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Antibiofilm properties of lactic acid bacteria and their metabolites against *Salmonella enterica* serotype Enteritidis on eggshell surface

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ABSTRACT

Salmonella enteritidis (SE) is a pathogenic bacterium commonly found on the surface of eggshells. In this study, we investigated the biofilm formation of a specific strain of *S. enteritidis*, CIDCA 115 (SE 115) on eggshells. Additionally, we examined the impact of *Lentilactobacillus kefiri* strains 83113 and 8321, as well as *Lactiplantibacillus plantarum* 83114, and their metabolites present in the supernatant on the biofilm formation of SE 115. Scanning electron microscopy revealed that SE 115 formed a mature biofilm structure on the eggshell. During co-incubation and pre-incubation, lactic acid bacteria strains significantly reduced the formation of SE 115 biofilm ($p < 0.05$ and $p < 0.01$, respectively) compared to SE 115 grown alone. The cell-free supernatants of lactic acid bacteria also exhibited a reduction in SE 115 biofilm formation and modified its structure. Co-incubation with SE 115 induced alterations in the composition of biofilm matrix components, notably in the levels of fimbria curli and cellulose. The qPCR analysis revealed that, after 48 h of incubation, the expression of the *csgD* gene, a critical regulator of biofilm formation, remained unchanged compared to planktonic cells. However, genes associated with the production of biofilm matrix components, curli (*csgA*) and cellulose (*bcsA*), exhibited heightened expression in the presence of lactic acid bacteria compared to the planktonic state. This study highlights the potential of lactic acid bacteria strains and their metabolites as innovative strategies for managing *Salmonella* biofilm formation in the context of poultry production.

Abbreviations

Baps	Biofilm-associated proteins
LAB	Lactic acid bacteria
SE 115	*S. enteritidis cidca 115*
SEM	Scanning electron microscopy
PBS	phosphate saline buffer

1. Introduction

Salmonella infection is a leading cause of morbidity in humans and remains one of the most commonly isolated pathogens in foodborne illnesses. It poses a significant public health concern worldwide, accounting for approximately 153 million cases of gastroenteritis and 57,000 deaths globally each year (Popa and Papa, 2021). Eggs have been identified as the primary food source for *Salmonella*-related outbreaks in Europe, accounting for a significant proportion of cases reported between 2015 and 2019 across 34 countries, as documented by the European Food Safety Authority (EFSA) (Pinedo et al., 2022). The eggshell, a complex mineral surface with numerous pores, serves as a potential route for microbial contamination, allowing microorganisms to enter the egg content while facilitating gas and water exchange during chick embryo development (Hincke et al., 2012).

Studies have demonstrated that various *Salmonella* isolates can

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persist on the surface of eggshells for up to 35 days at room temperature (Kingsbury et al., 2019; Lee et al., 2020). This ability, combined with the presence of moist organic materials, provides a conducive environment for microbial survival and growth by offering nutrients (Park et al., 2018). Consequently, horizontal transmission or trans-shell contamination can occur when *Salmonella* penetrates the eggshell either during or after oviposition (Messens et al., 2007).

Salmonella has the capability to develop biofilms on various surfaces commonly found in the food industry, including stainless steel, plastics, cement, rubber, and glass (Steenackers et al., 2012; Merino et al., 2019a). This biofilm formation enables bacteria to persist in the environment, leading to potential cross-contamination of diverse food products. *Salmonella* bacteria have the ability to produce an extracellular matrix composed of curli and cellulose, which play crucial roles in several biological processes such as surface adhesion, cell aggregation, and the formation of biofilms. These components are also critical for the environmental persistence of the bacteria (Solano et al., 2002; Pradhan et al., 2023). The synthesis of both curli and cellulose is co-regulated by a complex regulatory network, with the LuxR-type regulator CsgD playing a central role (Römling, 2005). The coordinated expression of curli and cellulose leads to the formation of a highly hydrophobic film composed of densely packed cells (Speranza et al., 2011). The CsgD regulator activates the biosynthesis of the extracellular polymeric matrix, including cellulose exopolysaccharide, curli, and biofilm-associated proteins (Baps), facilitating the transition of bacteria from the planktonic stage to the multicellular state (Latasa et al., 2005; Liu et al., 2014). The *csgA* gene is involved in the synthesis of the A subunit of curli-type fimbriae, while the *bcsA* gene is responsible for the synthesis of cellulose synthetase (Aya Castañeda et al., 2015).

Lactic acid bacteria (LAB) strains, due to the production of several antimicrobial components, offer a natural alternative for the prevention, control of foodborne pathogens (Sharma et al., 2017; Ibrahim et al., 2021; Toushik et al., 2021), and can reduce the formation of biofilms by *Salmonella* spp. in several abiotic surfaces (Merino et al., 2019b). However, the specific biofilm formation capacity of these strains on eggshell surfaces remains an area of limited investigation, and the efficacy of biofilm eradication strategies in this specific context remains limited. It is hypothesized that *S. enteritidis* (SE 115) is capable of forming biofilms on eggshell surfaces, and that the presence of three lactic acid bacteria (LAB) isolated from kefir grains (*Lentilactobacillus kefiri* strains 83113 and 8321, and *Lactiplantibacillus plantarum* strain 83114) will influence the development of SE 115 biofilms on these surfaces. In addition, we investigated the impact of *L. kefiri* 8321 on the synthesis of curli and cellulose, as well as the transcriptional expression patterns of genes associated with these central components of the *Salmonella* biofilm matrix.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Lentilactobacillus kefiri strains CIDCA 83113 and CIDCA 8321 and *Lactiplantibacillus plantarum* strain CIDCA 83114 were isolated from kefir grains and previously characterized and shown ability to inhibit the formation of *Salmonella* biofilm on several surfaces (Merino et al., 2019a). LAB were grown under aerobic conditions in De Man-Rogosa-Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France) for 48 h at 30 °C. *S. enteritidis* CIDCA 115, was isolated from poultry houses and kindly delivered by Dr. Dante Bueno (INTA, Concepción del Uruguay, Argentina). SE 115 strain was stored at -80 °C in cryogenic cultures and routinely subcultured in Luria Bertani (LB) broth (Biokar Diagnostics, Beauvais, France) for 18 h at 37 °C. The biofilm assays were performed in Brain Heart Infusion (BHI) medium (Biokar Diagnostics, Beauvais, France), in which both *Salmonella* and LAB grow properly (Merino et al., 2019a). Conditions of biofilm formation are described above.

2.2. Disinfection of the eggshell samples

The surface of individual eggs (small size), purchased locally, was marked with pencil (area of 1 cm^2) and disinfected by dipping them in 15 % w/v sodium hypochlorite solution for 5 min, then rinsed with sterile water to immerse them in 40 % v/v formalin solution for 5 min. Finally, the eggs were immersed for 5 min in 70 % v/v ethanol solution and allowed to dry in the laminar flow cabinet.

