Selection of Bacillus subtilis for animal and chicken embryo supplementation

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Abstract

Bacillus subtilis (B.S.) has been used as an excellent probiotic; however, some B.S. strains seem to be opportunist pathogens or do not present inhibitory effects in the pathogenic bacterium, so the characterization of B.S. strains for use in animals is mandatory. This study aimed to select nonpathogenic strains of B.S., which have beneficial effects on birds and can inhibit *Salmonella* spp., avian pathogenic *Escherichia coli coli* (APEC) and *Campylobacter jejuni* (C.J.). We tested nine (9) strains of B.S. isolated from several sources (named A to I) in *in vitro* by tests of mucin degradation activity, haemolytic activity, apoptosis, and necrosis in fibroblasts from chickens. After the *in vitro* test, we tested the remaining seven (7) strains (strains A to G) in a chicken embryo (C.E.) as an *in vivo model* and target animal. We inoculated 3 log CFU/CE of each strain via allantoic fluid at the 10th day postincubation (DPI). Each treatment group consisted of eight C.Es. At the 17th DPI. We checked C.E. mortality, gross lesions, C.E. weight, and whether B.S. strains were still viable. To perform the cytokine, total protein, albumin, and reactive C protein analysis, we collected the C.E. blood from the allantoic vessel and intestine fragments in the duodenum portion for histomorphometric analysis. After the results in C.Es., we tested the inhibition capacity of the selected B.S. strains for diverse strains of *Salmonella* Heidelberg (S.H.), *S.* Typhimurium (S.T.), *S.* Enteritidis (S.E.), *S.* Minnesota (S.M.), *S.* Infantis (S.I.), *Salmonella* var. monophasic (S.V.M) and *C. jejuni*. After the *in vitro* trial (mucin degradation activity, haemolytic activity, apoptosis, and necrosis), we removed two (2) strains (H and I) that showed β-haemolysis, mucin degradation, and/or high apoptosis and necrosis effects. Although all strains of B.S. were viable in C.Es. at the 17th DPI, we removed four (4) strains (A, B, D, F) once they led to the highest mortality in C.Es. or a high albumin/protein ratio. *C. jejuni* inoculated with strain G had greater weight than the commercial strain, which could be further used for egg inoculation with benefits to the C.E. Moreover, the cytokine analysis indicated that strains E and G have immunomodulatory effects on C.Es. From the tests in C.Es., we selected the strains C, E, and G for their ability to inhibit pathogenic strains of relevant foodborne pathogens. We found that the inhibition effect was strain dependent. In general, strains E and/or G presented better or similar results than commercial control strains in the inhibition of S.H., S.T., S.I., APEC and two (2) strains of C.J. In this study, we selected B.S. strains C, E and G due to their *in vitro* and *in vivo* safety and beneficial effects. In addition, we emphasize the value of C.E. as an *in vivo* experimental model for assessing B.S.'s safety and possible benefits for poultry and other animals.

1. Introduction

Probiotics are defined as live microorganisms that, when administered correctly, confer a health benefit to the host [1]. *Bacillus subtilis* (B.S.) is a Gram-positive rod that can be isolated from multiple terrestrial and aquatic environments, making it appear that this species is ubiquitous and broadly adapted to several settings on the planet [2].

Probiotics confer health benefits to poultry because they affect microbiota composition by restoring microbial homeostasis, reducing gut permeability due to the mucosal barrier, and modulating their immune response, which reduces local inflammation. B.S. is one of the most common bacterial species used in commercial probiotics in the U.S. [3], including in poultry [4, 5]. B.S. has a noticeable advantage over *Lactobacillus* and *Bifidobacterium* as probiotics because of its ability to sporulate to endure environmental stress, preparation conditions, and application processes. Moreover, B.S. tolerates low pH, bile salts, and other harsh conditions of the gastric environment. It also maintains viability and desirable characteristics within the gastric tract and has the ability to form biofilms to release biochemical compounds [6–12]. B.S. has been gaining more importance in recent years in poultry production, as several studies have shown its successful application in replacing antibiotic use to regulate gut flora. In addition, there is evidence that B.S. can improve growth performance, enhance immunity and gut health and reduce *Salmonella* spp. counts in challenged broilers [5, 13–15].

In the selection of B.S. strains to be used in feed for animals, one must address two factors regarding safety: the antibiotic resistance genes they could transfer and their toxin production capacity. Notably, the possibility of transferring genes of antibiotic resistance may pose a risk of increasing the presence of antibiotic resistance in bacteria of human and animal organisms. Previous research has shown B.S. strains harbouring mobile, extrachromosomal elements, such as plasmids with *erm(C)* or *tet(L)* genes, coding for macrolide or tetracycline resistance, respectively, and conjugative transposons Tn5397, carrying genes for tetracycline resistance *tet(M)* [16].

Bacillus sp. strains are well known to produce toxins, such as haemolysins, phospholipases, and other enterotoxins. Some of the toxins produced by *Bacillus* sp. are haemolysin, lecithinase, emetic toxins, diarrhoeal toxin, the B component, which is dermonecrotic, and the enterotoxins EntFM and CytK, which are associated with necrotic enteritis [17, 18]

Because of the factors cited above, the characterization of B.S. for probiotic use is mandatory. This manuscript aimed to select nonpathogenic strains of B.S. that have beneficial effects on birds and can inhibit *Salmonella* spp. and *Campylobacter jejuni* (C.J.) using chicken embryos (C.Es.) as an experimental model. Thus, this work makes a valuable contribution by proposing the selection of B.S. strains *in vitro* and in an *in vivo* experimental model and is valid for use in newborn animals and chicken embryos.

2. Methods

2.1. Ethics statement

The experiment occurred at the Laboratory of Infectious Diseases, Poultry Incubation, and Dr. Luiz Ricardo Goulart Filho Nanobiotechnology Laboratory of the Universidade Federal de Uberlândia (UFU). The Ethics Committee on Animal Use of the UFU certified under protocol number 11/2022/CEUA/PROPP/REITO, PROCESSO N°23117.023808/2022-77.

2.2. Strains of *Bacillus subtilis*

The trial consisted of 9 strains of B.S. isolated from diverse sources, deposited by Fundação Oswaldo Cruz (Fiocruz) and kindly donated by Dr. Leon Rabinovitch. The strains were isolated in the 1990s and named as follows: strain 220 (rabbit faeces), 32 (soil), 118 (soil), 122 (soil), 144 (soil), 207 (sand), 1273 (feathers and decomposing chicken feather meal), 1733 (soil), 1516 (coffee plantation) and one commercial probiotic of BS (DSM 17299) as a positive control. In this article, to facilitate the visualization of figures and tables, the strains were named in capital letters as follows: strain 220 (A), 32 (B), 118 (C), 122 (D), 144 (E), 207 (F), 1273 (G), 1733 (H), 1516 (I).

2.3. Mucin degradation activity

To test mucin degradation, we evaluated 9 strains of B.S. (A-I) and a commercial probiotic (DSM 17299) as a positive control, according to a previously published method [19] with few modifications. Briefly, we spotted 10 µL of each B.S. overnight-grown culture on Luria-Bertani agar plates (1.5% peptone, 1% yeast extract, 1% NaCl, and 1.5% granulated agar, pH 7) supplemented with 0.3% hog gastric mucin-type III (Sigma–Aldrich). We left the plates in the hood to dry and incubated them at 37°C for 48 h. Afterwards, we stained the plates by adding 1 mL of 0.1% amido black (Sigma–Aldrich) in 3.5 M acetic acid for 15 min and then discoloured them with 1.2 M acetic acid (Synth). We recorded mucin degradation activity as positive when we observed a clear lysis zone towards the grown colonies.

