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ORIGINAL PAPER

Chicken embryos are a valuable model for the selection of *Bacillus subtilis* for probiotic purposes

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Abstract

Bacillus subtilis (BS) has been used as an excellent probiotic; however, some BS strains seem to be opportunist pathogens or do not present inhibitory effects in the pathogenic bacteria, so the characterization of BS strains for use in animals is mandatory. This study aimed to select nonpathogenic strains of BS, which can inhibit *Salmonella* spp., avian pathogenic *Escherichia coli* (APEC), and *Campylobacter jejuni* (CJ) using a chicken embryo as a model. We tested nine (9) strains of BS 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 (CE) 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 CEs. At the 17th DPI we checked CE mortality, gross lesions, CE weight, and whether BS strains were still viable. To perform the cytokine, total protein, albumin, and reactive C protein analysis, we collected the CE blood from the allantoic vessel and intestine fragments in the duodenum portion for histomorphometric analysis. After the results in CEs, we tested the inhibition capacity of the selected BS strains for diverse strains of *Salmonella* Heidelberg (SH), *S.* Typhimurium (ST), *S.* Enteritidis (SE), *S.* Minnesota (SM), *S.* Infantis (SI), *Salmonella* var. monophasic (SVM), APEC 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 BS were viable in CEs at the 17th DPI, we removed four (4) strains (A, B, D, F) once they led to the highest mortality in CEs 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 CE. From the tests in CEs, 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 SH, ST, SI, APEC, and two (2) strains of CJ. In this study, we selected BS strains C, E and G due to their in vitro and in vivo safety and beneficial effects. In addition, we emphasize the value of CE as an in vivo experimental model for assessing BS's safety and possible benefits for poultry and other animals.

Keywords Probiotic · Salmonella · APEC · Campylobacter · Chicken embryo

Abbreviations

BS	*Bacillus subtilis*
APEC	Avian pathogenic *Escherichia coli*
CJ	*Campylobacter jejuni*
DPI	Day postincubation
SH	*Salmonella* Heidelberg
ST	*Salmonella* Typhimurium
SE	*Salmonella* Enteritidis

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Extended author information available on the last page of the article

SM	*Salmonella* Minnesota
SI	*Salmonella* Infantis
SVM	*Salmonella* Var. monophasic
DSM 17299	Commercial probiotic of BS
CLSI	Clinical and Laboratory Standards Institute
MIC	Minimal inhibitory concentration
EFSA	European Food Safety Authority
NC	Negative control
PC	Positive control
YP	Yo Pro-01
PI	Propide iodate
DAPI	4′,6-Diamidino-2-phenylindole

Introduction

Probiotics are defined as live microorganisms that, when administered correctly, confer a health benefit to the host (FAO/WHO 2002). *Bacillus subtilis* (BS) is a Gram-positive rod-shaped 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 (Earl et al. 2008).

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. BS is one of the most common bacterial species used in commercial probiotics in the U.S. (Joerger and Ganguly 2017), including in poultry (Heak et al. 2018; Jiang et al. 2021). BS 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, BS 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 (Elshaghabee et al. 2017; Mingmongkolchai and Panbangred 2018; Yahav et al. 2018; Elisashvili et al. 2019; Lee et al. 2019; Danilova and Sharipova 2020; Ugwuodo and Nwagu 2020). BS 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 BS can improve growth performance, enhance immunity and gut health, and reduce *Salmonella* spp. counts in challenged broilers (Heak et al. 2018; Hayashi et al. 2018; Cheng et al. 2018; Cruz et al. 2019).

In the selection of BS 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 BS 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)* (Gueimonde et al. 2007).

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 (Gray et al. 2005; Hwang and Park 2015).

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

Methods

Ethics statement

The experiment was conducted in 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.