2.3. Salmonella biofilm formation and quantification on eggshell samples

For the study of biofilm formation, the method described by Pande et al. (2016) was used, with some modifications. One cm^2 pieces of eggshell were cut, disinfected as described above, were placed in tubes containing 10 mL of BHI medium and inoculated with 100 μL of SE 115 culture in stationary phase (2×10^8 CFU/mL), and then tubes were incubated for 48 h at 28 °C. The pieces of egg were removed and then washed twice with sterile phosphate-buffered saline (PBS: KH_2PO_4 0.144 g/L; NaCl 9 g/L; Na_2HPO_4 0.795 g/L, pH 7.2) to release the non-adhered bacteria. This allows the removal of excess unattached cells under controlled pH conditions

The number of viable *Salmonella* in the biofilm was determined by the plate count method as described earlier by Castelijn et al. (2012), with some modifications. The pieces of eggshell over which biofilm was developed were placed in a Falcon tube containing 10 mL of 0.1 % w/v tryptone (Biokar Diagnostics, Beauvais, France) and sterilized glass beads. Tubes were gently shaken with vortex for 1 min and tenfold serial dilutions of the bacterial suspension obtained were made in 0.1 % w/v tryptone, 100 μL of suspension was plated on LB agar plates and incubated for 18 h at 37 °C. Results were expressed as colony forming units per cm^2 of eggshell (CFU/cm^2).

2.4. Biofilm architecture by scanning electron microscopy (SEM)

Salmonella biofilm structure on eggshell samples was evaluated by SEM. Following incubation at 28 °C for 48 h, the pieces of eggshell containing biofilm were washed twice with sterile PBS to remove non-attached bacteria. Biofilm developed on shell surface was fixed with 4 % v/v glutaraldehyde solution (Riedel de Haen, Seelze, Germany) in PBS for 24 h at room temperature. Then, eggshell samples were washed with PBS and dehydrated. Dehydration process involved successive incubations, of 15 min each one, in ethanol solutions of increasing concentration (50, 60, 80 and 100 % v/v ethanol in PBS). Then, samples were dried by a critical point inside the pressurized chamber at 10 °C. The samples were metallized with Au 24 and the observation was carried out in an electronic scanning electron microscope model FEI ESEM Quanta 200.

2.5. Effect of LAB and cell-free supernatants on Salmonella biofilm formation on eggshell sample

2.5.1. Separation of cell-free supernatant from LAB cultures

LAB a were cultivated in MRS broth medium, at 30 °C for 48 h, centrifuged for 10 min at 7000 × g and supernatants filtered through 0.22 μm membrane filter (Millipore Corporation, Milford, MA 01757, USA) in sterilized conditions to obtain the cell free supernatant at harvest pH (SNa). Alternatively, supernatant pH was adjusted to 7.0 using 5 M NaOH (Sigma Chemical Co., St. Louis, MO, USA) and then filtered to obtain neutralized supernatants (SNneu).

2.5.2. Effect of LAB and their metabolites on SE 115 biofilm formation on eggshell samples

Two different types of experiments were performed on eggshell samples: 1) Pre-incubation treatment: 1 cm^2 pieces of eggshell were put into tubes containing 10 mL of BHI medium, inoculated with LAB culture (2×10^8 CFU/mL) and incubated for 2 h at 30 °C. After that,

eggshell pieces were removed, washed twice with PBS, placed in a new tube containing 10 mL of BHI medium, inoculated with 100 µL of *Salmonella* culture (2×10^8 CFU/mL) and incubated for 48 h at 28 °C. 2). Co-incubation treatment: eggshell pieces were placed in tubes containing 10 mL of BHI medium and inoculated with 100 µl of LAB (2×10^8 CFU/mL). Then, tubes were added with 100 µL culture of *Salmonella* (2×10^8 CFU/mL) and incubated for 48 h at 28 °C. As *Salmonella* biofilm formation control, neither bacteria nor proteins were added. *Salmonella* biofilm formation was evaluated as described in Section 2.3. Each treatment was performed in duplicate in two independent experiments.

To study the effect of metabolites produced by LAB, eggshell samples were placed in Falcon tubes containing conditioned cultures media (BHI medium and equal volume of SNa or SNneu). Tubes were inoculated with SE 115 (2×10^8 CFU/mL) and incubated for 48 h at 28 °C. As a control, 50 % v/v of BHI medium and MRS medium was used. Then, viable counts on plate were performed as described above. Each treatment was performed in duplicate in two independent experiments.

2.6. Effect of L. kefiri 8321 on cellulose and curli production

2.6.1. Fimbria curli and cellulose production

The ability of SE 115 to produce fimbria curli and cellulose was characterized as described by Römling et al. (2003). Ten microliters of *Salmonella* culture were dropped on agar plates containing LB without NaCl and supplemented with 40 mg/L of Congo red (Sigma, Saint Louis, MO) and 20 mg/L of Coomasie brilliant blue (Sigma, Saint Louis, MO). The plates were incubated for 72 h at 28 °C. The colony morphology of *Salmonella* can be classified into three phenotypes: RDAR (red, dry, and rough), which expresses curli and cellulose; PDAR (pink, dry, and rough), which expresses cellulose; and SAW (smooth and wet), which does not express curli or cellulose. Cellulose production also was evaluated through fluorescence by means of visually under UV (light at 366 nm) on LB without NaCl plates containing 200 mg/L of the dye calcofluor (Sigma-Aldrich Co., St. Louis, MO) after 3 days at 28 °C of incubation.

Furthermore, we evaluated the effect of the presence of *L. kefiri* 8321 viable or not, in co-incubation condition on the production of these biofilm components. For this, the culture of *L. kefiri* 8321 grown in MRS for 48 h at 30 °C was centrifuged, washed twice with and resuspended in PBS. Non-viable cells were obtained by heating (5 °C for 30 min in a thermostatised bath) or UV-radiating (30 min in a UV lamp (ENG = 7.89 J/cm2, wav = 254 nm). Fifty microliters were taken of the suspension and co-incubated with 50 µL of SE 115 culture (10^8 CFU/mL) for 5 h at 28 °C. In all cases LABs and SE 115 ratio was 1:1. Ten microliters of each mixed were taken and seeded on LB agar plates with Congo red or calcofluor as explained previously.

2.6.2. Expression of Salmonella biofilm related genes (csgD, bcsA, and csgA)

The expression of genes associated to biofilm formation was evaluated in presence or not of *L. kefiri* 8321 by molecular techniques. *Salmonella* was grown in LB medium for 48 h at 28 °C in a 24-well plate, in the presence or absence of *L. kefiri* 8321.The RNA extraction was carried out from the cells in suspension (*planktonic* state) and from the film recovered from plate surface (*biofilm* state). Briefly, cells were dispersed by vortexing and homogenized by passage through a 22G needle. RNA was homogenized using TRIzol reagent (Invitrogen, Inc.) according to the manufacturer's protocol. RNA from biofilm samples was then extracted using the Direct-zol™ RNA kit MiniPrep Plus (Zymo Research, USA) according to the manufacturer's protocol.