2.4. Haemolytic activity

The haemolytic activity of the isolates was analysed in duplicate according to [20] with some modifications. We grew 10 µL of B.S. in overnight cultures and spotted colonies on tryptic soy agar plates (Kasvi) supplemented with 5% defibrinated sheep blood. The plates stayed open in the hood to dry; subsequently, we incubated them at 37°C for 48 h. We identified haemolysis activity by observing the presence of either a clear zone (β-haemolysis), greenish zone (α-haemolysis), or absence of haemolysis (γ-haemolysis) around the colonies.

2.5. Apoptosis and necrosis in a different strain of B.S. in cellular culture

We used a primary fibroblast culture from C.E. First, apoptosis and necrosis were verified. We removed the chorioallantoic membrane from C.E. Fourteen days post-incubation (DPI), the cells were obtained by cutting and placing them in a 0.25% trypsin solution under stirring for 10 minutes. Then, after the decantation of larger materials, we transferred the supernatant to a solution composed of 199 (Gibco™), 20% foetal bovine serum (FBS) (LGC), 1% of the antibiotic mixture (AM) composed of amphotericin B (250 µg/mL), gentamicin (50 mg/mL), streptomycin (10,000 µg/mL) and penicillin (10,000 IU/mL) (Sigma–Aldrich). We centrifuged the cells (1500xg for 10 minutes) (Eppendorf®), resuspended the pellets in a solution composed of 199 (Gibco™), 10% FBS, and 1% AM, and filtered the material through sterile gauze. The quantification of the filter was obtained in a Newbauer chamber. In each well, we seeded 1.5×10^3 cells. After 48 hours at 37°C and 5% CO2, we inoculated each strain of B.S. (A-I) and DSM17299. In parallel, we used a negative control (N.C.) without bacterium and positive control inoculated with 2 logUFC/well of Avian Pathogenic *E. coli* (APEC, serotype B2, isolated by us from a sick bird treated at the veterinary hospital of the Federal University of Uberlândia). For each bacterium or control, we performed eight replicates per strain. We incubated the cells again for 18 and 36 hours, washed them three times with PBS, and treated them with Yo Pro-01 (Y.P.) (Invitrogen) and propide iodate (P.I.) (Sigma) (1:1000 each) for 30

minutes at room temperature. Then, we washed the wells three times and fixed them with 4% formalin for 10 minutes. After that, we treated the cells with 4',6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich) to mark the cell DNA. To measure the intensity of fluorescence, we used a Fluorescence Microplate Reader (FLx800™, BioTek® Instruments, Inc.) using the following filters: DAPI (λex 360/40 nm; λem 460/40 nm), I.P. (λex 485/20 nm; λem 615/16 nm) and Y.P. (λex 485/20 nm; λem 428/20 nm). We subtracted the result of the intensity of fluorescence obtained from each sample from the results of the wells containing only buffer (negative control). We used the Gen5 program (BioTek® Instruments, Inc.) to evaluate the marked cells with DAPI (total cells), I.P., and Y.P. and registered cell destruction (dead and detached cells from the plaque) by the results of cells labelled with DAPI, in relative apoptosis by (YP-IP)/DAPI*100 and necrosis by (IP/DAPI)*100.

2.6. Virulence analysis of different B.S. strains in an *in vivo* model

To evaluate the harmlessness of the B.S. strains selected in the *in vitro* tests, we used 10-day incubation C.Es., a more elaborate organism than cells but more fragile than birth animals. Therefore, the test in this model allowed us to choose highly safe strains. Furthermore, the selected strains in C.Es. can be used as an *in ovo* preventive method in the poultry industry. In the poultry industry, C.Es. are inoculated at 18 DPI. In our manuscript, we used C.Es. at 10 DPI and collected at 17 DPI, so there was time to assess the response still during embryonic life. Thus, we propose, in addition to the use of B.S. in embryos, a model for probiotic selection.

Preparation and inoculation of the eggs

After the previous analysis, we excluded strains H and I from the following tests and evaluated the *in vivo* virulence of B.S. strains (A-G and the control DSM 17299) in C.E. based on different parameters. The C.E. virulence test also included a negative control (without infection) and positive control (inoculated with a strain of *Salmonella* Pullorum (S.P.) isolated by us from a chicken treated at the veterinary hospital of the Federal University of Uberlândia). We used 8 C.Es., and the eggs of laying hens (*Gallus gallus*), line Hy-Line W-36, were kindly donated by Hy-Line do Brazil (Uberlândia, Brazil). Before analyses, we submitted the eggs to a white-light ovoscopy at 10 DPI of incubation to guarantee their quality and embryogenic development. Then, in a laminar flow, we disinfected the eggs and inoculated 3 log CFU/CE of each strain of B.S. in the allantoic fluid. S.P. kills C.Es. at 10 DPI of incubation, and as the C.Es. must be functional for the biochemical and cytokine analysis, we inoculated the P.C. at 10 (positive death control) and 12 DPI (control of laboratory analysis). After inoculation, we incubated the C.Es. at 37.5°C and 58% relative humidity (R.H.). At 17 DPI, we checked the C.E. mortality, damage, weight, and whether the strains were alive and collected blood by the allantoic vessel to perform cytokine analysis, quantification of total protein, albumin, and reactive C protein. We fixed the collected fragment of the intestine (duodenum) in 4% formalin for further histomorphometric analysis.

2.6.1. Mortality and damage analysis in C.Es. inoculated with B.S.

The mortality was recorded as the ratio between the number of dead C.Es. and the total C.Es. incubated. We weighed the eggs at 10 DPI and C.Es. at 17 DPI using a high-precision balance (M214-AIH 0.0001 g). Their weight was adjusted to an initial weight of 50 g using the following equation:

aW = (ceW.50)/ieW

aW is the weight adjusted to 50 g, ceW is the C.E. weight at 17 days of incubation, and ieW is the initial egg weight at 10 DPI.

After euthanasia, we necropsied the C.Es. and performed the analysis of macroscopic damage.

2.6.2. Viability of the strains in allantoic fluid

We collected the allantoic fluid with a sterile swab to assess whether the B.S. strains remained viable 7 DPI. We inoculated the swab on nutrient agar and incubated the plates for 24 hours at 37°C with further identification by Gram staining to confirm the genus in P.C. using Bactray kit (Laborclin).

2.6.3. Quantification of biochemical markers of inflammation

We performed the biochemical analyses in an automatic biochemical analyser (ChemWell® 2910, Awareness Technology) using a Labtest diagnóstica® (Lagoa Santa, M.G., Brazil) kit. Before the test, we calibrated and standardized the equipment with universal

control serum. We centrifuged the blood to obtain the serum used for the total protein, albumin, and C-reactive protein analyses (1500xg 10 min).

