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Lactic Bacterium and Bacillus Sp. Biofilms Can Decrease the Viability of Salmonella Gallinarum, Salmonella Heidelberg, Campylobacter Jejuni and Methicillin Resistant Staphylococcus Aureus on Different Substrates

ABSTRACT

This study aimed to evaluate the efficacy of biofilms formed by lactic acid bacteria and Bacillus sp. (BLA) in preventing and controlling the formation of wild biofilms and/or planktonic forms of *Salmonella Gallinarum* (SG), *Salmonella Heidelberg* (SH), and methicillin-resistant *Staphylococcus aureus* (MRSA) on different surfaces. The SH and SG viability was evaluated in polystyrene plates, wood shavings, and soil samples. Two protocols were developed to examine the use of BLA in a preventive and control application. For analysis of *Campylobacter jejuni* (CJ) BLA was used only preventively in a polystyrene plate. Results showed that BLA was effective in preventing the growth of SG and SH in all matrices. The effectiveness of BLA for MRSA was lower than for SG and SH. The efficiency of BLA in preventing CJ growth seems to be related to the initial CJ contamination. BLA proves to be a potential alternative to control food-borne pathogens commonly encountered in animal production and food industry.

INTRODUCTION

Poultry production is one of the largest economic activities in the world. With affordable prices and practicality in preparation, chicken meat has convinced more and more consumers and is still gaining market share (ABPA, 2018). In this context of growing production and commercialization, quality control of poultry meat products and animal health requires stringent monitoring; indeed, birds' carriage of food-borne pathogens is a crucial food safety concern for this industry. *Salmonella* and *Campylobacter* cause the most frequent zoonotic diseases globally, and chicken is their primary vector (Domingues *et al.*, 2012). In addition to damage caused to animal and public health, these microorganisms also lead to a significant negative financial impact (ABPA, 2018).

Salmonellosis is one of the most complex zoonoses with the greatest impact on global public health and causes medical costs of up to \$356 million per year (CDC, 2011). *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum (SG), responsible for fowl typhoid, has been intensively isolated in recent years. Outbreaks of SG lead to high mortality of animals and the elimination of breeding stock and an increase in spending on vaccines and veterinary medicines (Alves, 2017). Also, significant rise in samples positive for *Salmonella enterica* subsp. *enterica* serovar Heidelberg (SH) from poultry production has recently been reported. SH has been proven to be difficult to control (Voss-Rech *et al.*, 2015).

Campylobacteriosis is an emerging zoonosis but still underreported and underdiagnosed worldwide. For example, in the European Union (EU), this disease is the most frequently reported food-borne illness, with around 200,000 cases per year. However, the number of cases is



believed to be approximately 9 million each year (EFSA, 2015). *Campylobacter jejuni* (CJ) is the most prevalent species in food poisoning in humans, leading to drug costs of up to \$ 1.56 billion (Scharff, 2011).

Staphylococci is also one of the leading zoonotic agents that causes harmful infection in humans. *Staphylococcus aureus* can contaminate meat from chickens and the skin and respiratory tract of food handlers (Montezani *et al.*, 2012). The control of this microorganism within poultry farming and the food industry is essential. Methicillin-resistant *Staphylococcus aureus* (MRSA) represents a threat to human health, and it has been isolated from raw chicken (Hasman *et al.*, 2010; Andrew *et al.*, 2011). Thus, its control of farms is essential because MRSA can pose a risk to human health.

Preventive action against the main microorganisms that cause zoonoses within poultry farming is a determinant factor for production success, significantly reducing economic losses and treatment costs (Lee *et al.*, 2015). Prevention is even more important for public health. Since the excessive use of antibiotics has been reduced in poultry production, searching for potential alternatives to control these food-borne pathogens is still necessary.

The use of antimicrobial disinfectants in organic matter is not effective and has been found unsafe for animals (Arayan *et al.*, 2017). Research using BLA has shown an inhibitory or reducing effect on gram-negative bacteria's microbial consortia (Castellano *et al.*, 2017). The competition for adhesion sites and nutrients between disease-causing microorganisms and lactic acid producers reduces biofilm production by pathogens (Jalilsood *et al.*, 2015).

Thus, applying a protective biofilm (as a biologic control tool), acting on the main zoonotic disease agents in poultry farming and the food industry, becomes a potential tool for preventing food-borne disease, reducing economic losses. This study aimed to evaluate the efficacy of biofilm formed by BLA in the prevention and control of biofilms and planktonic forms of SG, SH, MRSA and CJ in different matrices (commonly encountered within the poultry industry).

MATERIAL AND METHODS

The experiment was performed in the Molecular Epidemiology Laboratory of the Federal University of Uberlândia, Brazil. A pilot test was performed preceding this experiment to identify the different colonies of BLA in different agars (supplemental material).

Image of BLA biofilms

The BLA (containing Lactic acid bacteria, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus pumilus*, and *Pediococcus* spp) (LalfilmPro, Lallemand SAS®) was diluted in sterile distilled water at a concentration of 10.3 log CFU/g to monitor biofilm's formation in polystyrene plates. A total of 12 wells were inoculated at a dosage rate of 9.3 log CFU of BLA/m². At 24 and 48h post-inoculation the wells were fixed with 3.7% formaldehyde (Dinâmica®) for 20 minutes. A dose of 1ug of Calcofluor white (Sigma-Aldrich®) per well was applied for 40 minutes to stain the biofilms matrix and 1uG of propidium iodide (Sigma-Aldrich®) for 10 minutes at 37°C to stain the bacterium. Plates were studied using a fluorescence microscope (EVOS FL Cell Imaging System, ThermoFisher®).