Total RNA (600 ng per sample) was reverse transcribed with random primers using M- MLV Reverse Transcriptase (ThermoFisher Scientific). The expression of *csgD, bcsA, csgA*, and *gyrB* (endogenous normalizer) genes was determined by Real-time quantitative polymerase-chain-reaction (qPCR) and the amplification was performed in a StepOne Plus Real Time PCR System. PCR parameters were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles 15 s at 95 °C and 30 s at 60 ° Data was collected at the end of each cycle. After PCR, dissociation curves were assessed with the following conditions: 1.6 °C/second 95 °C 15 s, 1.6 °C/second 60 °C 1 min and +0.3 °C/second 95 °C 15 s. The relative quantification of gene expression was calculated with the average CT number of each triplicate according to the method ΔΔCt (Livak and Schmittgen, 2001).

Primers used for cDNA amplification for quantitative assessment of target genes are listed in Table 1.

2.7. Statistical analysis

Results were expressed as the mean and standard error of at least three independent assays. Significant differences associated with treatment were analyzed using one-way analysis of variance and Tukey test. The statistical differences between treatments were indicated at different significance levels. Graph Pad Prism 10 was used for data analyzes.

3. Results

3.1. Salmonella biofilm formation on eggshell surface

The ability of SE 115 to develop biofilms on the eggshell surface was visualized by SEM and quantified by plate count of the bacteria removed from the eggshell by swabbing.

SEM images of the eggshell showed an irregular structure with pores (Fig. 1A, indicated by white arrow). The incubation of SE 115 on this substrate showed a configuration with bacterial cells were conspicuously affixed in densely layered, overlapping arrangements (Fig. 1B). *Salmonella* exhibited the formation of compact cell clusters embedded in an extracellular matrix (Fig. 1C, denoted by dotted arrow), consistent with the characteristic of a mature biofilm. After 48 h at 28 °C in BHI medium, the SE 115 mature biofilm on the eggshell presented a count of $(2.2 \pm 0.9) \times 10^8$ CFU/cm^2.

3.2. Effect of lactic acid bacteria strains

The SE 115 biofilm formation on the eggshell surface subsequent to co-incubation or pre-incubation with the LAB strains was evaluated using viable count enumeration method. Among the three LAB strains examined, co-incubation with SE 115 exhibited a substantial reduction in biofilm formation compared to the control group inoculated solely with SE 115 ($p < 0.05$), as depicted in Fig. 2A. The highest inhibition was evidenced by *L. kefiri* 8321 strain that achieved a substantial reduction of two log CFU/cm^2 on biofilm formation. On the other hand, in pre-incubation conditions with *L. kefiri* 83113 and *L. kefiri* 8321 the ability of SE 115 to develop biofilm was significantly reduced ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 2B). In contrast, pre-incubation treatment with the *L. plantarum* 83114 strain did not induce any alteration in the extent of SE 115 biofilm on the eggshell surface. While the *L. kefiri* 8321 strain exhibited the highest inhibitory efficacy during co-incubation, the *L. kefiri* 83113 strain displayed comparable inhibitory capabilities in both co-incubation and pre-incubation conditions. Notably, the *L. plantarum* 83114 strain solely manifested the capacity to modulate

Table 1
Primers used for qPCR.

Gene target	Primer sequence (5′→3′)	Reference
csgD	GCCTCATATTAACGGCGTG (F) AGCGGTAATTTCCTGAGTGC (R)	Aya Castañeda (2015)
bcsA	GCCCAGCTTCAGAATATCCA (F) TGGAAGGGCAGAAAGTGAAT (R)	Aya Castañeda (2015)
csgA	AATGCCACCATCGACCAGTG (F) CAAAACCAACCTGACGCACC (R)	Aya Castañeda (2015)
gyrB	TGACTGCCGTGCTGCTCTTT (F) AGCCAACTCTGACCGAAGCC (R)	Cho (2015)

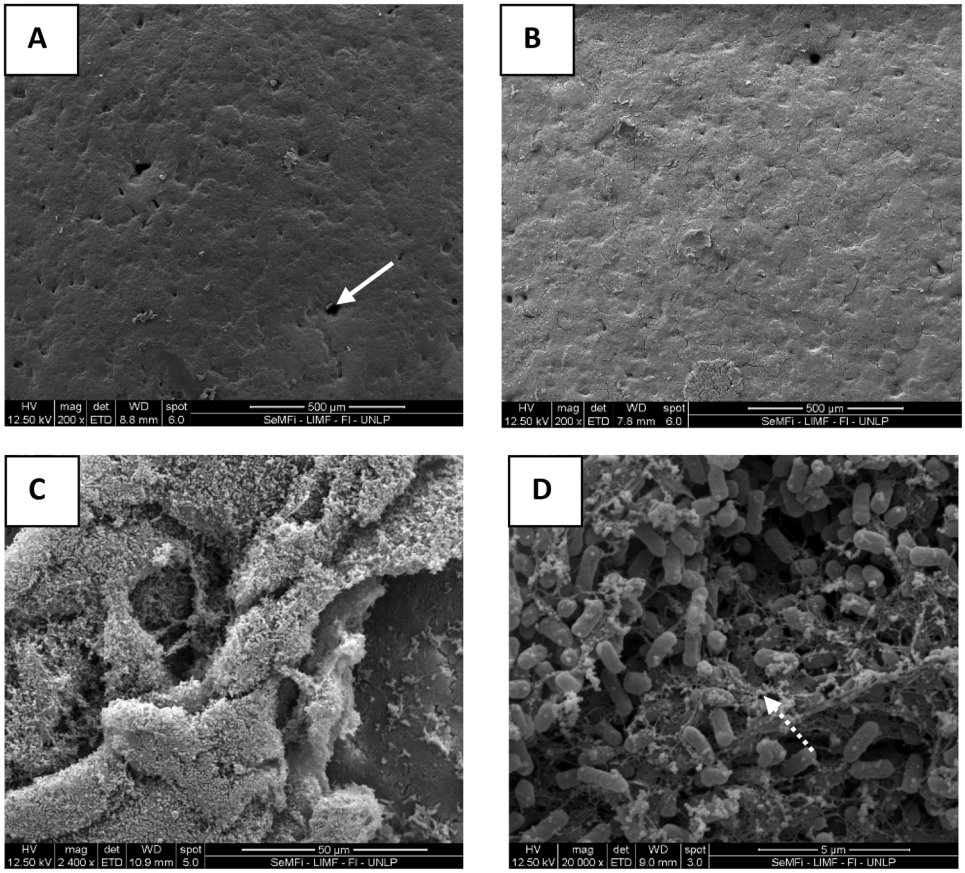

Fig. 1. Scanning electron microscopy images of *S. enteritidis* strain 115 biofilm formed on eggshell. A) untreated eggshell (200X, full arrow indicates pore); B), C) and D) *S. enteritidis* 115 biofilm formation in BHI broth medium 48 h at 28 °C in different magnifications (200X, 2400X and 20000X, dotted arrow indicates the extracellular matrix).