2.6.4. Effect on immunomodulation

To evaluate the presence of poultry-specific chicken interleukin (IL)-10 (ThermoFisher), IL-4 (AbeBio), IL-6 (ThermoFisher), and immunomodulation by the B.S., we used a ready-to-use microwell, strip-or-full-plate ELISA (enzyme-linked immunosorbent assay) kit according to the manufacturer's recommendation. From the absorbance values, we constructed a relative standard curve according to the dilutions specified by the manufacturer, expressed in pg/mL. Afterwards, we interpolated the data using GraphPad Prism.

2.6.5. Histopathologic and morphometric analysis

For histomorphometric analysis, we collected duodenum fragments from 17-DPI C.Es. First, we fixed the fragments in 4% buffered formalin and processed them to obtain histological slides stained with haematoxylin and eosin (HE) (Tolosa, E. M. C.; Rodrigues, C. J.; Behmer, O. A., and Freitas-Neto, 2003). Then, we examined duodenal villus height (from the tip of the villus to the villus–crypt junction) and villus width using ImageJ software (National Institutes of Health, USA).

2.7. B.S. in the inhibition of *Campylobacter jejuni*, *Salmonella* spp and avian pathogenic *E. coli* (APEC).

To assess the ability of B.S. strains to inhibit *Salmonella* spp. or APEC, we performed a triplicate analysis as follows: we grew 5 µL of BS at 8 log UFC/mL in a culture spot in the centre of the nutrient agar (Kasvi) plate at 37°C for 24 hours. Then, we inactivated the B.S. culture spot in the centre of the plate using chloroform vapour for 30 minutes. We added 5 µL of different serotypes of Salmonella *enterica* sub *enterica* (*S.* Enteritidis (S.E.), *S.* Heidelberg (S.H.), *S.* Minnesota (S.M.), *S.* Infantis (S.I.), *Salmonella* variant monophasic (S.V.M)) or APEC (serotype B2 isolated from sick bird) at 8 log UFC/mL in 10 mL of liquid AN at 42°C (+/-2), homogenized and added over the B.S. culture spot on the plate. We incubated the sample for 24 hours at 37°C and measured the inhibition area according to Santos et al., 1984. We considered a very strong inhibition zone to be larger than 20 mm, strong inhibition from 15 to 19 mm, moderate inhibition from 11 to 14 mm, and weak inhibition from 9 to 10 mm. No inhibition was registered when the inhibition zone was smaller than 9 mm. To standardize the size of the cultivation point, we adjusted all points to 65 mm. A similar procedure was performed on C.J. However, we used *Campylobacter* selective blood-free (CCDA) agar (oxoid) and incubated it for 48 hours at 37°C in a microaerophilic atmosphere.

2.8. B.S. strain susceptibility to antimicrobials

We performed antimicrobial susceptibility testing by the Kirby-Bauer disc diffusion method in triplicate [22]with strains C, D, and G. Each strain was inoculated on Muller Hinton agar, and the antibiotics were placed on the plate and incubated at 33°C for 24 hours. After incubation, we measured the inhibitory halos formed around each disc, and the values were compared to the Clinical and Laboratory Standards Institute (CLSI) table values for *Staphylococcus* sp..[23]. Since there are no specific values for *Bacillus* sp. in the CLSI tables, we used the parameters of the *Staphylococcus* sp. because both are Gram-positive bacteria, have considerable phylogenetic proximity and therefore present similar mechanisms of resistance acquisition [24]. The antimicrobials tested were amoxicillin + clavulanic acid, gentamicin, ceftiofur, enrofloxacin, sulfamethoxazole + trimethoprim, tetracycline, ceftriaxone, and norfloxacin. We tested the B.S. strains C, D, E, and G for tetracycline, erythromycin, gentamicin and vancomycin by minimal inhibitory concentration (MIC) to analyse possible antimicrobial resistance. By MIC, we used the microdilution method for tetracycline, erythromycin, and gentamicin as previously published [25], and we used a kit to perform the vancomycin MIC test (laborclin). We analysed eight (8) different dilutions, and each dilution was performed in triplicate.

2.9. Statistical analysis

We evaluated whether the data were parametric and then analysed variance (ANOVA) comparing each strain with controls, followed by Tukey's T test considering $p < 0.05$.

3. Results

3.1. *In vitro* selection of B.S.

3.1.1. Haemolysis and mucin degradation

We can see in Table 1 that strains H and I were β-haemolytic, and commercial probiotic (DSM 17299) and strain E were α-haemolytic. Strains A, B, D, F, G, H, and commercial probiotic (DSM 17299) were positive for mucin degradation (Table 1). Strains E and H and commercial probiotic presented haemolysis and mucin degradation. Because the commercial strain (DSM 17299) was positive for α-haemolysis and mucin degradation, we examined other strains with the same characteristics.

Table 1
Mucin degradation and haemolysis results of B.S. strains.

Strain ID of B.S.	Mucin Degradation	Haemolysis
A	Yes	γ
B	Yes	γ
C	No	γ
D	Yes	c
E	Yes	α
F	Yes	γ
G	Yes	γ
H	Yes	β
I	No	β
DSM	Yes	α

BS: *Bacillus subtilis;* DSM 17299: a commercial probiotic of BS (DSM 17299); A: strain 220; B: strain 32; C: strain 118; D: strain 122; E: strain 144; F: strain 207; G: strain 1273; H: strain 1733; I: strain 1516.

3.1.2. Necrosis and apoptosis test

After 18 hours of inoculation, we observed a decrease in the number of cells inoculated with strain I, which suggests cell destruction (Fig. 1). In addition, in the same cells, we also recorded an increase in IP/DAP (Fig. 1B) and YP/DAP (Fig. 1C), which suggests necrosis and apoptosis. After 36 hours, we observed increased necrosis in the cells inoculated with strain H (Fig. 1F) but not cell apoptosis (Fig. 1E) or a decrease in the number of adherent cells (Fig. 1D).
Because of the positive results obtained for the haemolysis, necrosis, and apoptosis tests, we excluded strains H and I from further analysis in this study.

3.2. B.S. selection in the C.E. model

3.2.1. Macroscopic changes and weight gain of C.Es.

C.Es. inoculated with strains A, B, and F had mortality rates above N.C. On the other hand, treatment with other strains and commercial probiotics (DSM 17299) resulted in C.E. mortality rates as low as those observed for the C.Es. inoculated with N.C. The C.Es. treated with strain G showed no mortality and had a higher weight than those inoculated with a commercial probiotic (DSM 17299), which made this strain a great candidate for further analysis (Fig. 2).

3.3.2. Viability of B.S. in the allantoic fluid

The bacterial culture revealed uniform colonies suggestive of B.S. in all samples except the negative control and P.C. We identified them as Gram-positive bacillus-shaped bacteria by Gram colouration analysis. The culture results for N.C. were negative; in the P.C., we confirmed colonies suggestive of S.P. using the Bactray system as belonging to the genus *Salmonella.*

3.2.3. Biochemical markers of inflammation in C.Es. inoculated with different strains of B.S.

While in the C.Es. inoculated with strains A, B, D, and F, the albumin-to-protein ratio results were similar to the P.C., in the C.Es. inoculated with strains C, E, G, DSM 17299, and N.C., they were lower than the P.C. results (Fig. 3A). We found no difference in the quantification of C-reactive protein between strains and the positive control (Fig. 3B).