Salmonella Gallinarum, Salmonella Heidelberg, and methicillin-resistant Staphylococcus aureus

In order to evaluate the BLA performance on the selected substrates (used in poultry production), 100 grams of soil or wood shavings were sterilized. To validate this set-up as a lab-scale experimental model for pathogen biofilm formation, parallel tests were performed on polystyrene plates (96 - well microplates). The thickness was standardized to 10 cm height for wood shavings to mimic the average litter height found on Brazilian broilers farms. The substrate was placed in sterile cylindrical containers (196 cm² area) and inoculated by applying the BLA solution (9.3 log CFU of BLA/m²) with a handheld sprayer. The layer's thickness was fixed at 2cm high for soil, as this is a denser substrate. The substrate was placed in sterile rectangular shape containers (357 cm² area). The same BLA dilution and application rate was performed as described for wood shavings.

For all substrates, analysis were performed in triplicate at a temperature of 30°C, with seven containers; being three treatment containers (tested pathogen plus BLA), three positive controls (PC) for pathogenic bacteria (only the tested pathogen), and one negative control for BLA biofilms (only BLA). In polystyrene plates, we analyzed each treatment in triplicate. For the pathogenic bacteria, the substrates (soil and wood shavings) were inoculated with each bacterium species separately (2 log CFU/g) diluted peptone water (Oxoid®). For the polystyrene microplates assays, 4 log CFU/well of each bacterium species, diluted in 200 uL of peptone water, were inoculated separately.



The experiment was conducted with two separate protocols for each bacterium:

- (i) Preventive use of BLA: Tested materials were inoculated with BLA, and the tested pathogen was applied 24h after applying BLA. The material was collected 24 and 48h post-challenge. Samples of 25g (soil or wood shaving) or 100uL (polystyrene plate) were diluted in peptone water 0.1%. Then, each sample was subjected to serial dilution for plate count in XLD (Xylose Lysine Deoxycholate) (Difco®) agar (SG and SH) at 35°C during 24 hours or BP (Baird-Parker) (Oxoid®) (MRSA) at 35°C during 48 hours. Specific colonies were evaluated and counted 24 and 48h post-challenge. Five colonies with typical morphological from each plate were selected for PCR evaluation for SG and SH or phenotypic validation for MRSA (Gram staining, catalase using hydrogen peroxide (Dinâmica®), and coagulase production (using Coaguloplasma, Laborclin®)). The PCR evaluation was done by extracting the DNA of five typical colonies from each plate and, when possible, five atypical colonies, using reagents, primer pairs, amplification cycles, time, and temperatures standardized in our laboratory. For the PCR evaluation, 10 pmol of primers located in the *ompC* gene (F 5'-ATCGCTGACTTATGCAATCG-3', R 5'-CGGGTTGCGTTATAGGTCTG-3') were used as a target to identify *Salmonella*. The PCR reaction was done using the GoTaq® Green Master Mix (Promega®) at an annealing temperature of 57°C (Silva *et al.*, 2019).

In parallel, the samples were cultured to evaluate the presence or absence of the tested microorganism. To assess SG and SH's presence/absence, samples were diluted in peptone water and incubated for 24 h at 36°C. After incubation, 1 mL of this culture was added in 9 mL of Tetrathionate broth (Merck®) (incubated at 36°C, for 24 h) and 0.1 mL in Rappaport broth (Merck®) (incubated at 42°C, for 24 h). After 24h, 0.1 mL of the cultures were inoculated in XLD agar, in parallel, and incubated for another 24h at 37°C.

- (ii) Control use of BLA: First, the tested materials were inoculated with the tested pathogen. After 24h, BLA was applied. Specific colony counts for SH, SG and MRSA were performed 24h and 48h post BLA application. Direct counts and confirmation of the species were performed as described above (i). In parallel, the samples were cultured to evaluate presence or absence.

Campylobacter jejuni

Campylobacter jejuni IAL2383 analysis

Due to the difficulty of recovering viable and cultivable cells of *Campylobacter* in low humidity substrate (soil and wood shaving), this microorganism was only studied on polystyrene plates. Thus, a strain of *C. jejuni* CJ IAL2383 (Fonseca *et al.* 2014) characterized and isolated from humans, was initially used. The experiment was conducted as follows:

- (i) Preventive use of BLA: Each well of polystyrene microplate (0.4cm²) was inoculated with BLA at the concentration of 9.3 log CFU of BLA/m²). After 24h, plates were inoculated with 5.43 log CFU/mL of CJ IAL2383 per well diluted in 200uL of Mueller-Hinton Broth (Merck®) supplemented with 5% of defibrinated sheep's blood (Laborclin®). The material was collected 24 and 48h post-challenge by scraping and submitted to serial dilutions for specific counts for CJ. Direct counts were made on CCDA (Oxoid®) agar in a microaerophilic atmosphere (Kit microaerobac, Probac®) at 37°C for 48h. Five colonies with typical morphological characteristics of each plate were selected for Gram staining and PCR evaluation.
- (ii) Control use of BLA: A total of 5.23 log CFU/mL of CJ IAL2383 diluted in 200uL of Mueller-Hinton Broth supplemented with 5% of defibrinated sheep's blood was inoculated and incubated for 24h before applying 9.3 log CFU of BLA/m². After 24 and 48h of BLA inoculation, the material was collected by scraping and submitted to serial dilutions for specific direct counts for *C. jejuni* on CCDA agar in microaerophilic atmosphere at 37°C for 48h. Five colonies with morphological characteristics of each plate were selected for Gram staining and PCR evaluation.