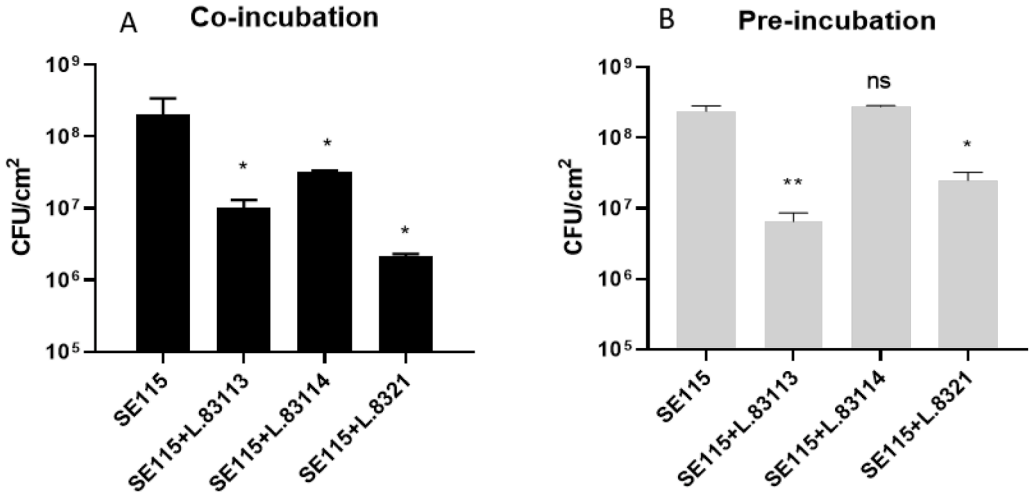

Fig. 2. Quantification by viable counts of *S. enteritidis* strain 115 biofilm formed on eggshell in presence of LAB strains. SE 115 was grown in BHI for 48 h at 28 °C in co-incubation (A) or pre-incubation (B) conditions in presence of LAB strains. Single cultivation of SE 115 was used as control. Significant differences from SE 115 used as control are indicated as ** ($p < 0.01$) and * ($p < 0.05$), ns: no significant differences.

biofilm formation during co-incubation.

The biofilm architecture of SE 115 developed with each LAB strain during co-incubation was additionally subjected to SEM analysis, as depicted in Fig. 3. The SE 115 biofilm appears as a dense cluster-shaped structure characterized by bacterial cells interconnected within a matrix (Fig. 3A). Upon co-incubation of SE 115 with *L. kefiri* 83113, bacterial cells exhibited a scattered distribution on the surface, forming not only small clusters but also extending linearly (Fig. 3B), indicated by full and dotted arrows, respectively. Co-incubation of SE 115 with *L. plantarum* 83114 produced a more homogenous distribution of the biofilm, covering almost the entire surface and no exposed shell surface was observed (Fig. 3C). When SE 115 was co-incubated with *L. kefiri* 8321 small clusters of bacteria scattered on the surface were observed and extracellular matrix was also present (Fig. 3D white arrow) and no

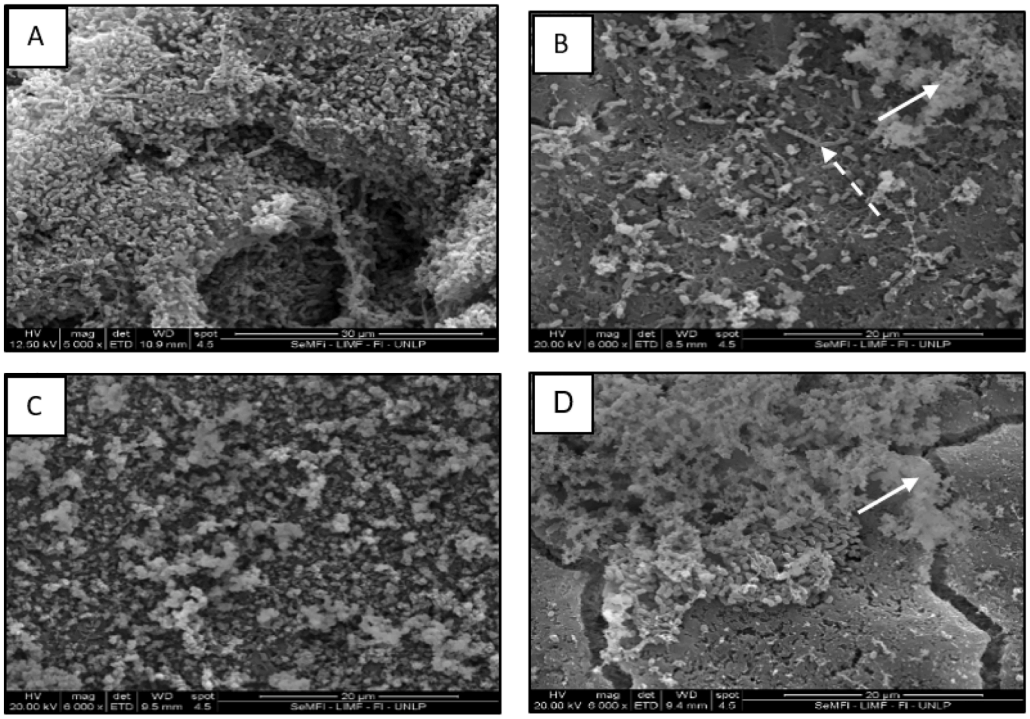

Fig. 3. Scanning electron microscopy images of *S. enteritidis* strain 115 biofilm formed on eggshell during co-incubation with LAB strains. SE 115 was co-incubated in BHI with lactobacilli strains for 48 h at 28 °C and photomicrographs were obtained at different magnifications (5000 to 6000X). A) SE 115 without LAB as control, B) SE 115 + *L. kefiri* 83113, C) SE 115 + *L. plantarum* 83114 and D) SE 115 + *L. kefiri* 8321. The solid arrow marks clusters adhered to the surface indicating the presence of the matrix, while the dotted line indicates the presence of long rods corresponding to lactobacilli.

isolated bacteria were observed.

