactobacillus* spp. inhibit *Salmonella* Heidelberg, it was not possible to correlate the data obtained, since each *Lactobacillus* spp. presented a halo measurement that did not correspond to the quantity of *Salmonella* Heidelberg actually inhibited, due to other factors involved.

Key words: Antagonism, chicken, microbiota, pathogen inhibition, probiotic

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The poultry industry has become more receptive to alternatives that allow reductions in production costs. Moreover, the industry also seeks to improve the quality of its final product, so as to meet consumer expectations. Consumers are increasingly becoming concerned about food safety and seek food that is free from antibiotics[1].

As explained by Martins[2], early use of probiotics is extremely important for sanitary management of poultry. Considering that these birds originate from the extremely clean and disinfected environment of an incubator, the first bacteria to come into contact with their gastrointestinal tract will tend to colonize the region more rapidly. This creates a competitive environment that is difficult for other bacteria to colonize. *Salmonella* is among the early colonizers.

Thus, the use of probiotics has steadily increased. Another reason for this increase is that probiotics improve the intestinal microbiota of poultry, which then leads to avoidance of indiscriminate use of antibiotics when rearing these birds[3].

The presence of *Lactobacillus* spp. in the microbiota of poultry has been proven to be beneficial[4]. In addition to decreasing colonization by pathogenic microorganisms such as *Salmonella* spp., *Lactobacillus* spp. can act in other ways in its environment. These characteristics make this microorganism a good probiotic for use in the poultry industry.

The mechanisms used by *Lactobacillus* spp. to inhibit other bacteria *in vitro* are believed to involve production of hydrogen peroxide, specific proteins known as bacteriocins and organic acids. These acids include lactic and acetic acids, which decrease pH and secrete lactate, acetate, succinate and ethanol, thereby aiding the proliferation of other beneficial bacteria. Moreover, *Lactobacillus* spp. adheres to the intestinal mucosa and limits multiplication of pathogenic bacteria such as *Escherichia coli*, *Salmonella* spp. and *Campylobacter* spp.[5].

According to Miyamoto *et al.*[6], the *Lactobacillus* species found in the intestinal microbiota of poultry are *Lactobacillus acidophilus*, *Lactobacillus salivarius* and *Lactobacillus fermentum*. Other species are rarely observed.

On the other hand, *Salmonella* spp. is an undesirable microorganism for the intestinal microbiota of poultry. Not only it is harmful to the birds' health, but also it is considered to be a public health risk and can trigger large outbreaks of food poisoning[7,8].

According to Matte[9], outbreaks of food poisoning related to *Salmonella* Heidelberg have increased considerably over recent years. Infection due to *Salmonella* Heidelberg has been shown to be more serious than other *Salmonella* infections and may lead to conditions of septicemia, myocarditis and extra-intestinal infections and even death.

Thus, these microorganisms started to be studied using methods and techniques to investigate the antagonism among them. One of the main techniques currently used is the Spot on the Lawn test, which has yielded the most significant results for research. In these studies, *Lactobacillus* spp. have gained the spotlight and have revealed positive results when challenged with pathogenic samples[10].

Concomitant cultures are another method used to observe antagonism. In addition to defining the degree of inhibition caused by *Lactobacillus* spp., it can also be measured every hour, which is an additional advantage of this method[11].

The objective of the present study was to correlate the results obtained using two methods for measuring antagonist activity between *Lactobacillus* spp. and *Salmonella* Heidelberg and to observe whether the correlation between the two methods tested was positive or negative.

MATERIALS AND METHODS

A total of 10 samples of *Lactobacillus* spp. and one sample of *Salmonella* Heidelberg were used. All the samples originated from birds and were taken from the bacterial collection of the Avian Pathology Laboratory of the School of Veterinary Medicine and Animal Science (FMVZ), located in the Municipality of Botucatu, São Paulo.

The first day of the technique consisted of enriching the samples of *Lactobacillus* spp. in DeMan-Rogosa-Sharpe (MRS)* agar in an incubator at 38°C for 18 h. After this period, on the second day, 10 µL were individually seeded at three points in a petri dish containing MRS agar. The petri dish was also placed in the incubator at 38°C for 18 h. On the same day, the *Salmonella* Heidelberg sample was enriched in a brain-heart infusion (BHI) broth and was placed in the incubator at 38°C for 18 h.

On the third day, 200 µL of *Salmonella* Heidelberg were transferred to a new tube containing BHI broth with 0.65% agar-agar at 40°C. This content was immediately placed on a dish that had been pre-cultured with *Lactobacillus* spp. After the agar had solidified, the dish was placed in an incubator at 38°C for 12 h. The inhibition halos were then measured.

*MRS medium composed of dextrose, peptonate, yeast extract, beef extract, ammonium citrate, sodium acetate, sorbitan complex, magnesium sulfate, manganese sulfate, disodium phosphate and water at pH 6.5[12]

The second part of the technique involved simultaneous mixtures of the two microorganisms (*Salmonella* Heidelberg and *Lactobacillus* spp.), in equal proportions of the culture mediums (BHI and MRS, respectively). These mixtures were kept in an incubator at 38°C for 9 h.