Campylobacter jejuni 596 and 33454/1 analysis

In this experiment, lower amounts of initial inoculum of CJ for the challenge and longer inoculation times for BLA was evaluated using two field strains (596 and 33454/1) isolated from chickens. The action of BLA was assessed at 24, 48, 72, and 96h post-treatment. The experiment was performed by separately inoculating 3.0 log CFU/mL of *C. jejuni* 596 and 33454/1 diluted in 200 uL per well, 24 h after inoculation of BLA.

In order to evaluate the presence/absence of CJ, the samples were diluted in Bolton broth (Merck®) supplemented with 5% of defibrinated sheep's blood



and incubated for 48h at 37°C in microaerophilic atmosphere. After 48h, the cultures were inoculated in CCDA agar and incubated in a microaerophilic atmosphere for another 48h at 37°C. The PCR evaluation was done by extracting the DNA of 5 typical colonies of each plate, and when possible, five atypical colonies; reagents, primer pairs, and temperatures were used according to protocols standardized in our laboratory. For the PCR evaluation, we used primers located in the *flaA* gene (F 5'-ATGGGATTTCGTATTAACAC-3, R5'-CTGTAGTAATCTAAAACATTTG-3') (Hänel *et al.*, 2004). The PCR reaction was done using the GoTaq® Green Master Mix. The conditions for the reaction were: 94°C for 10 minutes, followed by 30 cycles at 94°C for 1 minute, 47°C for 1 minute, 72°C for 1 minute, and a final stage of extension at 72°C for 10 minutes, with the primer at 10 pmol.

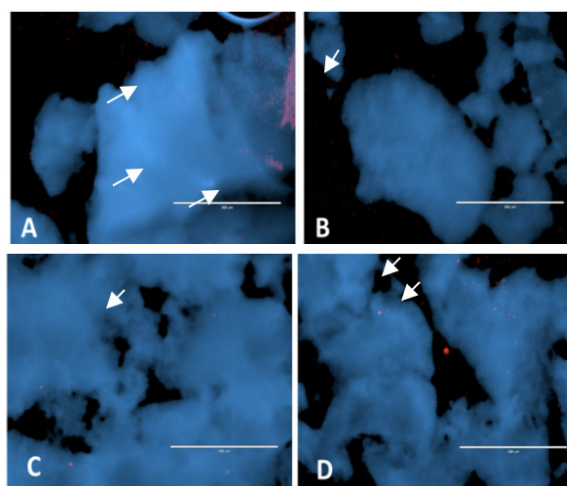
Statistical analysis

MRSA and CJ plates with 15 to 150 colonies and SG and SH plates with 25 to 250 colonies were considered for analysis. Data were transformed in logarithm base 10. The difference between the means was analyzed by test of variance followed by Tukey's Test ($p \leq 0.05$), using GraphPad Prism 7.04 program.

RESULTS

Formation of BLA biofilm

Biofilm formation on polystyrene plates was confirmed 24 or 48h after BLA inoculation by fluorescence (Figure 1). More specifically, a biofilm could already be detected within 24h, but its distribution throughout the surface was more significant after 48h.



Fluorescence microscopy of overlapping of the images of Lactic Bacteria and Bacilli biofilms grown for 24 h (A and B) and 48 h (C and D). The bacteria were stained with Propidium iodide (red) and the matrix of the biofilms was stained with Calcofluor white (Sigma-Aldrich®). It is possible to see a group of bacterium in biofilm (white arrow).

Salmonella Heidelberg analysis

In soil, SH counts increased to 8.92 log CFU/g after 48h (Table 1) in groups without BLA's preventive treatment, showing that SH can multiply in the soil. SH counts in the BLA treatment increased, compared to initial inoculum counts 24 h post-inoculation. However, SH did not increase compared to the initial inoculum counts 48h post-inoculation (table 1). In non-treated wood shavings (PC), SH counts increased compared to the initial inoculum 24 h after inoculation and continued to multiply after 48h up to 8.68 log CFU/g. In BLA pre-treated wood shavings, SH bacterium was below the detection limit at 24 and 48 h post-inoculation (Table 1). SH counts increased on polystyrene plates compared to the initial inoculum 24 and 48h post-inoculation up to 7.27 log CFU/g in PC group (table 1). On polystyrene plates pre-treated with

Table 1 – Means of *Salmonella* Heidelberg colony counts in different matrices with and without preventive treatment with BLA.