3.3. Effect of cell-free supernatants

The capacity of SE 115 to develop a biofilm on the eggshell surface in the presence of LAB cell-free supernatants (conditioned culture media), derived either at acidic harvest pH (SNa) or neutralized post-harvest (SNneu), was assessed through viable count enumeration and SEM analysis. For all examined LAB strains, the growth of SE 115 in the presence of their respective SNa or SNneu formulations led to a

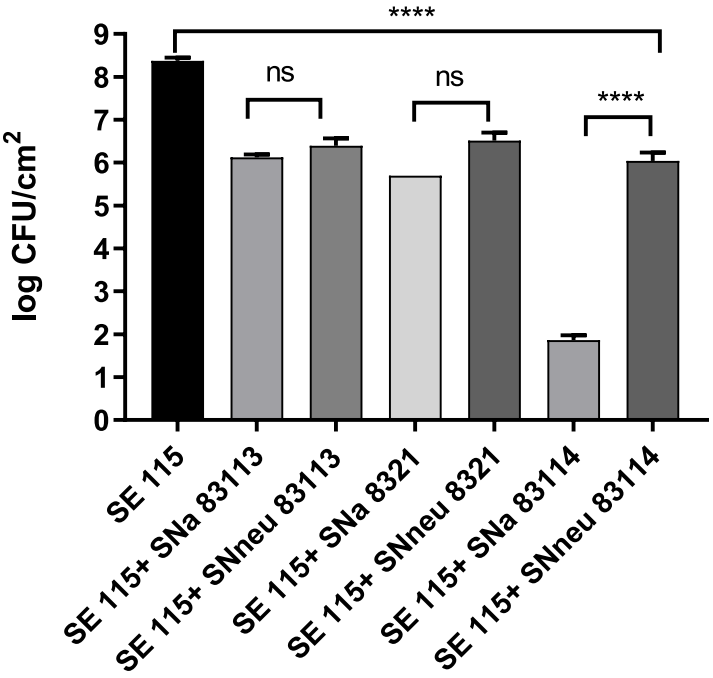

Fig. 4. Quantification by viable counts of *S. enteritidis* strain 115 biofilm formed on eggshell in presence of cell-free supernatants LAB strains at harvest pH (SNa) or neutralized (SNneu). SE 115 was grown in BHI:SNa (1:1), BHI:SNneu (1:1) of LAB strains or BHI:MRS (1:1) used as control. Incubation was performed for 48 h at 28 °C. Significant differences were indicated as **** ($p < 0.0001$). No significant differences were indicated as ns.

substantial reduction in the bacterial count within the biofilm, in comparison to the control group where SE 115 was subjected to an equal volume of fresh MRS medium ($p < 0.0001$). No statistically significant difference was observed between the effects of SNa and SNneu for both *L. kefiri* 8321 (SNa pH = 4.6) and L. *kefiri* 83113 (SNa pH = 5.0). However, in presence of *L. plantarum* 83114 SNa (pH = 3.8), the SE 115 count within the biofilm fell to below 100 CFU/cm^2. No differences were observed among the SNneu formulations derived from the diverse lactic acid bacteria strains investigated (Fig. 4).

SEM images showed that incubation of SE 115 with *L. kefiri* 83113 SNa (Fig. 5B) produced a scattered biofilm, consistent with a reduction in extracellular matrix material compared to the control (Fig. 5A). When SE 115 was incubated with *L. kefiri* 83113 SNneu (Fig. 5E), SEM images showed some cells adhered to the eggshell, with no discernible biofilm formation or extracellular matrix component production.

In the case of SE 115 incubated with *L. kefiri* 8321 SNa (Fig. 5C), a lower density of bacteria was evident, and no biofilm formation was observed. On the other hand, when SE 115 was incubated with *L. kefiri* 8321 SNneu (Fig. 5F), a diminished quantity and density of bacteria was apparent compared to the control, although extracellular polymeric substances were produced. SE 115 incubated with *L. plantarum* 83114 SNa (Fig. 5D) exhibited the formation of isolated irregular structures wherein adherent bacteria were indistinct. In contrast, incubation of SE 115 with *L. plantarum* 83114 SNneu (Fig. 5G) resulted in a high density of adherent bacteria accompanied by extracellular matrix production.

3.4. Effect of L. kefiri 8321 in the production of curli and cellulose by morphotyping

L. kefiri 8321 was selected for further study based on its demonstrated antibiofilm activity against SE115 in co-incubation assays (Fig. 2). Our subsequent experiments were designed to characterize the mechanisms by which this strain inhibits biofilm formation, focusing on morphotyping and the expression of relevant genes involved in matrix production. Morphotyping was used to investigate the effect of *L. kefiri* 8321 on the production of the main components of the *Salmonella* biofilm matrix. After 48 h of incubation at 28 °C on LB agar supplemented with Congo red, SE 115 exhibited an RDAR morphotype colony (Fig. 6a), indicating the simultaneous expression of fimbriae curli and cellulose. When *Salmonella* was incubated with *L. kefiri* 8321 or its SNneu supernatant (Fig. 6d and 6b respectively) a PDAR morphotype was observed, indicating that under these conditions, curli production was inhibited while cellulose production was not. On the other hand, in the presence of SNa derived from *L. kefiri, Salmonella* exhibited a SAW morphotype in Congo red (Fig. 6c), implying a potential negative influence on both curli and cellulose production.

To evaluate cellulose production growth of SE 115 in LB agar medium supplemented with Calcofluor was evaluated. As shown in Fig. 6e, the morphotype observed reveals that co-incubation with LAB results an increased fluorescence pattern spanning both the periphery and central region of the colony (Fig. 6h). This pattern characterises cellulose