Strains of *Bacillus subtilis*

The trial consisted of 9 strains of BS isolated from diverse sources, deposited by Fundação Oswaldo Cruz (FIOCRUZ) and kindly donated by Dr. Leon Rabinovitch. FIOCRUZ identified the species (BS) by biochemical tests, and we confirmed by Maldi-Tof mass spectrometry (Maldi Biotyper). 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), and 1516 (I).

Mucin degradation activity

To test mucin degradation, we evaluated 9 strains of BS (A-I) and a commercial probiotic (DSM 17299) as a positive control, according to a previously published method (Zhou et al. 2001a) with few modifications. Briefly, we spotted 10 µL of each BS 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.

Haemolytic activity

The haemolytic activity of the isolates was analysed in duplicate according to Xu et al. 2021 with some modifications. We grew 10 µL of BS 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.

Apoptosis and necrosis test in cells challenged with different strains of BS

We used a primary fibroblast culture from CE. First, apoptosis and necrosis were verified. We removed the chorioallantoic membrane from CE. Fourteen days postincubation (DPI), the cells were obtained by cutting and placing them in a 0.25% trypsin solution under stirring for 10 min. 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 (1500×*g* 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 h at 37 °C and 5% CO2, we inoculated each strain of BS (A-I) and DSM17299. In parallel, we used a negative control (NC) without bacteria 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 bacteria or control, we performed eight replicates per strain. To evaluate the apoptosis, we used Yo Pro-01 (YP) (Invitrogen) and propide iodate (PI) (Sigma). YP only stains cells in earlier stages of apoptosis still metabolically active, in which the D.N.A. fragmentation has not yet occurred but with compromised plasma membranes (Fujisawa et al. 2014). PI detects cells that are already dead by necrosis. We incubated the cells again for 18 and 36 h, washed them three times with PBS, and treated them with YP and PI (1:1000 each) for 30 min at room temperature. Then, we washed the wells three times and fixed them with 4% formalin for 10 min. After that, we treated the cells with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) to stain the cell D.N.A. 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 YP (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), IP, and YP and registered cell destruction (dead and detached cells from the plaque) by the results of cells labelled with DAPI. Cells in relative apoptosis were calculated by the ratio (YP-IP)/DAPI*100) and necrosis by (IP/DAPI)*100.

Virulence analysis of different BS strains in an in vivo model

To evaluate the harmlessness of the BS strains selected in the in vitro tests, we used 10 day incubation CEs, 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 CEs can be used as an in ovo preventive method in the poultry industry. In the poultry industry, CEs are inoculated at 18 DPI. In our manuscript, we used CEs 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 BS 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 BS strains (A-G and the control DSM 17299) in CE based on different parameters. The CE 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 CEs, 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 BS in the allantoic fluid. S.P. kills CEs at 10 DPI of incubation, and as the CEs must be functional for the biochemical and cytokine analysis, we inoculated the PC at 10 (positive death control) and 12 DPI (control of laboratory analysis). After inoculation of bacteria, we incubated the CEs at 37.5 °C and 58% relative humidity (RH). At 17 DPI, we checked the mortality rate, damage and weight of CE. Besides, we evaluated the viability of the strains 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.

Mortality and damage analysis in CEs inoculated with BS

The mortality was recorded as the ratio between the number of dead CEs and the total CEs incubated. We weighed the eggs at 10 DPI and CEs 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:

$$aW = (\text{ceW}.50)/\text{ieW},$$

aW is the weight adjusted to 50 g, ceW is the CE weight at 17 DPI, and ieW is the initial egg weight at 10 DPI

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

Viability of the strains in allantoic fluid

We collected the allantoic fluid with a sterile swab to assess whether the BS strains remained viable 7 days after incubation of the bacteria. We inoculated the swab on nutrient agar and incubated the plates for 24 h at 37 °C with further identification by Gram staining to confirm the bacillus format. To confirm the genus in PC we used Bactray kit (Laborclin).

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 (1500×*g* 10 min).

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 BS, 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.