	Initial inoculums	PC	NC	24h				48h			
				T	PC	NC	T	PC	NC	T	
Soil (log CFU/g)	2.00 ^a ± 0.00	8.74 ^b ± 0.32	0.00 ^c ± 0.00	8.47 ^b 0.47	8.92 ^b 0.47	0.00 ^c ± 0.00	2.9 ^a ± 0.97				
Wood shavings (log CFU/g)	2.00 ^a ± 0.00	7.53 ^b ± 0.19	0.00 ^c ± 0.00	1.00 ^{*d} ± 0.00	8.68 ^b 0.23	0.00 ^c ± 0.00	1.00 ^{*d} ± 0.00				
Polystyrene Plates (log CFU/well)	4.00 ^a ± 0.00	6.01 ^b ± 0.50	0.00 ^c ± 0.00	3.42 ^a ± 0.43	7.27 ^b ± 0.11	0.00 ^c ± 0.00	3.54 ^a ± 0.31				

Different letters on the same line represent statistical difference ($p < 0.05$). PC = positive control; NC = negative control; T= treatment. *CFU = 1: There was no bacterial count at all dilutions. This means that we standardize a number of bacteria considered below the detection limit = 1 logCFU/g.

Table 2 – Means of *Salmonella* Heidelberg colony counts with the use of BLA after SH inoculation in different matrices.

	Initial inoculum	PC 24h	NC 24h	48h			
				T 24h	PC 48h	NC 48h	T 48h
Soil(log CFU/g)	2.00 ^a ± 0.00	9.46 ^b ± 0.03	0.00 ^c ± 0.00	9.07 ^b ± 0.06	8.93 ^b ± 0.06	0.00 ^c ± 0.00	9.16 ^b ± 0.25
Wood shavings (log CFU/g)	2.00 ^a ± 0.00	10.58 ^b ± 0.26	0.00 ^c ± 0.00	10.79 ^b ± 0.11	10.83 ^b ± 0.12	0.00 ^c ± 0.00	10.51 ^b ± 0.04
Polystyrene Plates (log CFU/well)	4.00 ^a ± 0.00	7.08 ^b ± 0.00	0.00 ^c ± 0.00	7.23 ^b ± 0.82	8.95 ^b ± 0.00	0.00 ^c ± 0.00	7.91 ^b ± 0.53

Different letters on the same line represent statistical difference ($p < 0.05$). PC = positive control; NC = negative control; T= treatment.



BLA, SH colony count did not increase after 24h (3.42 log CFU/well) or 48h (3.54 log CFU/well) (table 1).

Table 2 shows the results of the application of BLA 24h after the inoculation of SH. The results show that SH colonies' counts increased both in the challenged positive control and treatment groups versus the negative control group for soil and wood shavings. For polystyrene plates, at 48h post-inoculation, a difference between the positive control and the treated plates was observed (8.95 vs. 7.91 log CFU/well).

Salmonella Gallinarum (SG) analysis

Table 3 shows the results for the use of BLA in preventing the growth of SG. In soil, the SG counts increased compared to the initial inoculum 24 and 48h post-inoculation in the PC group and the BLA-treated

group 24 h post-inoculation. However, 48h after BLA inoculation, the SG colony count was lower for the BLA treated substrate than the positive control (8.49 vs. 6.86 log CFU/g).

In the wood shavings, SG colony count also increased compared to the initial inoculum after 24 and 48h for the positive control. The BLA was adequate to prevent the growth of SG, which remained below the limit of detection in the treated groups at 48 h post-inoculation (Table 3).

The bacteria also increased on polystyrene plates compared to the initial inoculum 24 and 48h post-inoculation in the PC group. However, when BLA was applied 24 h before SG, it was below the detection limit 48 hours after the challenge for this group (table 3).

Table 3 – Mean of *Salmonella Gallinarum* colony counts with preventive treatment of BLA in different matrices.

	Initial inoculum	PC 24h	NC 24h	T 24h	PC 48h	NC 48h	T 48h
Soil (log CFU/g)	2.00 ^a ±0.00	8.56 ^b ±0.19	0.00 ^c ±0.00	7.78 ^b ±0.19	8.49 ^b ±0.34	0.00 ^c ±0.00	6.86 ^d ±0.77
Woods shavings (log CFU/g)	2.00 ^a ±0.00	4.64 ^b ±0.13	0.00 ^c ±0.00	3.58 ^b ±0.32	4.68 ^b ±0.60	0.00 ^c ±0.00	1.00 ^{*d} ±0.00
Polystyrene Plates (log CFU/well)	4.00 ^a ±0.00	6.05 ^b ±0.12	0.00 ^c ±0.00	3.17 ^d ±0.24	8.50 ^e ±0.17	0.00 ^c ±0.00	1.00 ^{*f} ±0.00

Different letters on the same line represent statistical differences ($p < 0.05$). PC = positive control; NC = negative control; T= treatment. *CFU = 1 log: There was no bacterial count at all dilutions. This means that we standardize a number of bacteria considered below the detection limit = 1 log CFU/g or well.

When BLA was applied as a treatment measure, i.e., 24 hours after the inoculation of the pathogen (Table 4), the results showed less difference related to positive control than the preventive approach. In soil, results show that SG counts increased both in the challenged positive control group and treatment group compared to the negative control. The bacteria multiplied in the wood shavings compared to the initial inoculum after

24 h, and it continued to multiply after 48 h post-inoculation in PC. BLA product reduced SG's growth since the number of bacteria was below the positive control at 24 h and 48h post-inoculation. Inoculation with BLA did not control the SG on polystyrene plates when applied after the pathogen challenge (table 4).