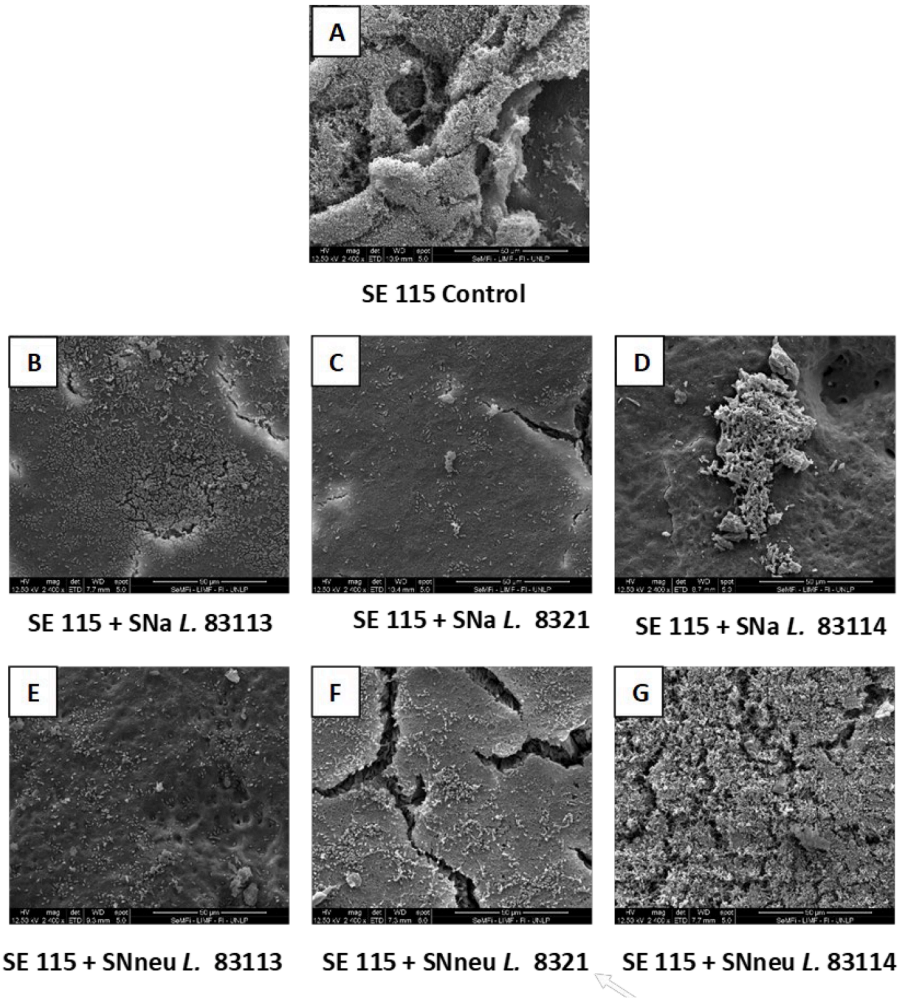

Fig. 5. Scanning electron microscopy images of *S. enteritidis* strain 115 biofilm formatted on eggshell in conditioned culture media by supernatants cell free of LAB strains at harvest pH (SNa) or neutralized post-harvest (SNneu). SE 115 was grown for 48 h at 28 °C in BHI:SNa (1:1), BHI:SNneu (1:1) of LAB strains or BHI:MRS (1:1) as control. Each treatment is indicated below the photomicrograph (magnification 2400X).

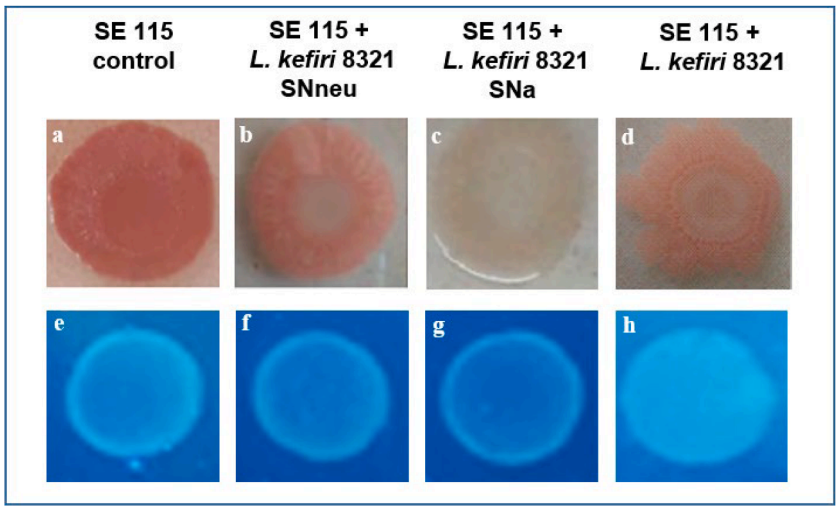

Fig. 6. *S. enteritidis* strain 115 colony morphology. Congo red agar: control *S. enteritidis* 115 (a) showed a RDAR morphotype wich indicates curli and cellulose production; incubation of SE 115 with SNneu (b) or *L. kefiri* 8321 (d) display a PDAR, indicating cellulose production but not curli; incubation with SNa (c) displays a SAW morphotype, indicating not curli or cellulose production. Calcofluor: Control SE 115 (e); SE 115 treated with *L. kefiri* 8321 SNneu (f); SE 115 treated with *L. kefiri* 8321 SNa (g); co-incubation of SE 115 in the presence of *L. kefiri* 8321 (h).

production. Conversely, incubation with SNneu and SNa (Fig. 6f and g respectively) yielded a morphology similar to that of the control SE 115, indicating that cellulose production remained unaltered under these conditions.

As observed on the eggshell surface, the SNa of *L kefiri* 8321 led to a notable reduction in bacterial density and no biofilm formation was observed, possibly due to loss of curli and cellulose production. In the case of co-incubation with either *L. kefiri* 8321 or their SNneu, a discernible reduction in both the quantity and density of bacteria within the *Salmonella* biofilm was noted, concomitant with the presence of extracellular matrix components. This may be due to the absence of curli production, while cellulose production appears to be maintained, in line with the colony morphotype outcomes.

3.5. Expression of biofilm related genes

The expression of *csgD, csgA* and *bcsA* genes associated with biofilm formation in *Salmonella* was studied by reverse transcription and RT qPCR. As shown in Fig. 7A, after 48 h of incubation at 28 °C, no differences were observed in the expression levels of the *csgD* gene in the cells of SE 115 growth in biofilm with respect to the planktonic (basal) state.

An upregulated expression of the *csgA* gene, involved in the synthesis of subunit A within the curli-type fimbriae, and the *bcsA* gene, responsible for cellulose synthetase enzyme synthesis, was observed in biofilm-associated cells in contrast to their planktonic counterparts. Moreover, the co-incubation of SE 115 with *L. kefiri* 8321 resulted in a significant elevation in the expression levels of *csgD, csgA*, and *bcsA* ($p < 0.05$). Specifically, we observed a three-fold increase in *csgD* and *csgA* gene expression in the presence of LAB strain, alongside a two-fold increase in *bcsA* gene expression compared to SE 115 cultivated without LAB strain.

4. Discussion

The present study investigated the biofilm-forming capabilities of *S. enterica* serovar Enteritidis CIDCA 115 (SE 115), an isolate from