The samples were then removed from the incubator and serial decimal dilutions were performed to count colony forming unit (CFU) every hour. This technique consists of transferring the mixture of bacteria to a phosphate-buffered saline solution at proportions of 1:10. The other dilutions were obtained with serial decimal dilutions, with subsequent plating of all dilutions in brilliant green agar (BGA). This same process was repeated every hour over a period of 9 h using the mixture of microorganisms.

After the plating process, the plates were stored in an incubator for a period of 12 h at 37°C. This enabled counting of the *Salmonella* Heidelberg present, which was expressed in CFU mL^{-1} of culture medium.

RESULTS

The antagonistic action of *Lactobacillus* spp. towards *Salmonella* Heidelberg in the Spot on the Lawn method is shown in Table 1.

All samples of *Lactobacillus* spp. submitted to the Spot on the Lawn test, showed inhibition against the indicator microorganism (*Salmonella* Heidelberg), but with different potentials of inhibition. As for example, sample 1 and 2 had the minimum and the maximum potential of inhibition against *Salmonella* Heidelberg, respectively.

Table 2 lists the colony-forming unit vales obtained by concomitantly culturing *Lactobacillus* spp. and *Salmonella* Heidelberg over a 9 h period of mixture, starting from bringing these two microorganisms together.

Table 2 shows the amount of colony forming units of *Salmonella* Heidelberg after concomitant culturing with the different strains of *Lactobacillus* spp. for 9 h.

With this test, it was clear that the different strains of *Lactobacillus* spp. act in different ways, as demonstrated by strain 3, which obtained the lowest count, strains 2, 4, 5 and 10 with intermediate counts and strains 1, 6, 7, 8 and 9 with the highest *Salmonella* Heidelberg count number.

It is suggested in this case that the inhibition may have occurred mainly by the production of lactic acids, which decrease the pH of the medium and make the environment not ideal for the development of *Salmonella* Heidelberg. However, there are several other factors that could have influenced this result, which may increase or decrease the inhibitory capacity of *Lactobacillus* spp.

DISCUSSION

Use of probiotics in the poultry industry is not recent, given that several authors have already reported their use since the beginning of this century. The main outcomes from their use are improvements of economic and zootechnical indexes among the animals, thereby increasing weight gain and improving food conversion[7,6].

Several studies have proven that *Lactobacillus* spp. are potential inhibitors of *Salmonella* spp. and other microorganisms that are considered pathogenic to the gastrointestinal tract of poultry[13,14,4].

Pereira and Gomez[1] reported that the strain *Lactobacillus acidophilus* was able to inhibit strains of *Escherichia coli* and

Table 1: Antagonistic effect of *Lactobacillus* spp. against *Salmonella* Heidelberg through the Spot on the Lawn method

Lactobacillus spp.	1	2	3	4	5	6	7	8	9	10
Inhibition halo *	0.7	2.0	1.1	1.4	1.8	1.1	1.5	1.3	1.4	1.2

*Measured in centimeters from the inhibition halo obtained from the Spot on the Lawn test

Table 2: Colony-forming units from concomitant culturing of *Lactobacillus* spp. and *Salmonella* Heidelberg over a 9 h period of mixture

		Hour								
Lactobacillus spp.	Inhibition halo*	1	2	3	4	5	6	7	8	9
1	0.7	1.6×10^{9}	6.0×10^{8}	9.0×10^{8}	1.8×10^{9}	6.0×10^{8}	3.0×10^{8}	4.0×10^{8}	2.0×10^{8}	6.0×10^{8}
2	2.0	1.1×10^{9}	5.0×10^{8}	2.0×10^{9}	2.5×10^{8}	2.8×10^{7}	1.0×10^{6}	0	0	0
3	1.1	0	0	0	0	0	0	0	0	0
4	1.4	3.0×10^{8}	1.1×10^{9}	1.5×10^{9}	7.0×10^{8}	5.0×10^{7}	1.8×10^{5}	0	0	0
5	1.8	6.0×10^{8}	1.3×10^{9}	6.0×10^{8}	1.6×10^{8}	8.0×10^{6}	3.0×10^{4}	0	0	0
6	1.1	2.7×10^{9}	2.1×10^{9}	1.3×10^{9}	1.5×10^{9}	2.7×10^{9}	2.8×10^{9}	9.0×10^{9}	1.9×10^{9}	2.8×10^{9}
7	1.5	1.8×10^{9}	1.9×10^{9}	2.0×10^{9}	1.6×10^{9}	1.9×10^{9}	1.9×10^{9}	8.0×10^{9}	1.4×10^{9}	6.0×10^{9}
8	1.3	2.2×10^{9}	5.0×10^{8}	1.8×10^{9}	2.5×10^{9}	8.0×10^{8}	1.0×10^{9}	8.0×10^{6}	9.0×10^{4}	1.0×10^{3}
9	1.4	1.9×10^{9}	1.0×10^{9}	1.5×10^{9}	1.8×10^{9}	2.2×10^{9}	1.7×10^{9}	1.5×10^{9}	5.0×10^{8}	1.2×10^{8}
10	1.2	1.1×10^{9}	7.0×10^{8}	1.2×10^{8}	2.4×10^{7}	1.6×10^{6}	2.1×10^{4}	0	0	0