3.2.4. Effect on immunomodulation

To assess the immunomodulatory effect of the tested strains, we measured the cytokines IL-6, IL-4, and IL-10. A greater amount of IL-6 was found in P.C., while all other C.Es. did not express this cytokine (Table 2). Except for the results observed in C.Es. inoculated with strain A, all C.Es. had lower IL-10 values than P.C. The C.Es. inoculated with strains D, E, G, and DSM 17299 presented similar values to IL-4 compared to N.C. and P.C. (Table 2). Only the positive control expressed Il-6 (Table 2).

Table 2
Quantification of the cytokines IL-10, IL-4, and IL-6 (pg/mL) in the serum of C.E. inoculates with different strains of B.S.

	A	B	C	D	E	G	DSM	NC	PC
IL-10	23.3	20.6	19.9	16.1	20.5	21.1	17.1	16.4	30.0
	(+/-2.3)ab	(+/-4.7)a	(+/-4.7)a	(+/-1.9)a	(+/-9.8)a	(+/-2.3)a	(+/-6.8)a	(+/-2.7)a	(+/-3.6)b
IL-4	156.5	3.0	10.8	30.6	40.1	13.0	42.1	3.8	98.7
	(+/-151.7)a	(+/-4.8)b	(+/-17.2)b	(+/-62.9)ab	(+/-44.7)ab	(+/-14.7)ab	(+/-40.7)ab	(+/-8.8)b	(+/-12.8)a
IL-6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	103
	(+/-0.0)a	(+/-0.0)a	(+/-0.0)a	(+/-0.0)a	(+/-0.0)a	(+/-0.0)a	(+/-0.0)a	(+/-0.0)a	(+/-84.5)b

Mean (standard deviation). A–G: Different strains of B.S. DSM: commercial probiotic of B.S. (DSM 17299); A: strain 220; B: strain 32; C: strain 118; D: strain 122; E: strain 144; G: strain 1273; NC: negative control, P.C. : positive control (C.E. inoculated with *Salmonella* Pullorum at 12 days postincubation (DPI)). We did not analyse strain F because we did not have enough serum. We used ANOVA and Tukey's test to compare the mean of each group with the mean of P.C. IL–10: We used ANOVA and Tukey's test to compare the mean of each group with the mean of the control group (DSM, NC, P.C.). IL–4 and IL–6: We used the Kruskal–Wallis test to compare each group's mean with the control group's mean (N.C., P.C.).

3.2.5. Histomorphometric analysis

Interestingly, the histomorphometry showed that C.Es. inoculated with strain G presented a longer villus length than P.C., NC, and DSM 17299. Strain E presented a longer villus length than DSM 17299 and P.C. There was no difference in the width of the villi (Fig. 4).

3.2.6. Inhibition of *Salmonella*, APEC and *Campylobacter* by selected B.S. strains

After the *in vivo* model test, we excluded strains A, B, F, and D from further tests due to the high albumin-to-protein ratio and/or higher mortality rate. Furthermore, we tested the inhibitory effect of the strains C, E, and G for different strains of S.H., S.T., S.M., S.E., SI, S.V.M, and C.J. As strain G showed better results in the *in vivo* test compared to others, some tests were performed only for this strain and the positive control.

The results show that the inhibition effect appears to be isolated dependent. For three (3) S.Hs. tested, strain G showed a moderate or strong inhibition effect superior to the E strain in inhibiting SH2. Although the C strain presented a result that classifies it as weak inhibition or without inhibition for the SH1 or SH3 isolates, there was no significant difference between the G strains and the strain control strain (DSM 17299). B.S. strain E did not inhibit SH2 (Table 2).

For S.E., the B.S. strains (C and G) showed moderate inhibition similar to the strain control strain (DSM 17299). There was no significant difference between the other strains of B.S. (Table 3). The B.S. strain E resulted in a weak inhibition of S.E.

Strains E and G showed a moderate or strong inhibitory effect on S.T. Strains E and G showed more significant inhibitory effects than the control strain (DSM 17299) on ST2, and strain E had a more significant inhibitory effect than the DSM 17299 control strain

on ST4 (Table 3).

Only strain G was tested to evaluate the inhibitory effect on the S.M. and S.V.M serotype isolates. The inhibitory effect for S.M. was moderate, similar to the DSM 17299 control strain, and it was strong for S.V.M (Table 3).

The inhibitory effect on the S.I. was very strong, strong, and moderate in all strains, except for DSM 17299, which showed moderate inhibitory effects. It should be noted that strain E and strain G had the best results. The inhibitory effect on C.J. IAL was moderate (E strain), strong (C and G), and very strong (DSM control strain), although there was no significant difference. The evaluation of the inhibitory effect on CJ1 and CJ2 was performed only for the G strain, which showed a very strong inhibitory effect for both C.J. strains, and in CJ1, the effect was greater than that of the DSM control, which did not inhibit CJ1 (Table 3).

The strain C didn't inhibit APEC, strains E showed moderated inhibition and strain G strong inhibition. Strains had E, G and DSM 17299 showed a greater inhibitory effect when compared to the C strain. The G strain had a greater inhibitory effect on APEC when compared to the DSM 17299 strain.

Table 3
Inhibitory effect (measured by the mean inhibition halo) of B.S. strains on *Salmonella* spp. and *Campylobacter jejuni.*

Strain	C	E	G	DSM
SH 1	**IA** 0.88 (+/-0.20)a	**M** 1.11 (+/-0.04)a	**M** 1.28 (+/-0.35)a	**M** 1.32 (+/-0.21)a
S.H. 2	**M** 1.23 (+/-0.08)a	**IA** 0.65 (+/-0.00)b	**M** 1.28 (+/-0.14)a	**M** 1.33 (+/-0.08)a
SH3	**M** 1.06 (+/-0.15)ab	**S** 1.74 (+/-0.43)a	**S** 1.50 (+/-0.35)ab	0.93 (+/-0.15)b
S.E.	**M** 1.28 (+/-0.12)a	**W** 1.08 (+/-0.37)a	**M** 1.25 (+/-0.07)a	**M** 1.26 (+/-0.04)a
ST1	**W** 1.00 (+/-0.30)a	**M** 1.18 (+/-0.50)a	**M** 1.18 (+/-0.06)a	**M** 1.29 (0.03)a
ST2	**M** 1.02(+/-0.01)ab	**M** 1.03 (+/-0.04)a	**M** 1.22 (+/-0.06)c	**W** 0.89 (+/-0.07)b
ST3	**M** 1.1(+/-0.21)a	**M** 1.12(+/-0.28)a	**M** 1.21(+/-0.06)a	**W** 0.9(+ 0.07)a
ST4	**M** 1.05(+/-0.17)ab	**S** 1.75(+/-0.44)a	**S** 1.50(+/-0.35)ab	**W** 0.94(+/-0.16)b
ST5			**M** 1.47 (+/-0.31)a	**W** 1.10 (+/-0.15)a
SM			**M** 1.30 (+/-0.10)a	**M** 1.15 (+/-0.05)a
S.V.M			**S** 1.50 (+/-0.28)a	**S** 1.68 (+/-0.40)a
SI1	**VS** 1.15(+/-0.0)a	**VS** 2.48(+/-0.15)b	**VS** 1.28(+/-0.15)a	**W** 0.58(+/-0.07)c
SI2	**M** 1.72(+/-1.0)a	**VS** 3.17(+/-0.19)b	**M** 3.33(+/-0.35)b	**W** 0.58(+/-0.07)a
APEC	**IA** 0.00 (+/-0.0)a	**M** 1.24 (+/-0.21)bd	**S** 1.508 (+/-0.18)b	**W** 1.03(+/-0.01)cd
CJ IAL	**S** 1.65 (+/-0.43)a	**M** 1.44 (+/-0.35)a	**S** 1.69 (+/-0.16)a	**VS** 2.1 (+/-0.61)a
C.J. 1			**VS** 2.12 (+/-0.10)a	**W** 0.66 (+/-0.01)b
C.J. 2			**VS** 2.97 (+/-0.05)a	**VS** 2.96 (+/-0.33)a

