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ARTICLE

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INHIBITORY EFFECT OF DIFFERENT PROBIOTIC FORMULATIONS ON *SALMONELLA* HEIDELBERG, AVIAN PATHOGENIC *ESCHERICHIA COLI* AND *CAMPYLOBACTER JEJUNI*

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ABSTRACT

A wide variety of microorganism species have been used as probiotics to improve the intestinal microbial balance, control and prevent the colonization of pathogenic bacteria and promote growth in animal production. Based on the various beneficial factors of probiotic agents, this study aimed to evaluate the effectiveness of three different formulations of probiotics strains through the analysis of antagonistic capacity against common pathogens from chickens gastrointestinal tract. Three formulations composed by *Bacillus, Lactobacillus, Saccharomyces, Enterococcus, Bifidobacterium* and *Pediococcus* were tested *in vitro* by the method of probiotic culture spot in plates seeded with inocula of *Escherichia coli, Salmonella* Heidelberg and *Campylobacter jejuni*. The inhibition halos were measured and classified according to the degree of pathogenic bacteria inhibition. The formulation containing an association among *Bacillus, Lactobacillus* and *Saccharomyces* presented better results when compared to the other formulations.

KEYWORDS: Lactic acid bacteria, yeasts, microbiota, poultry, *Salmonella, Escherichia coli, Campylobacter jejuni*

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1. INTRODUCTION

The term "probiotic" derives from Latin and means "for life" and is used to name live microorganisms that, when administered in adequate amounts, are used to improve the intestinal microbial balance, conferring health benefits to humans and animals. (FAO/WHO, 2002).

The resident microbiota, also called the normal microbiota of the gastrointestinal tract, is composed of bacteria, fungi, and protozoa and is permanently established without causing diseases in healthy individuals. However, disturbances of this balance can cause abnormal growth of these microorganisms, leading to the development of diseases (MACARI, LUNEDO e PEDROSO, 2014).

There are basically two ways in which probiotics act to maintain animal health: the exclusion of pathogens by competition and immunomodulation (YANG et al, 2009). Several studies show that competition for substrates and binding sites and the production of antimicrobial metabolites that inhibit pathogens produced by probiotics were effective in controlling Salmonella colonization in chickens (HIGGINS *et al*., 2007; STERN *et al*., 2001; PASCUAL *et al*., 1999). Probiotics interact directly with the immune system present in the intestinal mucosa and can modulate innate and adaptive immunity (DALLOUL *et al*, 2003; KOENEN *et al*, 2004; HAGHIGHI *et al*, 2005).

Lactic acid bacteria (LAB) have been commonly used as a probiotic. They are gram positive, non-spore forming, and facultative anaerobes or those that grow under microaerophilic conditions. This group includes the genera *Lactobacillus*, *Enterococcus*, *Pedicoccus*, and *Bifidobacterium*, with *Lactobacillus* being so far considered the largest group, containing more than 250 species (MOKOENA, 2017), but in 2020 a new classification of the genus *Lactobacillus* was released after genomic analyses, grouping only 38 species in this group (ZHENG et al, 2020). The bacteria of this genus come from various habitats, and can be found on plant surfaces, fermented food products and in various parts of the body of animals (DUAR et al, 2017).

The genus *Enterococcus* is related to cheese fermentation processes and has antimicrobial compounds responsible for food preservation (MORENO et al, 2006). Bacteria of the genus *Pediococcus* exert antagonism against other microorganisms by producing lactic acid and antimicrobial peptides known as pediocins (PAPAGIANNI and ANASTASIADOU, 2009). Regarding the genus *Bifidobacterium*, Gibson and Roberfroid (1995) emphasize its ability to control the proliferation of pathogenic bacteria by producing lactic acid and acetic acid, restore the intestinal microbiota after the use of antibiotics, and the production of vitamins, especially B vitamins.