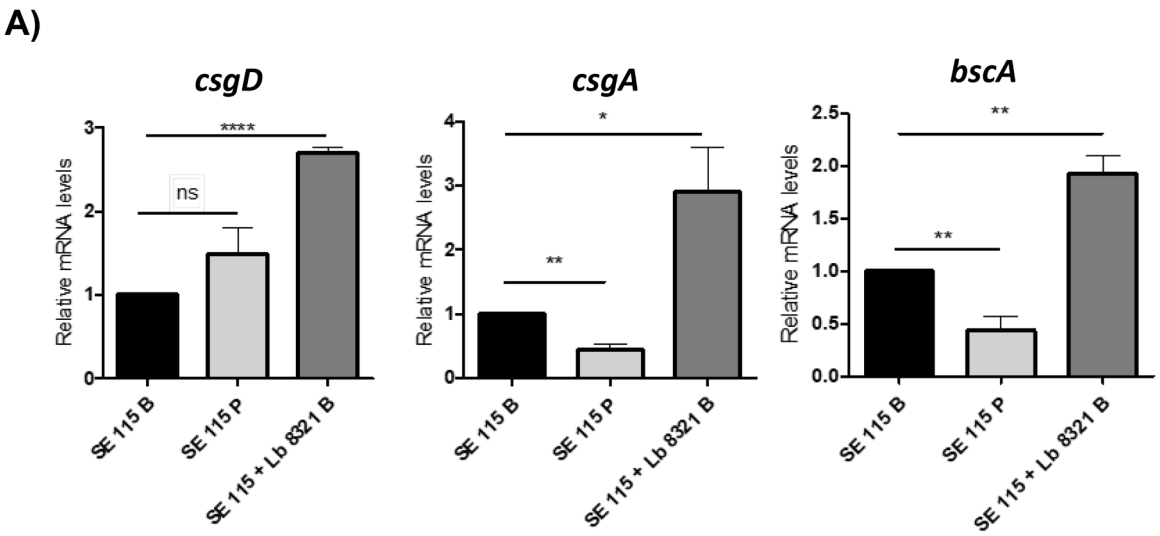

Fig. 7. Relative expression of genes related to biofilm production determined by qPCR. Total mRNA was harvested from *S. enteritidis* strain 115 in biofilm state (black bars, SE 115 B), in planktonic state (light-grey bars, SE 115 P), or in biofilm state in the presence of *L. kefiri* 8321 (dark-grey bars, SE 115 + Lb. 8321). Significant differences from control are indicated as * ($p < 0.01$).

poultry farms, specifically on the surface of commercial chicken eggshells. Furthermore, we explore the impact of three LAB strains and their cell-free supernatants, SE 115 biofilm formation, as well as the production and expression of major matrix components.

In *Salmonella*, the process of biofilm formation depends not solely on surface structures but also on the contribution of extracellular products. The predominant proteinaceous matrix component comprises curli fimbriae, crucial for initial substrate adherence and macrocolony development (Steenackers et al., 2012), while cellulose plays a role in supporting cell-cell interactions and safeguarding the biofilm against mechanical and chemical challenges (Vestby et al., 2009). Our findings reveal that after a 48-hour incubation period, SE 115 assembles a mature biofilm on eggshells, characterized by a dense bacterial mass enveloped within a substantial web-like matrix. These results strongly suggest the presence of curli fimbriae and cellulose as primary extracellular components within the matrix.

Salmonella spp. can persist on eggshell surfaces for up to 3 to 4 weeks post-contamination (Gole et al., 2014a, 2014b). Notably, *S.* serovar Typhimurium has demonstrated the ability to infiltrate both washed and unwashed eggs and survive within the egg albumen for up to 21 days (Gole et al., 2014a), underscoring a substantial food safety concern. In this context, the development of strategies to prevent *Salmonella* adhesion and persistence on surfaces is imperative. LAB have been extensively employed as pathogen control agents and biofilm inhibitors on various surfaces (Hossain et al., 2020; Pang et al., 2022). Therefore, their potential as inhibitors of *Salmonella* biofilm formation on eggshells holds promise. Prior research indicates that the three LAB strains selected for this study possess a co-aggregating ability with SE 115, indicating a surface interaction with *Salmonella*. Additionally, these LAB strains have exhibited antagonistic activity against *Salmonella* biofilm formation on abiotic surfaces commonly found in the food industry (Merino et al., 2019a). In the present work, both co-incubation and pre-incubation treatments with *L. kefiri* 83113, 8321, and *L. plantarum* 83114 have proven effective in reducing biofilm formation with a noticeable disruption of their structure.

The mechanisms underlying the inhibition of pathogen growth and/or biofilm formation may encompass several factors, including the production of organic acids, EPS, or biosurfactant-like substances (Cadieux et al., 2009). Furthermore, competition for nutrients and surface adhesion/attachment sites also plays a key role (Sikorska and Smoragiewicz, 2013), with some instances involving a combination of these mechanisms (Hibbing et al., 2010). In the present study, we observed a significant inhibition to SE 115 biofilm development on eggshells upon incubation with cell-free supernatants derived from LAB. Prior investigations by our research group have revealed that the supernatants of *L. kefiri* 83113 and 8321 strains contain organic acids (containing mainly lactic acid at concentrations around 70 mM) and other metabolites capable of inhibiting the planktonic growth of *Salmonella* (Golowczyc et al., 2007). The notable reduction in SE 115 biofilm formation in the presence of *L. kefiri* supernatants, particularly those with an acidic pH, may be attributed to the presence of dissociated organic acids within the supernatants and the potential involvement of other metabolites such as exopolysaccharides (EPS) or biosurfactants known to be active under acidic pH conditions.

Numerous investigations have demonstrated the efficacy of combined chemical and physical decontamination approaches, such as ultraviolet irradiation, sodium hypochlorite, X-ray irradiation, and chlorine dioxide, in diminishing pathogenic biofilms on eggshells (Park et al., 2018; Jung et al., 2018). However, the practical implementation of these methodologies for biofilm control encounters certain limitations. In particular, the intrinsic toxicity associated with some of the components makes them unsuitable for treating products intended for human consumption. Within the spectrum of generally recognized safe methods, encompassing cleaning and disinfection, bacteriophages, antibiofilm enzymes, natural products, lactic acid bacteria and their bacteriocins have been widely used to control biofilm formation in related contexts with food (Yuan et al., 2020). Eggshell disinfection using acids (acetic, citric, lactic, etc.) is a common practice aimed at reducing microbial contamination on eggs. It has been investigated that lactic acid spray (2 %) effectively reduced *S. enteritidis* count on the eggshell surface and is generally recognized as safe (Li, et al. 2019). Effective acid disinfection of eggs requires careful control of acid concentration to minimize eggshell damage while ensuring sufficient microbial load reduction. Optimal results depend on appropriate concentrations, application methods, and adequate rinsing to preserve egg quality. Culture of lactic acid bacteria (or supernatants alone), with their high lactic acid content, could be used as an alternative approach to remove *Salmonella* from eggshells, either as an individual treatment or integrated with existing biofilm control methods. In this regard, the findings of the present study provide a strong support for the use of LAB as natural barriers to control the formation of *Salmonella* biofilm on eggshell, without representing a risk to the health of consumers.

As described by Solomon et al. (2005), a correlation exists between biofilm formation and bacterial morphotype discernible on Congo red agar. Specifically, a RDAR (red, dry, and rouge) colony morphology serves as an indicator of curli and cellulose production. Serra and Hengge (2017) have expounded upon this correlation, highlighting its fundamental role in agar-based macrocolony morphology assays. These assays serve as a qualitative measure of amyloid curli and exopolysaccharide cellulose production, which are the two predominant extracellular polymeric substance (EPS) constituents in macrocolony biofilms of *Salmonella*. In this context, the co-incubation of SE115 with *L. kefiri* 8321 induces a detrimental impact on curli production while enhancing cellulose production. This leads to a modification of the matrix, where these two elements play a crucial role in structural integrity (Serra et al., 2013).