Mean (standard deviation). SH1, SH2, SH3: a different strain of *Salmonella* Heidelberg, SE: *Salmonella* Enteritidis, ST1, ST2, ST3, ST4, ST5: different strains of *Salmonella* Typhimurium, S.M.: *Salmonella* Minnesota. S.V.M: *Salmonella* variant monophasic. SI1 and SI2: different strains of *Salmonella* Infantis. APEC: Avian Pathogenic *E. coli* isolated of sick peacock. CJ IAL: *C. jejuni* IAL. CJ1 and CJ2: *Campylobacter jejuni*. *Salmonella*, CJ1, and CJ2 I were isolated from broiler chickens. CJ IAL was isolated from humans (Fonseca et al., 2014). DSM: commercial probiotic of B.S. (DSM 17299); C: strain 118; E: strain 144; G: strain 1273; NC: negative control. The bold text indicates different levels of inhibition. **VS**: very strong inhibition halo above 2.0 cm width; **S**: strong inhibition from 1.5 to 1.9 cm; **M**: moderate inhibition from 1.1 to 1.4 cm; **W**: weak inhibition from 0.9 to 1 cm; **IA**: inhibition absent or smaller than 0.9 cm (Santos et al., 1984). We performed ANOVA and Tukey's test ($p < 0.05$). Different letters in the same line indicate significant differences.

3.2.7. Sensitivity to antimicrobials

By the disk diffusion method, all strains tested were sensitive (Table 4).

Table 4
Mean inhibition halos formed after inoculation with different antibiotics in the different selected B.S. strains

Antimicrobial	Inhibition halos (cm)			
	C	E	G	DSM
Amoxicillin + clavulanate	2,9	3	2,7	2,6
Ceftiofur	3,3	3,2	3	4
Ceftriaxone	2,5	3,5	3	4
Enrofloxacin	3,5	3,7	2,8	3,1
Gentamicin	2,5	3,5	2,7	2,7
Norfloxacin	3	3,5	3	3,1
Sulfametoxazol + Trimetoprim	2,8	3	2,9	3
Tetraciclin	2,6	3,1	2,5	3,1

C, E, G: Different strains of B.S. DSM: commercial probiotic of B.S. (DSM 17299); C: strain 118; E: strain 144; G: strain 1273. All strains were sensitive to all antibiotics tested.

The MIC analysis performed for strains E, C, and G and the commercial strain showed that strains E and C were sensitive to gentamicin, erythromycin, tetracycline, and vancomycin. Strains G and commercial probiotic were sensitive to tetracycline and vancomycin and intermediate to gentamicin and erythromycin (Table 5)

Table 5
Mean minimum inhibitory concentration (µg/mL) of gentamicin, erythromycin, tetracycline, and vancomycin on selected strains of B.S.

	Gentamicin	Eritromicin	Tetraciclin	Vancomicin
C	0.5 (+/-0.0) S	0.25 (+/-0.0) S	0.25 (+/-0.0) S	0,125 (+/-0.0) S
E	0.5 (+/-0.0) S	0.25 (+/-0.0) S	0.25 (+/-0.0) S	0,125 (+/-0.0) S
G	8.0 (+/-0.0) I	1.0 (+/-0.0) I	1.0 (+/-0.0) S	0,25 (+/-0.0) S
DSM	8.0 (+/-0.0) I	1.0 (+/-0.0) I	1.0 (+/-0.0) S	0,25 (+/-0.0) S

Mean (standard deviation). A-C: Different strains of B.S. DSM: commercial probiotic of B.S. (DSM 17299). S. sensible. I. Intermediate.

4. Discussion

We analysed the safety, immunomodulatory effects, antibiotic resistance, and capacity to inhibit *Salmonella* spp. and C.J. of B.S. strains isolated from diverse sources.

The evaluation of haemolytic activity is recommended if the isolated bacteria are intended to be used in food products [27]. Lack of haemolytic activity is essential during the selection of probiotic strains because a lack of haemolysin ensures that virulence will not appear among the bacterial strains [1]. Strains A, B, C, D, F, and G were γ haemolytic, while strains E and DSM 17299 were α haemolytic, and strains H and I were β haemolytic (Table 1). Although the E strain showed alpha haemolysis, this haemolysis was very discreet. Thus, as the DSM 17299 strain was also α haemolytic, the E strain was not excluded only by this attribute.

For mucin degradation, all strains were positive except strains C and I (Table 1). Since the commercial strain (DSM 17299) used as a control was positive for mucin degradation (Table 1) and we found an absence of necrosis and apoptosis in the primary culture of fibroblasts in strain C (Fig. 1), we considered that these strains should be further analysed. Commensal bacteria may penetrate the intestinal mucus barrier without harming the host [28]. However, severe changes in the intestinal barrier structure can affect its

function. Commensal strains of *Bifidobacterium* used in the food industry for decades have the potential to degrade mucin *in vitro* [29]. Thus, as an isolated feature, mucin degradation is not a risk indicator in some cases.

Assessment of the apoptosis and necrosis index is appropriate for candidate strains for probiotics. We inoculated high doses of BS (5 log CFU/well) in chicken fibroblasts and evaluated the total number of adherent cells, necrosis, and apoptosis index. Interestingly, 18 hours postinoculation, strain I led to a decrease in cells marked with DAP (Fig. 1), indicating that many dead cells had detached from the plate. The YP/DAP and IP/DAP increased (Fig. 1), showing an increase in apoptosis and necrosis, respectively. This result indicates that strain I is not safe because it kills cells by mechanical necrosis and apoptosis. Strain H increased necrosis but not apoptosis at 36 hours after inoculation (Fig. 1). As strains H and I were β haemolytic, leading to more significant cell death 18 and/or 36 hours after inoculation in chicken fibroblasts, these strains were not considered safe and were excluded from further analysis.

Strains A, D, and F led to higher mortality in C.Es. The C.Es. inoculated with strain G presented no deaths (Fig. 2). As C.Es. in early and intermediate incubation stages are more sensitive, even nonpathogenic bacteria in high doses can probably lead to death. Therefore, we considered an acceptable mortality rate similar to that of the commercial control. In this way, we evaluated mortality and the subsequent results to assess the selection of strains.

Regarding the biochemical results, the selected strains had a lower albumin-to-protein ratio but a C-reactive protein concentration similar to the P.C. (Fig. 3). The level of C-reactive protein increases in blood in response to inflammation, infection, or tissue damage [30] and is an important marker of inflammation in dogs and humans. However, few recent studies have evaluated C-reactive protein in birds or C.Es. Although previous studies have shown that C-reactive protein can be an inflammation marker in chickens [31] C-reactive protein does not rise in chickens as quickly as it does in humans [32].