In addition to the commonly exploited strains of the LAB group, spore-forming bacteria of the genus *Bacillus* also carry probiotic attributes (ELSHAGHABEE et al, 2017). Other microorganisms widely used for their probiotic actions are yeasts, mainly of the genus *Saccharomyces*. Yeasts are

not normal inhabitants of birds' gastrointestinal tract and are found in cereals, vegetables, and citrus fruits (REVINGTON, 2002). The yeasts also act as prebiotics, because they promote the growth of microorganisms of probiotic action, helping in the increase of these beneficial bacteria in the gastrointestinal tract of the host (GIBSON and ROBERFROID, 1995).

The indiscriminate use of antibiotics seeking their effects as performance enhancers in poultry production has favored the emergence of antimicrobial resistance. Thus, each time more prebiotic and probiotic agents have been suggested to replace antibiotics in poultry farming (PERALTA-SÁNCHEZ et al, 2019).

Based on the various beneficial factors of agents of probiotic action in the body, this paper aimed to evaluate the efficacy of strains of three different probiotic formulations by analyzing the inhibitory capacity of the metabolites produced against common pathogens of the gastrointestinal tract of chickens.

2. MATERIAL AND METHODS

2.1. FORMULATIONS OF PROBIOTICS USED.

The species used in each formulation tested and their respective amounts are shown in table 1.

Table 1. Compositions of probiotic bacteria used.

Formulation	Species	Amount
FA	*Bacillus subtilis*	5 X 10^8 CFU/g
	Bacillus licheniformis	5 X 10^8 CFU/g
	Bacillus coagulans	5 X 10^7 CFU/g
	Lactobacillus acidophilus	5 X 10^7 CFU/g
	Saccharomyces cerevisiae	2 X 10^7 CFU/g
F1	*Bifidobacterium animalis*	1 X 10^{11} CFU/kg
	Enterococcus faecium	6 X 10^{11} CFU/kg
	Lactobacillus reuteri	2,5 X 10^{10} CFU/kg
	Lactobacillus salivarium	2,5 X 10^{10} CFU/kg
	Pediococcus acidilactici	2,5 X 10^{11} CFU/kg
F2	Competitive Exclusion probiotic microorganisms (NAGF)	Unspecific strains
	Probiotic Compounds	

The microorganisms that compose the formulations were received lyophilized and the hydration of the strains was performed at the time of the tests.

2.2. PATHOGENIC STRAINS USED.

Three different Avian pathogenic *Escherichia coli* (APEC) isolates were used, one

from pet chicken (APEC 1), another from peacock (APEC 2) and another APEC from chickens kindly donated by Dr. Terezinha Knöbl from the University of São Paulo (USP, São Paulo, Brazil) (APEC 3).

The three *Salmonella* Heidelberg (SH) isolates used were from broiler strains and for *Campylobacter jejuni*, strain IAL.

2.3. AVIAN PATHOGENIC ESCHERICHIA COLI (APEC) AND SALMONELLA HEIDELBERG (SH).

Petri dishes containing nutrient agar (NA - Kasvi) were prepared previously and pre-inoculation of the probiotics was done by adding 2g of the probiotic formulation to be tested in 10 mL of saline solution at pH 3 and homogenizing every 5 minutes with the aid of a vortex. After 30 minutes, the mixture was centrifuged for 5 minutes at 8,000 rpm and the supernatant was removed completely with the aid of a pipette. Next, 10 mL of saline solution at pH 7 was added and the homogenization process was repeated. Using a pipette, 5 µL of each of the formulations was carefully deposited on the surface of the NA (bacterial culture spot), leaving the plate open in laminar flow for 10 min for drying of the inoculum. The plates were then incubated at 37°C for a period of 24 h in a bacteriological oven.

In parallel, the APEC and SH pathogenic strains were streaked on nutrient agar and grown overnight at 37°C in a bacteriological oven.