Histopathologic and morphometric analysis

For histomorphometric analysis, we collected duodenum fragments from 17-DPI CEs. First, we fixed the fragments in 4% buffered formalin and processed them to obtain histological slides stained with haematoxylin and eosin (HE) (Tolosa et al. 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).

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

To assess the ability of BS strains to inhibit *Salmonella* spp. or APEC, we performed a triplicate analysis as follows: we grew 5 µL of BS at 8 log U.F.C./mL in a culture spot in the centre of the nutrient agar (Kasvi) plate at 37 °C for 24 h. Then, we inactivated the BS culture spot in the centre of the plate using chloroform vapour for 30 min. We added 5 µL of different serotypes of Salmonella *enterica* sub *enterica* [*S.* Enteritidis (SE), *S.* Heidelberg (SH), *S.* Minnesota (SM), *S.* Infantis (SI), and *Salmonella* variant monophasic (SVM)] isolated from chicken or APEC (serotype B2 isolated from sick bird) at 8 log U.F.C./mL in 10 mL of liquid AN at 42 °C (+/−2), homogenized and added over the BS culture spot on the plate. We incubated the sample for 24 h at 37 °C and measured the inhibition area according to Coelho-Rocha et al. (2022). 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 CJ isolated from chicken or humans (IAL 2383) (Fonseca et al. 2014). However, we used *Campylobacter* selective blood-free (CCDA)

agar (oxoid) and incubated it for 48 h at 37 °C in a microaerophilic atmosphere.

BS strain susceptibility to antimicrobials

We performed antimicrobial susceptibility testing by the Kirby–Bauer disc diffusion method in triplicate (Clinical and Laboratory Standards Institute 2015) 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 h. 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..(CLSI 2012). 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 (Zhang et al. 2020). The antimicrobials tested were amoxicillin + clavulanic acid, gentamicin, ceftiofur, enrofloxacin, sulfamethoxazole + trimethoprim, tetracycline, ceftriaxone, and norfloxacin. We tested the BS 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 (Anadón et al. 2006), 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.

Statistical analysis

We evaluated whether the data were parametric and then analysed variance (ANOVA) comparing each strain with controls, followed by Tukey's or Kruskal–Wallis test to nonparametric data considering $p < 0.05$.

Results

In vitro selection of BS

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 1f Mucin degradation and haemolysis results of BS strains

Strain ID of B.S	Mucin degradation	Haemolysis
A	Yes	γ
B	Yes	γ
C	No	γ
D	Yes	γ
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.

Necrosis and apoptosis test

After 18 h of inoculation, we observed a decrease in the number of cells inoculated with the strain I, which suggests cell destruction (Figure 1). In addition, in the same cells, we also recorded an increase in IP/DAPI (Fig. 1B) and YP/DAPI (Fig. 1C), which suggests necrosis and apoptosis. After 36 h, 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.

BS selection in the CE model

Macroscopic changes and weight gain of CEs

CEs inoculated with strains A, B, and F had mortality rates above NC. On the other hand, treatment with other strains and commercial probiotics (DSM 17299) resulted in CE mortality rates as low as those observed for the CEs inoculated with NC. The CEs 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 promising candidate for further analysis (Fig. 2).

Viability of BS in the allantoic fluid

The bacterial culture revealed uniform colonies suggestive of BS in all samples except the negative control and PC. We identified them as Gram-positive bacillus-shaped