Chicken embryos infected with infectious bronchitis virus do not have increased C-reactive protein levels[33] Our study did not find an increase in C-reactive protein even in the positive control. The dynamics of the release of this marker from inflammation may be different in C.Es., and other acute phase proteins should be indicated for study in chickens [34]

In inflammatory processes, there is an increase in total plasma proteins because globulins rise and occasionally decrease albumin, causing a decrease in the albumin/globulin ratio. Often, the total proteins may be in normal ranges, although the albumin/globulin ratio decreased, so this relationship has greater clinical significance. In our study, there was no decrease in the albumin/protein ratio, perhaps because the phase of acute inflammation had passed. This hypothesis should be considered since there was an increase in IL-4 and IL-10 in the positive control (Table 2), showing a phase of the immune system's resilience. On the other hand, in dehydrated birds, an increase in albumin is evident because albumin increases, while total protein can be low. The increased dehydration can be explained by the fact that the injured C.E. has increased energy requirements or respiratory rate, losing more water than the others[35]

We quantified Il-6, a proinflammatory cytokine generated by innate and adaptive responses. It is interesting to study this cytokine because, in the intestine, it modifies the expression of different tight junction proteins and increases tight junction permeability [36–39]. Even as a proinflammatory cytokine, IL-6 may indicate an immunomodulatory effect when it increases concomitantly with anti-inflammatory cytokines such as IL-10 [40]. Our study only found IL-6 in the C.Es. inoculated with S.P. (positive control), indicating that inflammation was not induced in the strains tested.

The positive control exhibited increased IL-6, IL-10, and IL-4 (Table 2) because S.P. caused inflammation, and the immune system tried to modulate the inflammation caused by S.P., similar to what occurs in the newborn animal[41, 42]. None of the strains studied increased IL-6, strain A increased IL-10, and the IL-4 level was similar to that in P.C. (Table 2). The increased concentration of IL-4 in strains A, C, D, E, G, and DSM 17299 at similar levels of P.C. may indicate an immunomodulatory effect of the strains inoculated. However, strains C, D, E and G showed levels of the cytokine IL-4 similar to those in the positive control but also in the negative control.

The safe and probable immunomodulatory effects of the B.S. strains should be interpreted in conjunction with several results. Since strains A, B, D, and F increased the embryo mortality rate and/or the albumin-to-protein ratio, we excluded these strains from our work; our objective justifies using B.S. with high safety in both newborn animals and C.Es.

C.Es. are a valuable *in vivo* model to evaluate probiotic safety, and the results obtained with the C.Es. inoculated with strain G indicate that this strain could bring benefits to C.E. growth (Fig. 2) in addition to other beneficial effects. Moreover, the villus height of C.Es. inoculated with strains G and E was higher than that of the commercial strain and the negative control (Fig. 4).

Previous studies have shown that whether B.S. causes beneficial or detrimental effects in C.Es. is strain dependent. Seeking to understand the beneficial effects of B.S. in hatchability, chick performance, and intestinal microflora, studies have shown that early B.S. probiotics inoculated *in ovo* can colonize the small intestine and create a deleterious environment for pathogenic bacteria that could impair chick health. As beneficial effects are obtained when probiotics are added to the feed, early inoculation *in ovo* could induce earlier stimulation of the immune system to confer protection as soon as the chicks reach the poultry houses [43, 44].

From the nine initial strains, we selected three strains (C, E, and G) to test the inhibitory effect against S.E., S.H., SI, S.M., S.T., S.V.M, APEC and C.J. In this study, we found that the B.S. strains tested have diverse degrees of inhibitory effects, and the effect is strain dependent. For the trial with 12 *Salmonella* spp., APEC and C.J., B.S. strains C and E had some degree of inhibition for 75% (9/12) and 91,6% (11/12), respectively, and strain G had moderate, strong, or very strong inhibitory effects in all pathogenic strains.

The first and second most commonly reported zoonoses in humans in the European Union in 2018 were campylobacteriosis and salmonellosis, respectively. C.J., S.T., S.V.M, S.E., and S.I. are among the most common species and serovars associated with disease and are prevalent and associated in poultry meat [45]. Several studies have tested the dietary effect of B.S. in chickens challenged with distinct *Salmonella* spp. and found an exclusion effect [46–50]. Similarly, the B.S. anti-*Campylobacter* effects in poultry are well documented but are variable and strain specific [51], as confirmed in the results found in this study. Strain G presented strong inhibitory effect on APEC that is an important pathogen to poultry production [52]. Our work makes it clear that the selected strains have action on different *Salmonella* serotypes, APEC and C.J. in addition to being safe. Since antibiotics are considered harmful chemicals and lead to increased antibiotic-resistant bacteria, dysbacteriosis, and drug residues in food products, the use of probiotics in the poultry industry has become popular in recent years. A probiotic included in commercial formulations, such as GalliPro (Chr Hansen) and Alterion (Novozymes), can improve chicken feed conversion and body weight, reduce lesions caused by *Clostridium perfringens*, elongate intestinal villi and modulate the microbiota to improve intestinal *Lactobacillus* concentration and reduce pathogens such as *Salmonella* and *Campylobacter* by competitive exclusion and other mechanisms. In this study, we have found secure and efficient strains of B.S. to inhibit *Salmonella* and *Campylobacter in vitro*. Further studies must be performed to understand the *in vivo* effects of the selected strains, either in feed or *in ovo*.

The European Food Safety Authority (EFSA) establishes specific parameters for testing antimicrobial resistance in all microorganisms used as food additives for humans and animals through the MIC and tetracycline, erythromycin, gentamicin, and vancomycin[27] antimicrobials of choice. Our results showed that the selected strains did not resist these antibiotics (Table 4), increasing the safety of inserting these strains as additives for animal production. In addition, we analysed the antibiotic sensitivity of the main antibiotic classes, and there was no resistance (Table 3).

5. Conclusion

Our study revealed 3 highly safe B.S. probiotic strains with the ability to inhibit *Salmonella* spp., APEC or C.J. tested in C.Es. that proved to be an appropriate experimental model for the selection of probiotic strains. Therefore, the selected strains can be used in the poultry industry *in ovo* or in newborn animals with a high level of security.

Abbreviations

Bacillus subtilis: B.S.

Avian pathogenic *Escherichia coli*: APEC

Campylobacter jejuni: C.J.

Day postincubation: DPI

Salmonella Heidelberg: S.H.

Salmonella Typhimurium: S.T.

Salmonella Enteritidis: S.E.

Salmonella Minnesota: S.M.

Salmonella Infantis: S.I.

Salmonella var. monophasic: S.V.M

Campylobacter jejuni: C.J.

Commercial probiotic of BS: DSM 17299

Clinical and Laboratory Standards Institute: CLSI

Minimal inhibitory concentration: MIC

European Food Safety Authority: EFSA

Negative Control: N.C.

Positive Control: P.C.

Yo Pro-01: Y.P.

Propide iodate: P.I.

4',6-diamidino-2-phenylindole: DAPI

Declarations

Ethics approval and consent to participate

The Ethics Committee on Animal Use of the UFU certified under protocol number 11/2022/CEUA/PROPP/REITO, PROCESSO N°°23117.023808/2022-77.