The next day, the plate-grown formulations were inactivated with chloroform to assess the production of extracellular substances capable of diffusing into the agar and preventing the growth of adjacent pathogenic bacteria. For this, the plates were opened in a laminar flow hood and the colonies were exposed to chloroform vapor (Synth®) by adding 1 mL of chloroform on filter paper in the lid of the plate and closing it upside down for 30 min to promote cell death. Then, the plates were opened, still in the flow, for another 30 min, to evaporate the residual chloroform. After this time had passed, a solution of the pathogenic bacteria was made at a concentration of approximately 108 CFU using the Mc Farland scale as the turbidity standard, and 10 µL of this sample was added to 10 mL of warm, still liquid NA. The agar with the added pathogenic bacteria sample was poured over each plate containing the probiotic strains, and the plates were incubated in a bacteriological oven at 37°C for 24h.

The halo of inhibition formed around the bacterial culture spot was measured with the help of a millimeter ruler, adjusting it to 6.5 mm in diameter, similar to an antibiotic disk for antibiogram, and the determination of the antagonist capacity of the isolates was performed according to Santos (1984), being considered as very strong inhibition the zones above 20 mm in diameter; strong inhibition, from 15 to 19 mm; moderate, from 11 to 14 mm; weak, from 9 to 10 mm; and no inhibition, less than 9 mm.

This methodology was sufficient for APEC but not for SH. Thus, the analyses for both pathogenic bacteria tested with the

probiotic culture spots were repeated, choosing to incubate the spots for 48 hours.

All the work was done in triplicate with the evaluation of the diameters in 8 different directions. Negative control and positive controls were inserted at all times during the analysis, in addition to the antibiotic controls.

2.4. CAMPYLOBACTER JEJUNI STRAIN IAL

Petri dishes containing Charcoal Cefoperozone Deoxycholate agar (CCDA - Kasvi) were previously prepared and the probiotics were also prepared and treated in the same way as described in item 4.2. The probiotic culture spots were submitted to the inactivation process after 48 hours of growth, however, one of the microorganisms composing the probiotic formulation grew under *Campylobacter jejuni* conditions (microerophilic). Thus, a pilot test was conducted in order to verify if the inactivation after incubation of only 24 hours of the probiotic spots would be efficient to inactivate the resistant microorganism. As the result was positive, the inactivation with 24 hours was standardized. After this period, the probiotic was inactivated and the *C. jejuni* prepared as described in the previous item and mixed in the same concentrations as in the first test to the warm and still liquid CCDA and inoculated over the inactivated culture spot. The plates were incubated for 48 hours in a micro-aerobic atmosphere.

The halo of inhibition formed around the probiotic culture spot was measured adjusted to 6.5 mm in diameter, similar to an antibiotic disc for antibiogram. The determination of the antagonistic capacity of the isolates was performed according to the method described for SH and APEC.

The whole experiment was performed in sextuplicate with the evaluation of the diameters in 8 different directions. Negative and positive controls were inserted at all times of the analysis in addition to antibiotic controls.

2.5. STATISTICAL ANALYSIS.

ANOVA followed by Tukey's test or T test was the statistical method used ($p < 0.05$) by Graph pad prism 9.1

3. RESULTS

3.1. APEC AND SH WITH SPOT PROBIOTIC INCUBATED FOR 24 HOURS

The results concerning the antimicrobial activity of the probiotic formulations F1, F2 and FA for APEC and SH after incubation for 24 hours are described in Table 2. The agar diffusion method to evaluate the antagonist activity of the strains that compose the studied formulations revealed various sizes of halos, measured in millimeters (mm) being highlighted in green the results that characterize very strong inhibition, with halos larger than 20 mm, in blue for strong inhibition, with halos between 15 and 19 mm, in pink for moderate inhibition, with halos from 11 to 14 mm, in yellow for weak inhibition, with halos from 9 to 11 mm, and finally in gray, indicating absence of inhibition, with halos smaller than 9 mm.

Table 2. Results of APEC and SH inhibitions (halos in mm) after incubation of the probiotic spot for 24 hours.