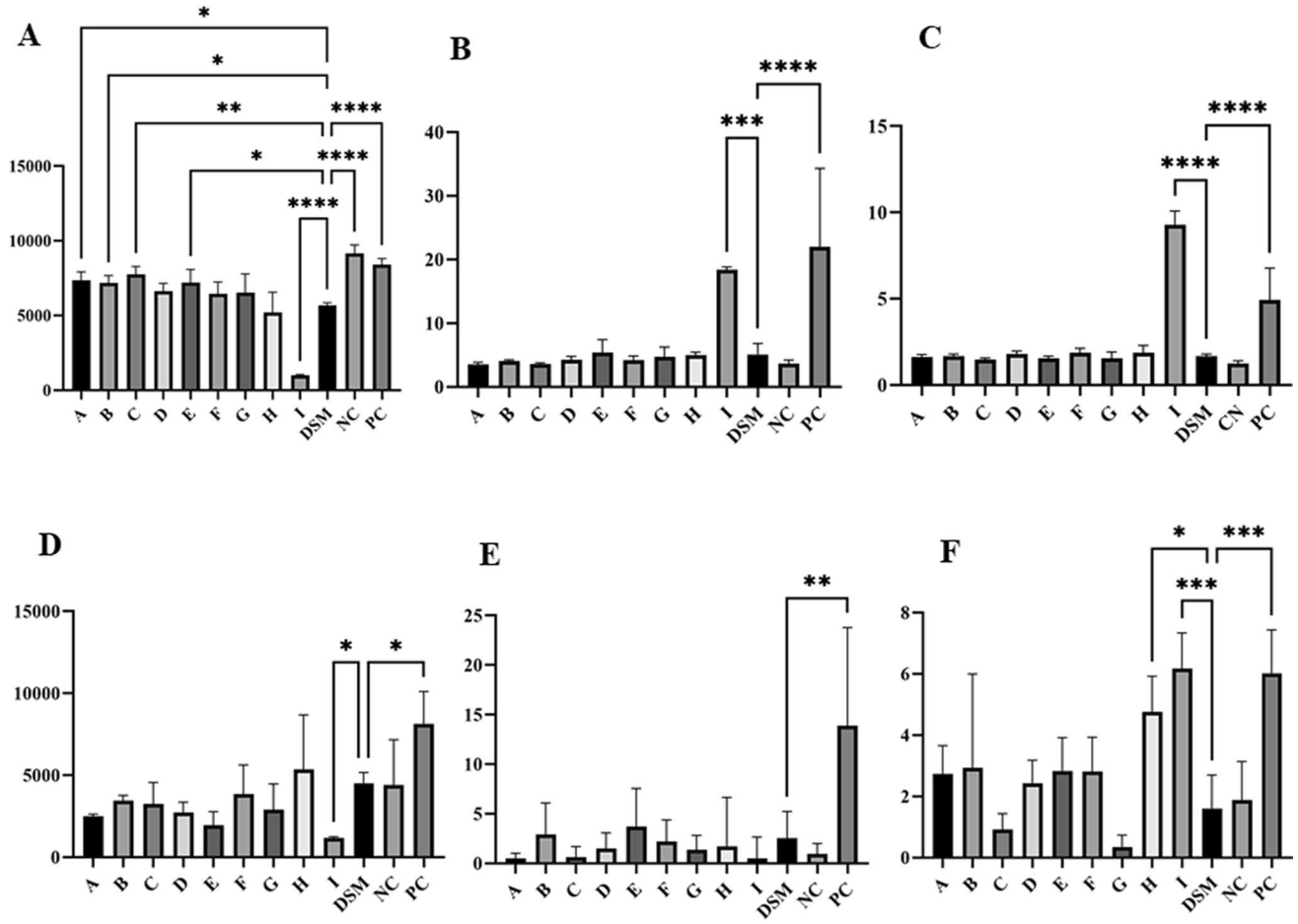

Fig. 1 Relative fluorescence intensity level for total cell number, apoptosis, and necrosis in fibroblasts treated with different BS strains after 18 and 36 h. **A** Quantification of cells stained by DAPI 18 h postinoculation of bacteria, **B** relative apoptosis: relation cells stained according to the equation: (YP−IP)/DAPI*100) 18 h postinoculation, **C** relative necrosis: Relation of cells stained by IP/DAPI 18 h postinoculation, **D** DAPI 36 h postinoculation, **E** YP/DAPI 36 h postincubation, and **F** I/DAPI 36 h postinoculation. A−I Different strains of *B. subtilis* (BS). DSM.: 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. PC: 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*).* DAPI: stain total cells. (YP−IP)/DAPI*100: stain cells in relative apoptosis. IP/DAPI*100: stain cells in necrosis

bacteria by Gram colouration analysis. The culture results for NC were negative; in the PC, we confirmed colonies suggestive of S.P. using the Bactray system as belonging to the genus *Salmonella.*