Bacterial biofilm formation is a complex and coordinated process that requires the sequential activation of genes governing various stages. These stages encompass initial surface adhesion, subsequent microcolony growth, extracellular matrix production, maturation, and eventual biofilm dispersal (Tolker-Nielsen, 2015). The main regulator in biofilm formation, the curli subunit D (*csgD*) gene, activates the biosynthesis of the extracellular polymeric matrix components, cellulose exopolysaccharide, curli and biofilm-associated proteins (Baps). Our study on SE 115 biofilm formation, reveals that *csgD* exhibits comparable expression levels to the control when in the planktonic state after a 48-hour incubation period. This suggests that, at this time, a mature biofilm has been established with the *csgD* gene effectively "turned off." Grantcharova et al. (2010) have posited that this phenomenon may correspond to bistable *csgD* expression. Biofilm formation is considered energetically demanding for cells, primarily due to the extensive production of matrix components. Consequently, *csgD* expression occurs within a subpopulation of bacteria, which is subsequently shared with the broader population within the biofilm. This minimizes energy expenditure and maximizes collective biofilm benefits, constituting a bistable expression pattern. Consequently, two discernible subpopulations of cells arise based on their CsgD content: a high-expression population (*csgD*-ON) and a low-expression population (*csgD*-OFF). Consequently, this bimodal expression of *csgD* contributes to the emergence of phenotypic diversity during biofilm development. Notably, in *Salmonella*, inactivation of *csgD* expression does not diminish the quantity of *csgDEFG* operon mRNA (Römling et al., 2000). This bistable *csgD* expression equips *Salmonella* with adaptability to rapidly respond to environmental fluctuations, especially in stressful or antagonistic conditions. Our results demonstrate that gene expression in the *Salmonella* biofilm is clearly affected by *L. kefiri* 8321. These observations strongly suggest that the presence of this LAB generates increased production of matrix components, although at the expense of a reduced SE 115 bacterial population, a phenomenon consistent with our viable count assays.

The expression of crucial genes, *CsgA* for curli production and *bcsA* for cellulose production, plays a significant role in biofilm formation, as

demonstrated in various in vitro assays (Chen et al., 2021). Our findings reveal a three-fold increase in the relative expression of the curli-associated gene (*csgA*) and a two-fold increase in the cellulose production gene (*bscA*) when SE115 is incubated with *L. kefiri* 8321 compared to the basal state. This upregulation could be attributed to the stress induced by the presence of *Lactobacillus*. Evaluation of morphotypes using Congo red and cellulose indicates that the presence of *L. kefiri* 8321 or its metabolites disrupts curli production while promoting cellulose production. The observed genotypic and phenotypic changes suggest a potential post-transcriptional effect on these biofilm components. Göksel et al. (2022) reported that bacteriocins from certain lactic acid bacteria exhibit antibiofilm activity against *Salmonella typhimurium* 14028.

While *Salmonella* biofilm gene regulation is not fully understood, research indicates that lactic acid bacteria (LAB) can modulate biofilm formation in pathogens. These mechanisms include nutrient competition and interference with quorum sensing (QS), a bacterial communication system regulating biofilm-related genes. Lee and Kim (2014) reported that *Lactobacillus rhamnosus* reduces *Streptococcus mutans* biofilm formation by decreasing the production of glucan, essential components of the extracellular matrix. Wasfi et al. (2018) demonstrated that *Lactobacillus* sp. supernatants reduce the expression of exopolysaccharide production, acid tolerance, and quorum sensing genes in *Streptococcus mutans* biofilms.

Given the significant role of eggs as vehicles for *Salmonella* and its associated public health implications, a comprehensive control strategy is essential in commercial laying flocks. Scientific research and practical experience have consistently shown that the most effective approach involves implementing multiple interventions throughout the egg production cycle (Gast et al., 2024). Lactic acid bacteria and their metabolites have emerged as potential control agents for this pathogen, offering a promising avenue for complementary interventions within a multi-faceted control strategy.

While our review of the literature has uncovered investigations into the influence of LAB supernatants or metabolites on the biofilm-forming ability of *Salmonella*, there is a paucity of publications elucidating the impact of LAB on the expression of *Salmonella* genes associated with biofilm formation. Consequently, our results acquire importance as they contribute to the understanding of the mechanisms underlying the inhibition of LAB-mediated biofilm formation in *Salmonella*. Further research will be imperative to identify the specific bioactive components and molecular pathways responsible for the anti-biofilm properties exhibited by these LAB strains. Furthermore, the development of practical application methodologies adapted to real-world food processing settings will be a promising avenue for future research.

5. Conclusion

Salmonella strains possess the capability to establish biofilms on the surface of eggshells, thus increasing the potential for internalization inside the egg and, consequently, constituting a significant human health hazard. To mitigate microbial populations on eggshells and ensure their sanitary status, various decontamination strategies have been examined. While numerous extant physical and chemical treatments exhibit potent pathogen-killing potential, a drawback exists in some of these methods, characterized by the use of toxic compounds or an inadvertent facilitation of *Salmonella* ingress into the egg interior. In this context, our present study underlines the efficacy of applying LAB cultures, in conjunction with their metabolites, directly onto the eggshell surface for controlling biofilm-forming pathogens such as *Salmonella*. This approach not only enhances food safety but also aligns with the principles of multiple barrier technology. This study has provided additional insights into the influence of the presence of *L. kefiri* 8321 on the expression profiles of critical genes involved in *Salmonella* biofilm formation. Additional research is essential to thoroughly understand how LAB can influence the regulatory mechanisms that govern *Salmonella* biofilm formation across different processing environments. In this work, the effect that co-incubation of *L. kefiri* with *Salmonella* has on the expression of genes that participate in biofilm formation is studied and observed for the first time.

In this scenario, the utilization of lactic acid bacteria and their metabolites can be harmoniously combined with various preventative and preservative measures applied to food products and environmental surfaces. These measures may encompass heat treatments, refrigeration, radiation, essential oils, bacteriophages, and a spectrum of other methods, all of which act synergistically to impede pathogen proliferation across diverse fronts.

Ethics approval and consent to participate

Ethics committee is “Not applicable” in this study.

CRediT authorship contribution statement

Lina E. Merino: Investigation, Formal analysis, Writing – original draft. **Mariángeles Noto Llana:** Investigation, Formal analysis. **Ailín Garófalo:** Investigation, Formal analysis. **Fernando M. Trejo:** Conceptualization, Writing – review & editing. **Marina A. Golowczyc:** Conceptualization, Writing – original draft, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2025.100384.

Data availability

Data will be made available on request.

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