	APEC 1		APEC 2		SH 1		SH 2	
	Average	PD	Average	PD	Average	PD	Average	PD
F1			14,41a	7,64	Not inhibited		Not inhibited	
F2			7,67b	1,83	Not inhibited		Not inhibited	
FA	19,07a	3,25	12,9a	2,52	Not inhibited		Not inhibited	
Gentamicin Control			6,5c	0,00	Not inhibited		Not inhibited	
Enrofloxacin control	8,875b	0,640			Not inhibited		Not inhibited	
Test	T test		ANOVA (Kruskal -Wallis)					

Different letters in the same column indicate statistical difference. A blank cell means that the test was not performed. Cells in green color indicate very strong inhibition, in blue strong inhibition, in pink moderate, in yellow weak and in gray no inhibition.

For APEC 1, only formulation FA was tested, showing strong inhibition, with a halo of 15 to 19 mm, being statistically smaller when compared to the control made with the antibiotic enrofloxacin. For APEC 2, the formulations F1 and FA showed moderate inhibition, with a halo of inhibition from 11 to 14 mm and F2 showed no inhibition, because the halo formed was smaller than 9 mm for APEC2. F1 and FA were statistically similar and larger than F2, which was larger than the control made with the antibiotic gentamicin. None of the formulations showed inhibition for SH 6 and 7, nor the controls made with the two antibiotics mentioned above.

3.2. APEC AND SH WITH PROBIOTIC SPOT INCUBATED FOR 48 HOURS

Due to the results presented in the previous item, in which none of the formulations and also none of the antibiotics used as controls promoted inhibition in the growth of SH, a new test was performed, with analysis after incubation for 48 hours.

Table 3 shows the results concerning the antimicrobial activity of the probiotic formulations F1, F2 and FA for APEC 1, APEC 2 and standard APEC after incubation for 48 hours. The colors used, identify the same classifications presented previously, being green, blue, pink, yellow and gray to indicate very strong, strong, moderate, weak and absent inhibitions, respectively.

Table 3. Results of APEC inhibitions (halos in mm) after incubation of the probiotic spot for 48 hours.

	APEC 1		APEC 2		APEC 3	
	Average	PD	Average	PD	Average	PD
F1	11,6a	1,95	12,58a	3,20	13,33	2,49
F2	8,33b	1,30	9,58a	1,31	9,83	1,40
FA	24,00c	11,65	25,33b	8,76	12,42	2,46
Enrofloxacin control	24,3c	0,81	21,5c	1,56	20,5	0,00
Test	ANOVA (Kruskal -Wallis)		ANOVA (Tukey)		ANOVA (Tukey)	

Different letters in the same column indicate statistical difference. Cells in green color indicate very strong inhibition, in blue strong inhibition, in pink moderate, in yellow weak, and in gray no inhibition.

Formulation F1 caused moderate inhibition halos for all APEC, with halos between 11 and 14 mm, and all were statistically smaller compared to the control with the enrofloxacin antibiotic. For APEC 1, there was no inhibition in formulation F2, as the halo produced was smaller than 9 mm, while for APEC 2 and standard APEC, there was weak inhibition as they produced halos between 9 and 10 mm. The result was statistically lower for APEC 1 compared to APEC 2 and standard APEC, and all were smaller than the control with enrofloxacin. The FA formulation caused very strong inhibition for APEC 1 and 2, with halos greater than 20 mm, and moderate for standard APEC, with a halo between 11 and 14 mm. The inhibition on APEC 1 was statistically similar to the control with enrofloxacin, but lower than the inhibition on APEC 2. The inhibition on standard APEC was statistically lower than the control with the antibiotic.

The results for the test of antagonistic ability of formulations F1, F2 and FA to the growth of SH1, SH2 and SH3 after 48 hours of incubation are presented in Table 4.

Table 4. Results of SH inhibitions (halos in mm) after incubation of the probiotic spot for 48 hours.