Biochemical markers of inflammation in CEs inoculated with different strains of BS

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

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 PC, while all other CEs did not express this cytokine (Table 2). Except for the results observed in CEs inoculated with strain A, all CEs had lower IL-10 values than PC The CEs inoculated with strains D, E,

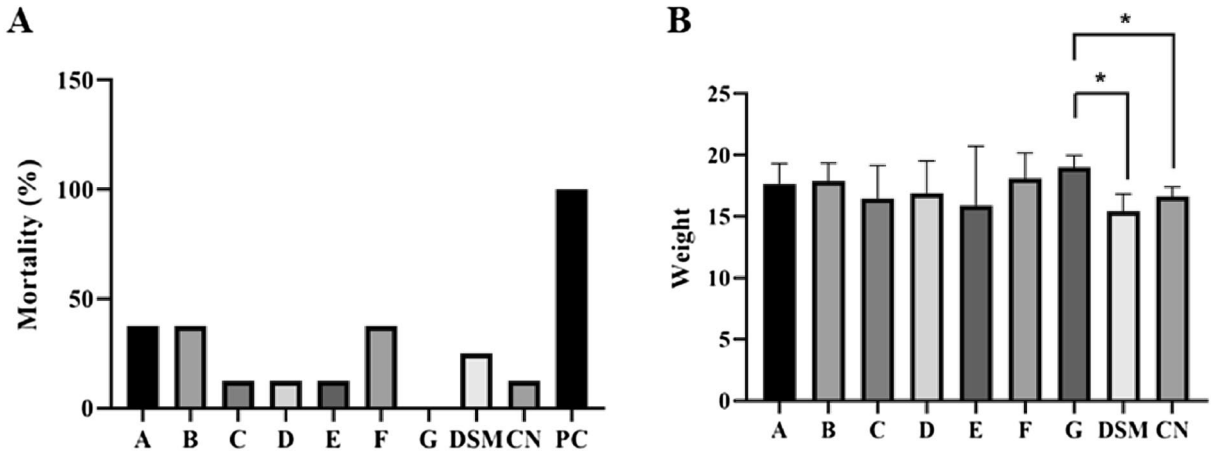

Fig. 2 Mortality rate and weight gain of CEs inoculated with diverse BS strains A. Mortality (%) B. Weight (g) of chicken embryos inoculated with different strains of *B. subtilis* (BS). A−G: Different strains of BS DSM.: commercial probiotic of BS (DSM 17299); A: strain 220; B: strain 32; C: strain 118; D: strain 122; E: strain 144; F: strain 207; G: strain 1273; NC: negative control; PC positive control (*Salmonella* Pullorum inoculated at 10 DPI. (**A**) *Salmonella* Pullorum inoculated at 12 DPI. (**B**). **B** does not contain the weight data of positive control results, because all chicken embryos (CEs) 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