All typical colonies were confirmed to be *Salmonella* spp. by PCR (supplementary figure 1A).

Table 4 – Means of *Salmonella Gallinarum* colony counts with the use of BLA after SG inoculation.

	Initial inoculums	PC 24h	NC 24h	T 24h	PC 48h	NC 48h	T 48h
Soil (log CFU/g)	2.00 ^a ±0.00	8.32 ^b ±0.56	0.00 ^c ±0.00	8.10 ^b ±0.24	8.32 ^b ±0.56	0.00 ^c ±0.00	7.34 ^b ±0.67
Wood shavings (log CFU/g)	2.00 ^a ±0.00	6.32 ^b ±0.31	0.00 ^c ±0.00	4.06 ^d ±0.06	7.04 ^b ±1.08	0.00 ^c ±0.00	3.67 ^d ±0.18
Polystyrene Plates (log CFU/well)	4.00 ^a ±0.00	6.64 ^b ±0.80	0.00 ^c ±0.00	6.40 ^b ±0.34	8.52 ^d ±0.33	0.00 ^c ±0.00	8.31 ^d ±0.17

Different letters on the same line represent statistical differences ($p < 0.05$). PC = positive control; NC =negative control; T= treatment.

Methicillin-resistant *Staphylococcus aureus*

Table 5 shows the results of the use of BLA in preventing the growth of MRSA. The bacteria multiplied compared to the initial inoculum 24h and 48h post-inoculation in the PC group on all substrates. MRSA also multiplied in the BLA treated group, but its growth was lower than in PC 48h post-inoculation (7.16 vs. 6.68 log CFU/g) in soil. The BLA reduced MRSA's growth compared to the PC at 24h

post-inoculation in the wood shavings. In polystyrene plates, BLA decreased MRSA's growth compared to the PC at 24 and 48 h post-inoculation, respectively (Table 5).

When the MRSA challenge was applied 24h before applying BLA, the bacteria increased compared to the initial inoculum 24h and 48h post-inoculation in all matrices (Table 6). All strains isolated from the tested substrates were confirmed as SA.



Table 5 – Means of MRSA colony counts with preventive treatment with BLA.

	Initial inoculum	PC 24h	NC 24h	T 24h	PC 48h	NC 48h	T 48h
Soil (log CFU/g)	2.00 ^a ± 0.00	7.31 ^b ± 0.18	0.00 ^c ± 0.00	7.73 ^b ± 0.07	7.16 ^b ± 0.33	0.00 ^c ± 0.00	6.68 ^d ± 0.21
Wood shavings (log CFU/g)	2.00 ^a ± 0.00	4.65 ^b ± 0.41	0.00 ^c ± 0.00	3.38 ^d ± 0.24	5.45 ^b ± 0.09	0.00 ^c ± 0.00	4.42 ^{bd} ± 0.26
Polystyrene Plates (log CFU/well)	4.00 ^a ± 0.00	10.07 ^b ± 0.17	0.00 ^c ± 0.00	6.53 ^d ± 1.69	10.55 ^b ± 0.17	0.00 ^c ± 0.00	7.16 ^d ± 0.61

Different letters on the same line represent statistical differences ($p < 0.05$). PC = positive control; NC = negative control; T= treatment. *CFU = 1 log: There was no bacterial count at all dilutions. This means that we standardize a number of bacteria considered below the detection limit = 1log/CFU.

Table 6 – Means of MRSA colony counts with the use of BLA after *S. aureus* inoculation.

	Initial inoculum	PC 24h	NC 24h	T 24h	PC 48h	NC 48h	T 48h
Soil(log CFU/g)	2.00 ^a ± 0.00	9.64 ^b ± 0.36	0.00 ^c ± 0.00	9.60 ^b ± 0.13	8.65 ^d ± 0.69	0.00 ^c ± 0.00	8.87 ^d ± 0.36
Wood shavings (log CFU/g)	2.00 ^a ± 0.00	5.34 ^b ± 0.99	0.00 ^c ± 0.00	4.57 ^b ± 1.27	6.45 ^b ± 0.85	0.00 ^c ± 0.00	4.547 ^b ± 1.21
Polystyrene Plates (log CFU/well)	4.00 ^a ± 0.00	10.0 ^b ± 0.22	0.00 ^c ± 0.00	10.06 ^b ± 0.05	8.824 ^b ± 1.09	0.00 ^c ± 0.00	8.438 ^b ± 1.10

Different letters on the same line represent statistical differences ($p < 0.05$). PC = positive control; NC = negative control; T= treatment. *CFU = 1 log: There was no bacterial count at all dilutions. This means that we standardize a number of bacteria considered below the detection limit = 1 log/CFU.

Campylobacter jejuni

Table 7 shows the results of BLA's use in preventing and controlling the growth of CJ IAL2383 isolated from the human on polystyrene plates. CJ IAL2383 multiplied compared to the initial inoculum 24 and 48 h post-inoculation without difference between the control and treatment groups (table 7). When the amount of initial inoculum was decreased from log 5

CFU to 2 CFU/well and additional time points were added further in time (72 and 96 h post-inoculation), in the positive control groups, CJ strains increased with time (table 8). The preventive treatment with BLA decreased the colony count (5.64 vs. 3.90 log CFU/well) for the CJ 596 strain only 96 h post-inoculation. For the CJ 33454/1 strain, BLA treatments already showed a lower count of *Campylobacter* at 48, 72, and 96h post-inoculation, respectively (table 8).