	SH 1		SH 2		SH 3	
	Average	PD	Average	PD	Average	PD
F1	10,25a	1,48	12,25a	1,91	11,92	3,36
F2	7,33b	1,43	10,17a	1,73	9,33	1,66
FA	8,91ab	2,23	17,58b	2,12	12,75	2,9
Enrofloxacin control	24,5c	0,04	22,5c	0,012	22,5	0,07
Test	ANOVA (Tukey)		ANOVA (Kruskal -Wallis)		ANOVA (Tukey)	

Different letters in the same column indicate statistical difference. Cells in green color indicate very strong inhibition, in blue strong inhibition, in pink moderate, in yellow weak, and in gray no inhibition.

Formulation F1 produced weak inhibition for SH1 and moderate inhibition for SH2 and SH3 with halos between 9 and 11 mm for the first one and 12 and 14 for the last two, the first one being statistically smaller in relation to the last two and the latter being smaller than the control with the enrofloxacin antibiotic. For formulation F2, there was no inhibition of SH1, since the halo formed was smaller than 9 mm. For SH2 and SH3, formulation F2 produced weak inhibition, forming halos between 9 and 11 mm. Finally, formulation FA promoted strong inhibition for SH2 and moderate inhibition for SH3, with inhibition halos between 15 and 19 for the former and 12 and 14 for the latter. There was a statistical difference for all results compared to the control with the enrofloxacin antibiotic.

3.3. CAMPYLOBACTER JEJUNI WITH PROBIOTIC SPOT, INCUBATED FOR 24 HOURS.

The results of the measurements of the growth inhibition halos of *Campylobacter jejuni* by the probiotic formulations F1, F2 and FA after 24 hours of incubation in microaerophilic are described in Table 5.

Table 5. Results of *Campylobacter jejuni* inhibitions (halos in mm) after incubation of the probiotic spot for 24 hours.

	Average	PD
F1	22,60a	0,29
F2	13,84b	0,11
FA	17,00b	0,25
Enrofloxacin control	16,53b	0,005
Test	ANOVA (Tukey)	

Different letters in the same column indicate statistical difference. Cells in green color indicate very strong inhibition, in blue strong inhibition, in pink moderate, in yellow weak, and in gray no inhibition.

For *Campylobacter*, there was very strong, moderate and strong inhibition for formulations F1, F2 and FA respectively, with halos greater than 20 mm for F1, between 11 and 14 for F2 and between 15 and 19 for FA. There was no statistical difference between the results presented by F2 and FA in relation to the control used with the enrofloxacin antibiotic.

4. DISCUSSION

The indiscriminate use of antibiotics as growth promoters in animal production has become a serious public health problem, leading producers to a great challenge: to find alternative methods to control and prevent the colonization of pathogenic bacteria. An ideal alternative should have the same benefits as antibiotics used as performance enhancers, in other words, ensure optimal animal development and increase nutrient availability (ANADÓN et al, 2019).

The ability to produce antimicrobial compounds such as bacteriocins, hydrogen peroxide, volatile organic acids, lactic acid and acetic acid that inhibit the growth of potentially pathogenic bacteria (GIBSON and ROBERFROID, 1995; PAPAGIANNI and ANASTASIADOU, 2009; MOKOENA, 2017) makes microorganisms with probiotic activity an excellent alternative. Probiotics, in formulations composed of a single species or multi-species, have been widely used in animal productions as an alternative to the use of antibiotics (PREMAVALLI, SANGILIMADAN, OMPRAKASH, 2018).

The FA formulation, composed of microorganisms of the genus *Bacillus*, *Lactobacillus* and *Saccharomyces*, generated a very strong inhibition on two APEC strains. These results corroborate with the findings of Arreguin-Nava *et al* (2019), who inoculated *Bacillus* probiotics into *E. coli* infected embryos and observed a significant reduction in the total number of gram-negative bacteria in the gastrointestinal tract of these animals. The presence of yeasts of the genus *Saccharomyces* may have contributed to the good performance of this formulation because, besides the probiotic properties, they also have prebiotic properties, which enhance the effects of other probiotic agents present in the formulation (REVINGTON, 2002; GIBSON and ROBERFROID, 1995), which may explain its better action against the bacteria APEC strains 1 and 2 and SH strain 2, when compared to the other formulations, and good action against *Campylobacter*.