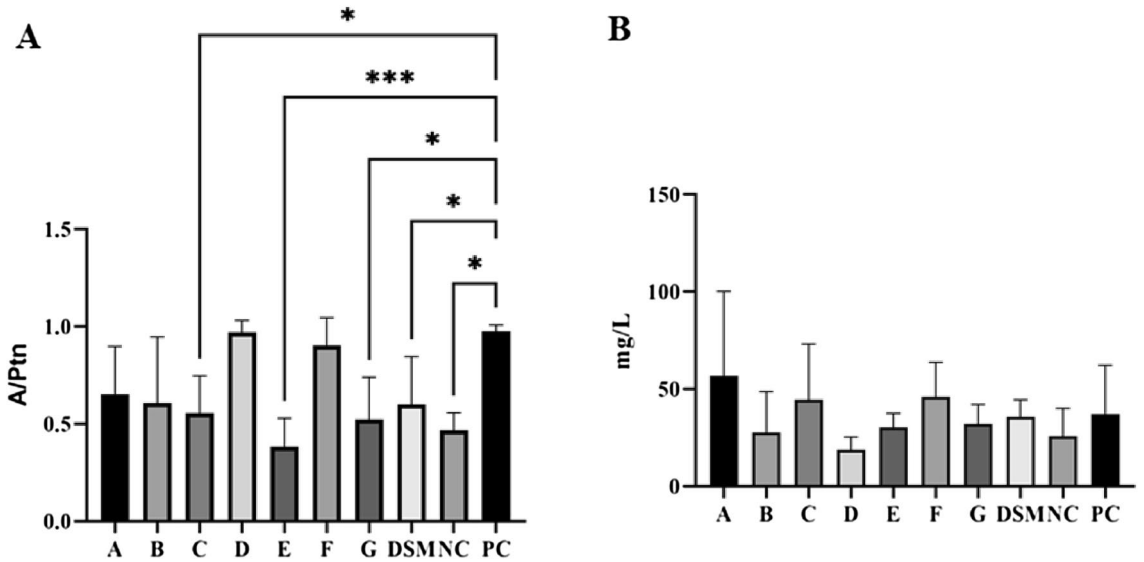

Fig. 3 Quantification of biochemical markers of inflammation in embryos inoculated with different strains of *B. subtilis (*BS). **A**. Albumin protein relationship. **B**. C−reactive protein levels of chicken embryos (CE) inoculated with different strains of BS. A−G: Different strains of BS DSM: commercial 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; NC: negative control; PC: positive control (CE inoculated with *Salmonella* Pullorum at 12 days postncubation (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 PC

Table 2 Quantification of the cytokines IL-10, IL-4, and IL-6 (pg/mL) in the serum of CE inoculates with different strains of BS

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

Mean (standard deviation). A-G: Different strains of BS DSM.: commercial probiotic of BS (DSM 17299); A: strain 220; B: strain 32; C: strain 118; D: strain 122; E: strain 144; G: strain 1273; NC: negative control, PC: positive control [CE 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 PC 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, PC). IL-4 and IL-6: We used the Kruskal–Wallis test to compare each group's mean with the control group's mean (NC, PC)