Table 7 – Mean of colony counts of *Campylobacter jejuni* IAL2383 inoculated before and after BLA in polystyrene plate.

	Initial inoculum	PC 24h	NC 24h	T 24h	PC 48h	NC 48h	T 48h
C.jejuni IAL2383 before BLA	5.43 ^a ± 0.02	8.3 ^b ± 0.82	0.00 ^c ± 0.00	8.26 ^b ± 1.16	8.00 ^b ± 0.32	0.00 ^c ± 0.00	7.93 ^b ± 0.12
C.jejuni IAL2383 After BLA	5.23 ^a ± 0.01	5.29 ^a ± 0.30	0.00 ^c ± 0.00	6.15 ^{ab} ± 0.53	6.65 ^b ± 0.69	0.00 ^c ± 0.00	6.89 ^b ± 0.55

Different letters on the same line represent statistical differences. PC = positive control; NC = negative control; T= treatment. C.jejuni IAL2383 before BLA: The strain was inoculated 24 hours before BLA treatment. C.jejuni IAL2383 after BLA: The strain was inoculated 24 after BLA treatment.

Table 8 – Mean of colony count of different strains of *Campylobacter jejuni* at 24, 48, 72 and 96h post preventive treatment with BLA in polystyrene plates.

	li	PC 24h	NC 24h	T 24h	PC 48h	NC 48h	T 48h	PC 72h	NC 72h	T 72h	PC 96h	NC 96h	T 96h
<i>C. jejuni</i> 596	3.0 ^a ± 0.00	4.73 ^{bd} ± 0.24	0.00 ^c ± 0.00	4.6 ^{bd} ± 0.41	5.75 ^b ± 0.80	0.00 ^c ± 0.00	5.95 ^b ± 0.59	4.69 ^{bd} ± 0.45	0.00 ^c ± 0.00	4.9 ^{bd} ± 0.65	5.64 ^b ± 1.20	0.00 ^c ± 1.00	3.90 ^{ad} ± 0.06
<i>C. jejuni</i> 33454/1	3.0 ^a ± 0.00	4.51 ^{bde} ± 0.41	0.00 ^c ± 0.00	4.12 ^{be} ± 0.16	5.17 ^d ± 0.16	0.00 ^c ± 0.00	3.78 ^{ae} ± 0.57	4.83 ^{bd} ± 0.37	0.00 ^c ± 0.00	3.73 ^{ae} ± 0.64	4.28 ^{bd} ± 0.00	0.00 ^c ± 0.00	3.26 ^a ± 0.07

Different letters on the same line represent statistical differences ($p < 0.05$). PC = positive control; NC = negative control; T= treatment; li: Initial inoculum.

DISCUSSION

Lactic Bacteria and Bacilli biofilms

In this study, biofilm formation is illustrated by immunofluorescence (Figure 1) 24 and 48 h post-application of the BLA using a polystyrene plate as an experimental model. Biofilm matrix was more extensive at 48 h compared to 24 h. This result aligns with our expectations because time is an essential factor for bacteria's organization in biofilms (Merino *et al.* 2019).

Biofilms are mainly formed by extracellular polymeric substances (EPS) self-produced by microorganisms. These EPS are mostly polysaccharides, proteins, nucleic acids, and lipids, which are essential for the stability of the biofilms, adhesion to surfaces, and responsible for the organized network formation of biofilm cells (Gialamas *et al.* 2010). Calcofluor white (CFW) binds β 1-3 and β 1-4 carbohydrate (Workman *et al.* 2005), common EPS within the biofilm matrix and cannot penetrate intact cell membranes so does not stain



viable cells (Mason *et al.* 1995). In figure 1 it is possible to see the biofilm of EPS formed with 24 and 48 hours.

Lactic acid bacteria can form protective biofilms on surfaces used in the food industry to control pathogenic microorganism colonization (Yang *et al.* 2014). Competition for nutrients and/or space and the production of antimicrobial compounds by lactic acid bacteria and *Bacilli* could explain the BLA biofilms' mode of action for inhibiting pathogenic bacteria growth (Ahmad *et al.*, 2017). In the current study, biofilm formation by BLA was visually confirmed before the challenge trials were performed.

Salmonella Heidelberg analysis

We used two strategies in this study; preventive and control use of BLA on SH's growth. Success was recorded mainly for BLA's prophylactic use since a large decrease in SH count was observed in all tested substrates, especially soil and wood shavings. It is interesting to mention that decreased growth was more pronounced after 48 h than 24 h (table 1). This event probably occurred because BLA needs at least 48h to form a stable biofilm, as was observed in the first part of this study.

The application of BLA 24 h after SH infection did not prove to be as efficient as the preventive strategy (table 2). It can be hypothesized that *Salmonella* had multiplied rapidly before the application of BLA and already formed a biofilm, as *Salmonella* is reported to do in adverse environments with low nutritional availability (Wang *et al.* 2013). The lesser effect of BLA on SH growth in polystyrene plates can be explained by the biofilm formation capacity in each substrate type (Dhakal *et al.* 2019).