*Measured in centimeters from the inhibition halo obtained from the Spot on the Lawn test

Staphylococcus aureus, *in vitro*. They found that the peak inhibitory activity of *L. acidophilus* was reached after 72 h of incubation at 37°C in a MRS broth under aerobiotic conditions. Moreover, they explained that these results were attained probably due to low pH values and to the action of lactic acid on the strains studied.

In turn, Poppi *et al.*[15] demonstrated the important role of acids produced by samples of *Lactobacillus*, particularly lactic acid, in the antagonistic effect of pathogenic samples. They observed that addition of sodium bicarbonate (acid neutralizer) hindered the inhibitory effect of some strains of *Lactobacillus*. However, not all samples in their study had the same result. Some strains presented inhibition even with addition of sodium bicarbonate, thus demonstrating that this effect is caused by a combination of various factors and products generated by *Lactobacillus*.

Regarding the method used, Shanthya *et al.*[16] and Cadirci and Citak[10] reported that the best method for evaluating antagonism between samples is the Spot on the Lawn method. However, Soomro *et al.*[11] disagreed with this and stated that the best technique was in fact the paper disc method, which is considered easy to apply. Nevertheless, all authors have agreed that the inhibition that occurs with the Spot on the Lawn method is largely due to the metabolites produced, such as lactic acid, acetic acid and bacteriocins[16,11].

Miyamoto *et al.*[6] suggested that *Lactobacillus* could present a protective effect for poultry against colonization by *Salmonella* Enteritidis, considering the inhibition halos obtained through the Spot on the Lawn technique. Similarly to what was obtained in the present study, halos were formed around the samples, measuring between 0.2 and 1.6 cm.

There are no data in the literature regarding use of the concomitant culturing technique to evaluate and quantify antagonism between samples. However, the samples that showed formation of large halos in the Spot on the Lawn method did not present the best performance in the concomitant culturing and vice versa.

According to Borowsky[17], in the specific case of *Salmonella* spp., quantification is not done routinely, which makes studying these microorganisms more difficult. Moreover, the results vary greatly according to the methods used for analyses.

This situation was observed in comparing sample numbers 2 and 3. While sample 2 presented the largest halo by means of the Spot on the Lawn technique, inhibition in this same sample using the CC technique was only completed after 7 h of contact. On the other hand, sample 3 presented an intermediate halo and was able to inhibit *Salmonella* Heidelberg within the first hour of contact.

The results obtained may have varied because the amount of *Lactobacillus* spp. inoculated in each sample was not assessed. Moreover, the pH of the environment, the concentration of the inoculum and the culture medium itself are all considered to be variables.

The medium used in the present study for culturing of *Lactobacillus* spp. differed from what was used by Lopes[12] in their study, different culturing mediums were compared and it was demonstrated that the one with greatest production of lactic acid had been enriched with tomato extract and had been agitated. Moreover, in comparison with the medium CSN 12**, the standard medium***, can be considered to be the second best medium for production of large amounts of lactic acid from *Lactobacillus* spp.

Differences among the results were expected, since different strains of *Lactobacillus* were used. This demonstrates that some strains are more resistant towards inhibiting *Salmonella* Heidelberg than others.

However, more studies are still needed, particularly regarding the inhibition promoted by *Lactobacillus* spp. *in vivo*. The tables demonstrated that samples with higher inhibition capacity through the Spot on the Lawn technique did not present high inhibition capacity when cultured together in the same medium. Thus, this result shows that there was neither a positive nor a negative correlation between the tests.

CONCLUSION

It is concluded that all *Lactobacillus* spp. inhibit *Salmonella* Heidelberg, it was not possible to correlate the data obtained, since each *Lactobacillus* spp. presented a halo measurement that did not correspond to the quantity of *Salmonella* Heidelberg actually inhibited, due to other factors involved.

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**CSN 12 medium composed of peptonate, yeast extract, beef extract, ammonium citrate and clarified sugar cane broth at pH 6.2[12].

***Standard medium composed of sucrose, yeast extract, magnesium sulfate (0.2 g L^{-1}), manganese sulfate (0.01 g L^{-1}), sodium chloride (0.01 g L^{-1}), ferrous sulfate (0.01 g L^{-1}) (Sol A), dibasic potassium phosphate (Sol B), calcium chloride (Sol C) and water at pH 6.2[12]

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