Although formulation F1 did not show as strong inhibition as FA on APEC, the probiotic composed of bacteria of the genus *Bifidobacterium*, *Enterococcus*, *Lactobacillus* and *Pediococcus*, produced a strong inhibition, also presenting a good antibacterial activity result. CAO *et al* (2013) used *Enterococcus faecium* as a probiotic in APEC-infected chickens and observed that *E. faecium* efficiently inhibited the adhesion of *E. coli* to the intestinal mucus, probably through competitive exclusion and pH alteration.

The pathogenic bacteria SH is one of the emerging serovars in Brazil and there is great concern in the poultry industry, because this microorganism is multidrug resistant to antibiotics. Many strategies focused on *Salmonella* control have limited the prevalence of these serovars, but there is a difficulty in controlling the Heidelberg serovar. In this experiment, the F1 formulation inhibited the growth of two SH strains moderately and the FA formulation promoted very strong inhibition in one of the tested strains. Da Silva Sabo *et al* (2020), in

an experiment conducted in Brazil, testing probiotics against SH *in vitro*, suggest that the antimicrobial activity produced by *Lactococcus* and *Enterococcus* isolates against SH is not provided by bacteriocins, but by organic acids produced during their growth. MENCONI *et al* (2011), using a formulation containing 11 *Lactobacillus* strains significantly decreased the amount of SH in cecal tonsil samples from infected birds.

Campylobacteriosis is an important foodborne disease that occurs worldwide. The chicken is an asymptomatic carrier of *Campylobacter,* being the production of this animal the site of primary contamination of the bacteria that mainly colonizes the cecum and small intestine of birds (GHAREEB *et al*, 2012). COX *et al* (2012) present several studies that confirm that vertical transmission of pathogenic bacteria such as bacteria of the genera *Campylobacter* and *Salmonella* from the parent to the chick occurs from the first days post-hatch.

Ghareeb *et al* (2012) observed that the inhibitory effects of *Enterococcus faecium*, *Pediococcus acidilactici*, *Lactobacillus salivarius* and *Lactobacillus reuteri* against *C. jejuni in vitro* could suggest that these strains could have potential to reduce *C. jejuni* in poultry. Indeed, in this study, the F1 formulation, which contains the aforementioned species, showed the best inhibitory response against *C. jejuni*. These results are also in agreement with the findings of Willis and Reid (2008), who showed a lower level of *C. jejuni* in broilers fed a standard diet supplemented with a mixed probiotic containing strains of *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*.

The inhibition of pathogenic strains of APEC, SH and *Campylobacter* produced by the probiotic formulations generated by the antimicrobial activity of these microorganisms proved to be strain dependent. Observing the results produced in all experiments, formulation FA (composed of *Bacillus subtilis*, *B. licheniformis*, *B. coagulans*, *Lactobacillus acidophilus* and *Saccharomyces cerevisiae*) showed a superior result when compared to formulations F1 and F2, which can be explained by the association of probiotic agents with prebiotic agents, which can potentiate the action of the formulation on pathogenic bacteria. Between F1 and F2, formulation F1 (composed of *Bifidobacterium animalis*, *Enterococcus faecium*, *Lactobacillus reuteri*, *L. salivarium*, and *Pediococcus acidilactici*) presented the best results.

The inhibition analyses performed in this work take into consideration the products produced by the probiotic bacteria against pathogens. The results were satisfactory, especially for formulations F1 and FA. This indicates that the use of these probiotics is efficient for the control of the bacteria studied and can be safely used in routine farm operations.

5. CONCLUSION

The inhibition of pathogenic strains of APEC, SH and *Campylobacter* produced by probiotic formulations generated by the antimicrobial activity of these microorga-

nisms is strain dependent. Overall, the FA formulation (composed of *Bacillus subtilis*, *B. licheniformis*, *B. coagulans*, *Lactobacillus acidophilus* and *Saccharomyces cerevisiae*) showed a superior result when compared to the F1 and F2 formulations.

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