Besides demonstrating the protective effect of BLA against SH growth, this study's results also show the survival and multiplication of SH in matrices present in farms such as soil and wood shavings (table 1 and 2). To our knowledge, this is the first time that this is demonstrated for SH. *Salmonella* may be challenging to control on broiler farms, especially SH, which has a high prevalence in poultry houses (Deblais *et al.* 2018). These bacteria may remain present on-farm via biofilms, even in harsh environments such as soil.

Salmonella Gallinarum analysis

The positive control results indicate that SG survived and multiplied in wood shavings, polystyrene plates, and soil (table 3). Although SG seems to be more adapted to the poultry gastrointestinal tract since it is a specific birds' pathogen (Foley *et al.* 2011), this

microorganism can form biofilms (Silva *et al.* 2019), making it more challenging to eliminate from the farm environment.

BLA prevented SG's multiplication very efficiently since, in the treated group, SG was below the detection limit in wood shavings and polystyrene plates within 48h post-inoculation. However, in soil, BLA was not so effective in preventing SG growth (table 3). This event probably occurred because soil might be an ideal environment for SG survival due to minerals such as iron (Andino & Hanning, 2015), making it more resistant to other bacterial competition such as biofilm bacteria. However, other studies are necessary to further understand these observations. In Brazil, many broiler farms still have soil instead of cement under the poultry litter, meaning vigilant monitoring for SG in this environment may be essential to control pathogen populations (Rogeri *et al.* 2016). When SG is inoculated 24h before applying BLA, the decrease of SG was only observed in wood shavings (table 4). As for SH, BLA's effectiveness as a preventative measure to reduce SG growth is more significant than a control measure. We speculate that this may have happened because establishing a BLA biofilm community before the entry of pathogenic bacteria does not allow for SG and SH's multiplication. However, further studies are needed to understand this mechanism better.

This study shows that BLA effectively prevents SG and SH growth on substrates commonly encountered on poultry farms (soil and wood shavings) and in polystyrene plates (experimental model). BLA effectively formed biofilm on the surfaces studied previously and decreased the pathogen's multiplication, indicating a protection mechanism and even a competition between these microorganisms (Gómez *et al.* 2016). The bacteria present in BLA can act synergistically with antimicrobial agents, enhancing bacteriocins production (Viedma *et al.* 2010; Gómez *et al.* 2012), controlling the pathogens. In practice, this means that BLA should be used as soon as possible after cleaning and disinfecting the environment to avoid SG or SH colonization.

Methicillin-resistant Staphylococcus aureus

When BLA was applied before inoculation with MRSA, a decrease in growth was observed in polystyrene plates, wood shavings, and soil, although not as effective as SG and SH reduction (table 5). BLA was not adequate for MRSA's control when the MRSA was applied before BLA for all studied substrates (table 6).



It is known that MRSA is an efficient biofilm forming (Wang *et al.* 2018). This bacterium builds its biofilm through surface molecules that promote the formation of an extracellular matrix, which protects them from harm and contributes to longer-lasting colonization (Otto, 2008). This could explain why BLA was less effective in preventing the growth of MRSA. The reduction of MRSA when BLA was inoculated before the pathogen is probably due to the competition between the microorganisms for adhesion/attachment sites, nutrients, and the secretion of inhibitory substances from BLA (Fahad & Radeef, 2011).

MRSA resists synthetic penicillins (methicillin, oxacillin, and nafcillin), cephalosporins, erythromycin, clindamycin, aminoglycosides, and quinolones (Sikorska & Smoragiewicz, 2013). Therefore, the use of alternatives to these compounds when treating and preventing MRSA is imperative. Using BLA within the poultry industry as a preventive method could allow better control over MRSA contamination.

Campylobacter jejuni

The effectiveness of BLA to prevent CJ growth using a polystyrene plate as an experimental model seems to be dependent on the initial quantity of inoculum and tested strain. When using one strain isolated from humans with an initial inoculum of approximately 5 log CFU/well, BLA could not prevent or control the growth of CJ (table 7). When CJ IAL2383 was inoculated after BLA growth, there was no bacterial multiplication after 24 hours. But when IAL was inoculated before the BLA, the CJ multiplied (table 7). This indicates that the presence of BLA inhibits the early multiplication of CJ. But this is not maintained because after 48 hours, CJ was able to multiply.

When fewer inoculum was used (3 log CFU/well) for two strains isolated from chickens, CJ decreased from 48 to 96h (table 8). The behavior of CJ seems to be strain-dependent in different situations (Melo *et al.* 2016). This bacterium is difficult to control in the farm and food industry (Doyle, 2018). So, normally different control strategies are necessary to decrease the contamination index (Techaruvichit *et al.* 2016).