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

BBF, NDC, SS, TFMR, GRS, IMC, DAR and PGH conducted the *in vitro* experiment. TFMR, GRS, MCS, AES, SS, IMC made the collections and analyses in embryos. FON, AAMR and IPC prepared the histopathological slides and performed the analysis. MSC performed the ELISA. PGH wrote the manuscript. BBF and VAC idealized, designed, analysed the data, and edited the article.

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Figures

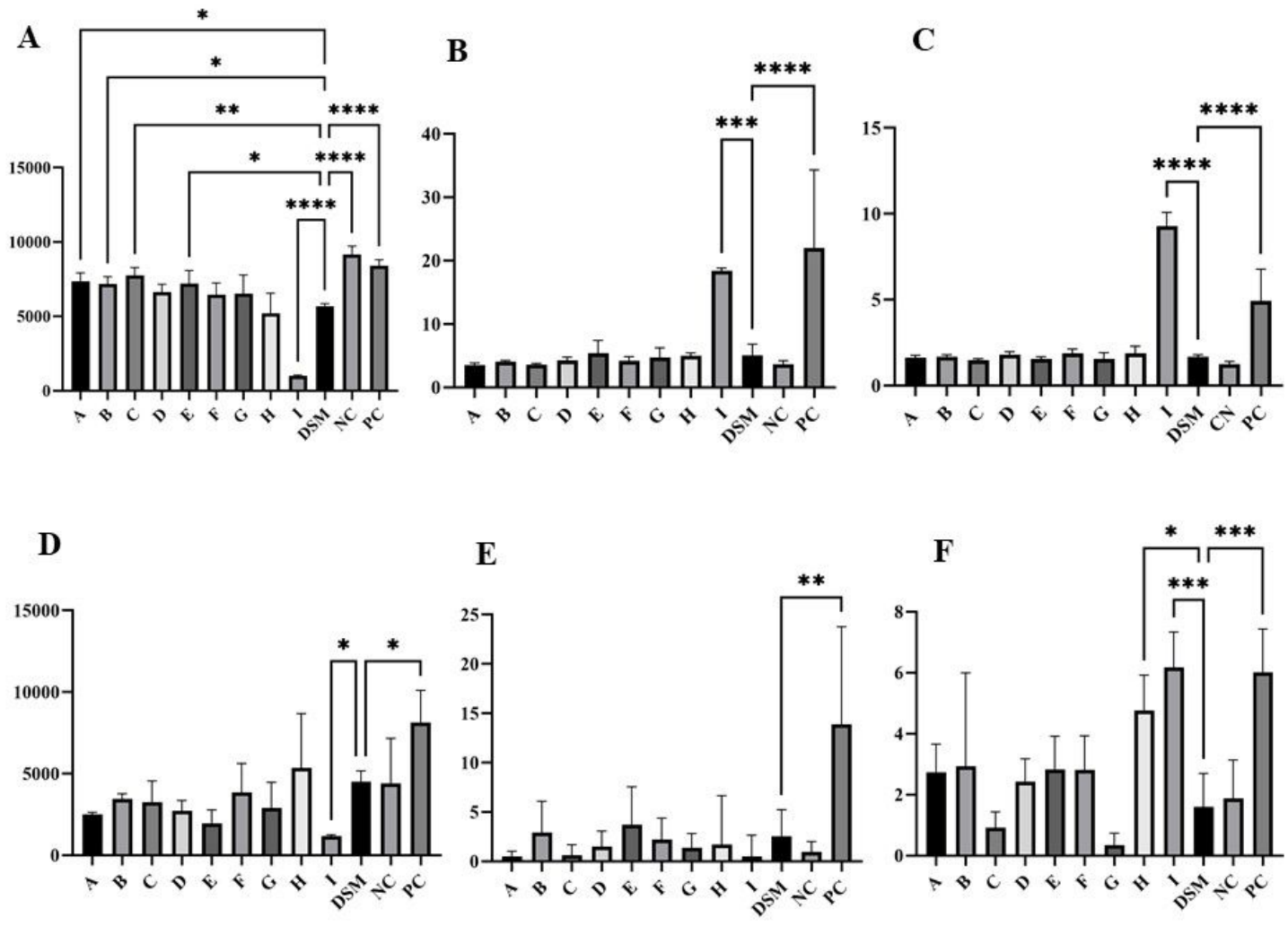

Figure 1

Relative fluorescence intensity level for total cell number, apoptosis, and necrosis in fibroblasts treated with different B.S. strains after 18 and 36 hours

A: DAD 18 hours postinoculation, B. YP/DAP 18 hours postinoculation, C. IP/DAP 18 hours postinoculation, D: DAP 36 hours postinoculation, E. YP/DAP 36 hours postinoculation, F. I/DAP 36 hours postinoculation. A-I: Different strains of *B. subtilis* (B.S.). DSM: commercial probiotic of B.S. (DSM 17299). A: strain 220; B: strain 32; C: strain 118; D: strain 122; E: strain 144; F: strain 207; G: strain 1273; H: strain 1733; I: strain 1516. P.C. : Positive control (avian pathogenic *E. coli* - APEC, serotype B2, isolated from an ill bird). NC: Negative control. Y axis: relative fluorescence intensity. We used ANOVA and Tukey's test to compare the mean of each group with the mean of a control group (DSM 17299*).*

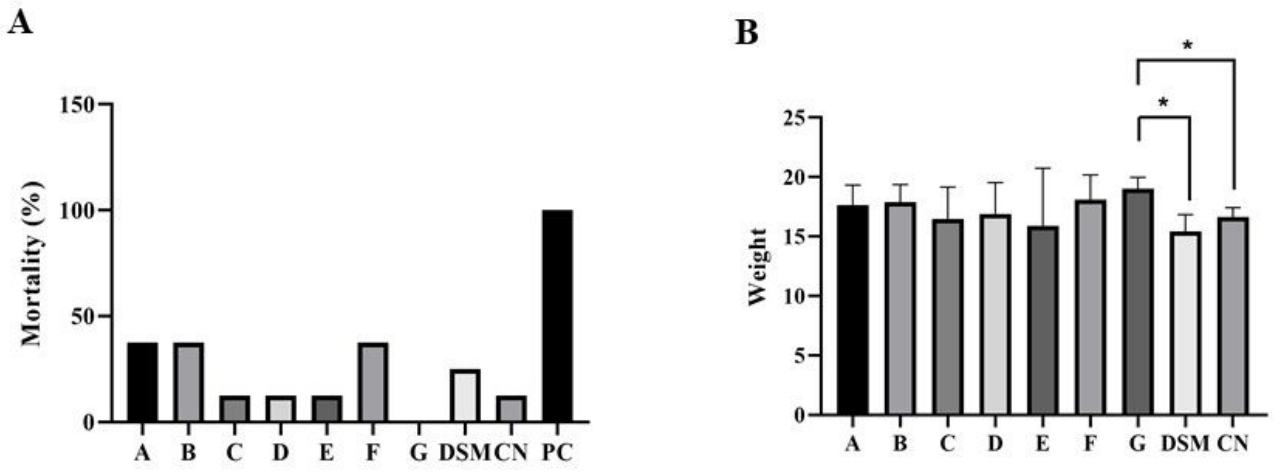

Figure 2

Mortality rate and weight gain of C.Es. inoculated with diverse B.S. strains.