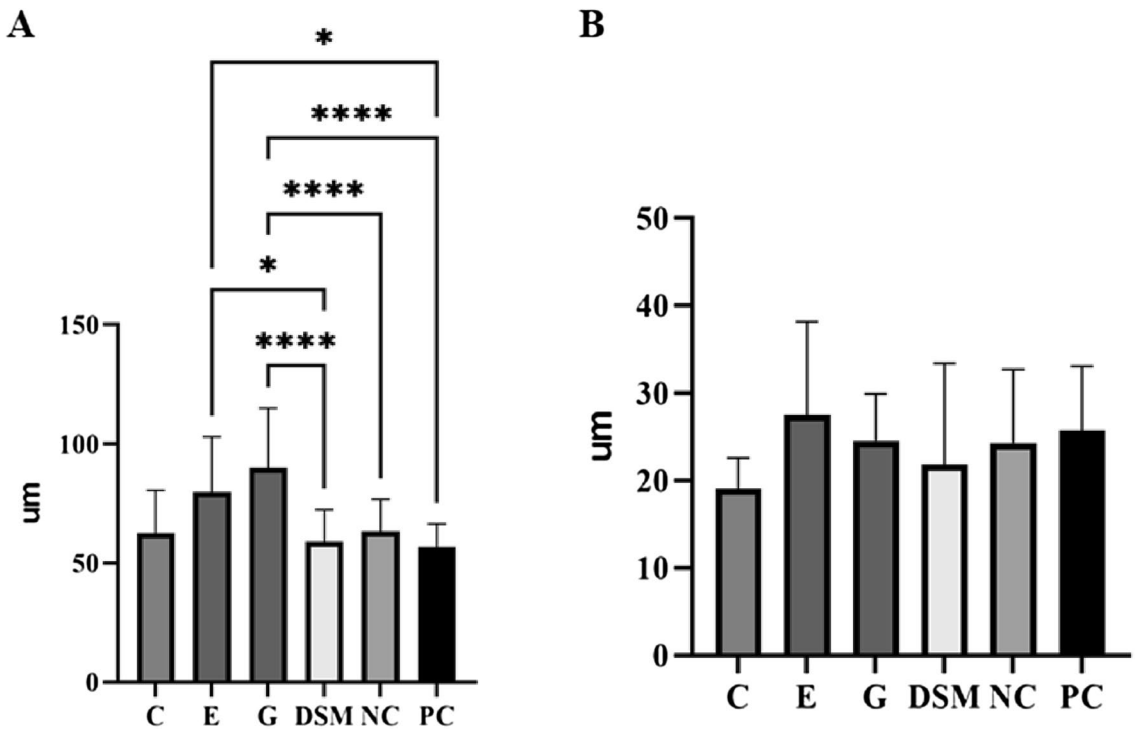

Fig. 4 Histomorphometric analysis of CE intestinal cells inoculated with different strains of BS. A. Villus length. B. Villus width of chicken embryos (CEs) inoculated with different strains of *B. subtilis* (BS). C, E, G: Different strains of BS. DSM: commercial probiotic of BS (DSM 17299); C: strain 118; E: strain 144; G: strain 1273; NC: negative control; PC positive control (CE 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, PC)

G, and DSM 17299 presented similar values to IL-4 compared to NC and PC (Table 2). Only the positive control expressed Il-6 (Table 2).

Histomorphometric analysis

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

Inhibition of *Salmonella,* APEC, and *Campylobacter* by selected BS 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 SH, ST, SM, SE, SI, SVM, and CJ. 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 isolate dependent. For three (3) SHs 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). BS strain E did not inhibit SH2 (Table 2).

For SE, the BS 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 BS (Table 3). The BS strain E resulted in a weak inhibition of SE

Strains E and G showed a moderate or strong inhibitory effect on ST. 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 SM and SVM serotype isolates. The inhibitory effect for SM was moderate, similar to the DSM 17299 control strain, and it was strong for SVM (Table 3).

The inhibitory effect on the SI 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 CJ 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 CJ strains, and in CJ1, the effect was greater than that of the DSM. control, which did not inhibit CJ1 (Table 3).

The strain C did not inhibit APEC, and 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 BS 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
SH 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	**W** 0.93 (±0.15)b
SE	**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
SVM			**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
CJ 1			**VS** 2.12 (±0.10)a	**W** 0.66 (±0.01)b
CJ 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. SVM: *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 2383 was isolated from humans (Fonseca et al. 2014). DSM: commercial probiotic of BS (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 (Coelho-Rocha et al. 2022). We performed ANOVA and Tukey's test ($p < 0.05$). Different letters in the same line indicate significant differences

Table 4 Mean inhibition halos formed after inoculation with different antibiotics in the different selected BS 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 BS DSM: commercial probiotic of BS (DSM 17299); C: strain 118; E: strain 144; G: strain 1273. All strains were sensitive to all antibiotics tested

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

	Gentamicin	Erythromycin	Tetracyclin	Vancomycin
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 BS DSM: commercial probiotic of BS (DSM 17299). S. sensible. I. Intermediate

Sensitivity to antimicrobials

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

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).

Discussion

We analysed the safety, immunomodulatory effects, antibiotic resistance, and capacity to inhibit *Salmonella* spp., APEC and CJ of BS strains isolated from diverse sources.