For all microorganisms studied, the results show that BLA should be used in the poultry industry as a preventive method to control the pathogens. The mechanisms involved with the inhibition of pathogens may be related to several factors, for example, the secretion of organic acids, bacteriocins, and biosurfactants by the BLA (Gálvez *et al.* 2010; Kanmani *et al.* 2013). BLA form biofilm within which

it can survive, multiply and produce bacteriocins such as niacin and pediocin (Henderson *et al.* 1991; Stevens *et al.* 1991; Fimland *et al.* 2000; Drider *et al.* 2006), subtilin and subtilisin (Jansen & Hirschmann, 1944; Zheng & Slavik, 1999; Joseph *et al.* 2013) which are active against pathogenic bacteria. Competition for binding sites and bacteriocins production in high quantity may be responsible for inhibiting or controlling the multiplication of pathogens studied in this research. Future studies are essential to know what mechanism BLA uses to impede the growth of the bacteria tested in this manuscript.

CONCLUSION

This study suggested that SH and SG can survive and multiply in substrates such as wood shavings and soil. The preventive use of BLA is efficient in controlling the multiplication of SH and SG. The efficiency of BLA in preventing CJ growth seems to be related to the initial CJ contamination. The use of BLA after traditional disinfection methods on farms or even in the food industry may help decrease the studied pathogens. Thus, BLA can become an excellent complement to disinfectant use in animal production and the food industry.

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SUPPLEMENTAL MATERIAL

Pilot test

The formulation *LalfilmPro* (Lallemand SAS®) containing Lactic acid bacteria, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus pumilus*, and *Pediococcus* spp. was used in this research. To evaluate bacteria's growth and characteristics in the agar and substrate used, the BLA was tested in different substrates, agars, and conditions for each bacterium pathogenic before the experiment. The agar tested were: CCDA, Preston (Oxoid®), BP, MRS(Man, Rogosa e Sharpe) (Merck®), XLD.



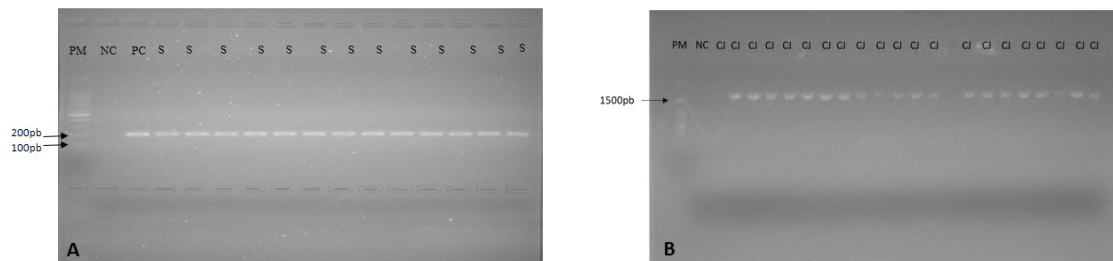
To assess biofilm formation capacity, the BLA was diluted in sterile water and inoculated in a polystyrene plate (96 wells) at a dosage of 9.3 log CFU of BLA/m² (200 μ L per well). A similar procedure was carried out with negative control using just sterile water. The analysis was performed in triplicate. After 24 and 48 hours, the water with BLA was discarded, and the polystyrene plates were washed three times (one minute each under agitation) to remove non-adherent bacteria. To collect the adherent bacteria, the plates were scraped for 90 seconds. A total of 100 μ L of the suspension was placed on the surface of the MRS (Merck®) agar for the colonies' count and Gram staining.

The BLA was diluted in sterile water and sprayed on previously sterilized soil and wood shavings (collected from a broiler chicken farm after seven production cycles during a period of sanitary vacuum) at a dosage of 9.3 log CFU of BLA/m². A similar procedure was carried out for the negative control using just sterile water. The test was performed in triplicate. After 24 and 48 hours, 25 g of each substrate was weighed and diluted in 225 mL of sterile water. Serial dilutions were performed and plated on agar CCDA and Preston (Oxoid®) and incubated at 42 °C for 48 hours under microaerophilic

conditions. The same procedure was performed on BP and XLD agars at 36 °C for 24 hours under anaerobic atmosphere. The colonies were identified based on a visual inspection and Gram staining.

On MRS (Merck®) agar, colonies of two shades, yellow or caramel, were detected, and by Gram staining, there were gram-positive cocci and *Bacilli*. On Preston (Oxoid®) agar, in microaerophilic condition, the colonies considered typical were those of grayish color, shiny, with the appearance of drops of water. This kind of colony was very similar to *Campylobacter* and by Gram staining, it was observed cocci gram-positive (probably *Pediococcus* that can grow in microaerophilic conditions). In CCDA agar, there is cefoperazone that is an antibiotic that inhibits Gram-positive bacterium, but in Preston (Oxoid®) agar, there are only polymyxin B and cycloheximide that inhibit gram-negative bacteria. In XLD and CCDA agar, no bacterium growth was observed probably because as well as in CCDA, XLD contains sodium deoxycholate. The colonies were small, light brown, and opaque on BP agar, which is a kind of colony different from MRSA. Due to these results, CCDA, XLD, and BP agar were used to isolate CJ, *Salmonella* (SH and SG) and MRSA respectively.

Supplementary figure 1



A. PCR to presence of genes *ompC* to *Salmonella* spp. Amplicon (PB): 204. PM = 100-bp molecular weight marker. B. PCR to the presence of genes *flaA* gene to *Campylobacter jejuni*. Amplicon (PB): 1728. PM = 100-bp molecular weight marker NC: negative control. PC: positive control. S: *Salmonella*. CJ: *Campylobacter jejuni*.

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