Mortality (%) B. Weight (g) of chicken embryos inoculated with different strains of *B. subtilis* (B.S.). A-G: Different strains of B.S. DSM: commercial probiotic of B.S. (DSM 17299); A: strain 220; B: strain 32; C: strain 118; D: strain 122; E: strain 144; F: strain 207; G: strain 1273; NC: negative control; P.C. positive control (*Salmonella* Pullorum inoculated at 10 DPI (Figure A) *Salmonella* Pullorum inoculated at 12 DPI (Figure B). Figure B does not weigh P.C. because all chicken embryos (C.Es.) died. We used only descriptive statistics for mortality (%). For the weight analysis, we used a T test to compare the mean of each group with the mean of a control group (DSM 17299) and NC.

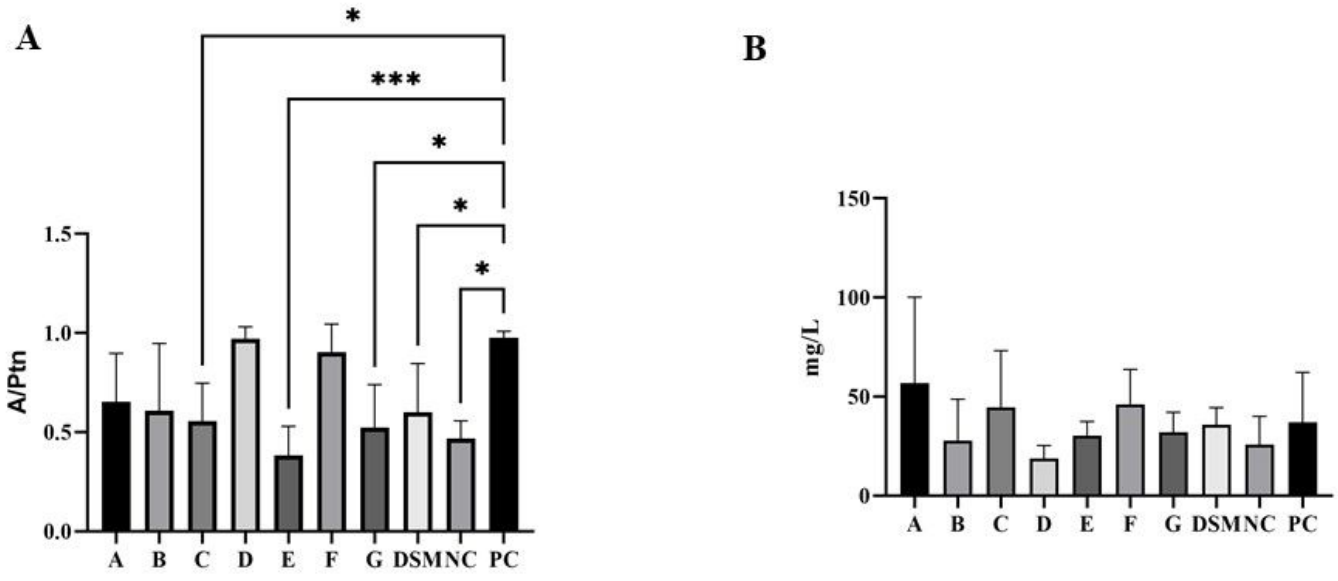

Figure 3

Quantification of biochemical markers of inflammation in embryos inoculated with different strains of B.S.

A. Albumin protein relationship. B. C-reactive protein levels of chicken embryos (C.E.) inoculated with different strains of *B. subtilis* (B.S.). A-G: Different strains of B.S. DSM: commercial probiotic of B.S. (DSM 17299); A: strain 220; B: strain 32; C: strain 118; D: strain 122; E: strain 144; F: strain 207; G: strain 1273; NC: negative control; P.C. : positive control (C.E. inoculated with *Salmonella*

Pullorum at 12 days pós incubation (DPI)). A/Ptn: Albumin (g/dL) protein (g/dL) relationship. We used ANOVA and Tukey's test to compare the mean of each group with the mean of P.C.

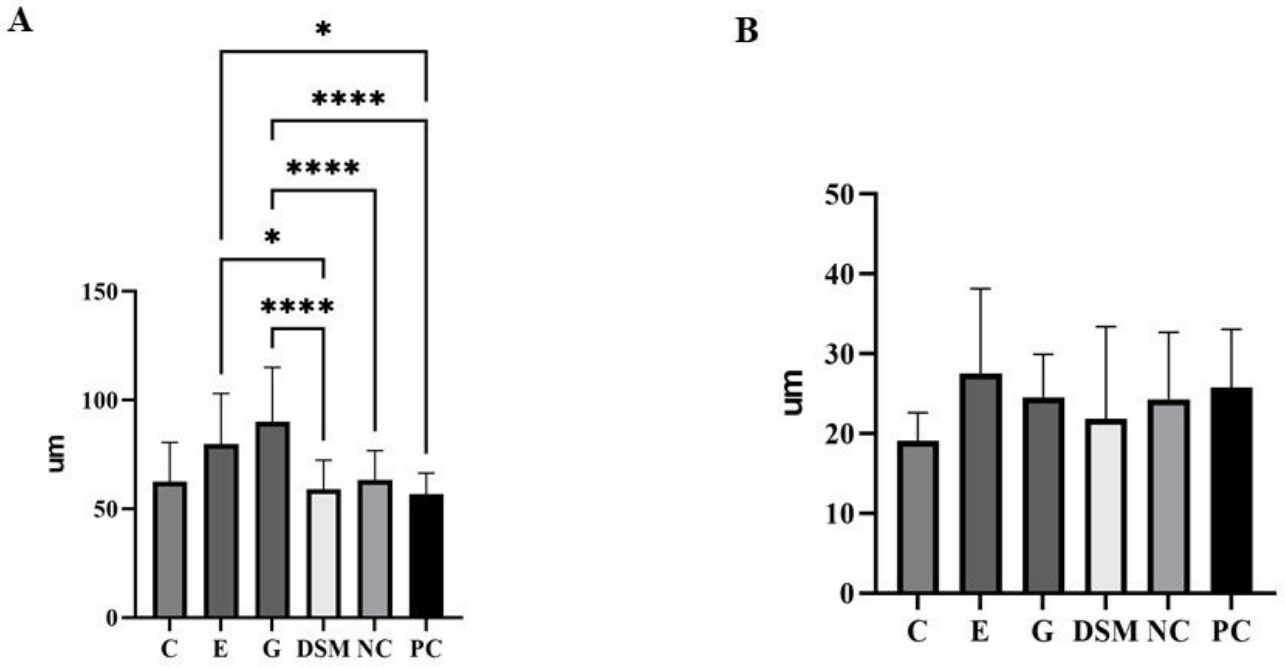

Figure 4

Histomorphometric analysis of C.E. intestinal cells inoculated with different strains of B.S.

A. Villus length. B. Villus width of chicken embryos (C.Es.) inoculated with different strains of *B. subtilis* (B.S.). C, E, G: Different strains of B.S. DSM: commercial probiotic of B.S. (DSM 17299); C: strain 118; E: strain 144; G: strain 1273; NC: negative control; P.C. positive control (C.E. inoculated with *Salmonella* Pullorum at 12 days postincubation (DPI)). We used ANOVA and Tukey's test to compare the mean of each group with the mean of the control groups (DSM, NC, P.C.).