The evaluation of haemolytic activity is recommended if the isolated bacteria are intended to be used in food products (EFSA 2012). 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 (FAO/WHO 2002). 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 (Zhou et al. 2001b). 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* (Ruas-Madiedo et al. 2008). 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 h postinoculation of bacteria, strain I led to a decrease in cells marked with DAPI (Fig. 1), indicating that many dead cells had detached from the plate. The YP/DAPI and IP/DAPI 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 h 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 CEs. The CEs inoculated with strain G presented no deaths (Fig. 2). As CEs 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 PC (Fig. 3). The level of C-reactive protein increases in blood in response to inflammation, infection, or tissue damage (Pepys and Hirschfield 2003), and is an important marker of inflammation in dogs and humans. However, a few recent studies have evaluated C-reactive protein in birds or CEs. Although previous studies have shown that C-reactive protein can be an inflammation

marker in chickens (Patterson and Mora 1964). C-reactive protein does not rise in chickens as quickly as it does in humans (Patterson and Mora 1965).

Chicken embryos infected with infectious bronchitis virus do not have increased C-reactive protein levels (Sommerfeld et al. 2022). 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 CEs, and other acute phase proteins should be indicated for study in chickens (O'Reilly and Eckersall 2014).

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 CE has increased energy requirements or respiratory rate, losing more water than the others (Lumeij et al. 1997)

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 (Zeissig et al. 2007; Al-Sadi et al. 2008; Suzuki et al. 2011; Smyth et al. 2012). Even as a proinflammatory cytokine, IL-6 may indicate an immunomodulatory effect when it increases concomitantly with anti-inflammatory cytokines such as IL-10 (Oakley and Kogut 2016). Our study, we only found IL-6 in the CEs inoculated with SP (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 SP, similar to what occurs in the newborn animal (Tang et al. 2018; Foster et al. 2021). None of the strains studied increased IL-6, strain A increased IL-10, and the IL-4 level was similar to that in PC (Table 2). Considering the high standard deviation, we could not assess immunomodulation's effect based on the IL-4 results. However, the IL-4 results for strains E and G were identical to the control strain (DSM) and E and G were the main strains with good results in the embryo infection tests.

The safe of the BS 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 BS with high safety in both newborn animals and CEs

CEs are a valuable in vivo model to evaluate probiotic safety, and the results obtained with the CEs 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 CEs 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 BS causes beneficial or detrimental effects in CEs is strain dependent. Seeking to understand the beneficial effects of BS in hatchability, chick performance, and intestinal microflora, studies have shown that early BS 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 (Oliveira et al. 2014; Pender et al. 2017).

From the nine initial strains, we selected three strains (C, E, and G) to test the inhibitory Effect against SE, SH, SI, SM, ST, SVM, APEC, and CJ In this study, we found that the BS strains tested have diverse degrees of inhibitory effects, and the effect is strain dependent. For the trial with 12 *Salmonella* spp., APEC and CJ, BS 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. CJ, ST, SVM, SE, and SI are among the most common species and serovars associated with disease and are prevalent and associated in poultry meat (EFSA 2017). Several studies have tested the dietary effect of BS in chickens challenged with distinct *Salmonella* spp. and found an exclusion effect (Knap et al. 2011; Oh et al. 2017; Khochamit et al. 2020; Nishiyama et al. 2021; Xing et al. 2021). Similarly, the BS anti-*Campylobacter* effects in poultry are well documented but are variable and strain specific (Saint-Cyr et al. 2016), 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 (Kathayat et al. 2021). Our work makes it clear that the selected strains have action on different *Salmonella* serotypes, APEC and CJ 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 BS to inhibit *Salmonella,* APEC 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 (EFSA 2012) 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).

Conclusion

Our study revealed 3 highly safe BS probiotic strains with the ability to inhibit *Salmonella* spp., APEC or CJ tested in CEs 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 and they could be tested in newborn animals with a high level of security.

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

Declarations

Conflict of interest The authors declare that they have no competing interests.

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