Immunological and bacteriological shifts associated with a flagellin-hyperproducing *Salmonella* Enteritidis mutant in chickens

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Abstract

Salmonella Enteritidis causes infections in humans and animals which are often associated with extensive gut colonization and bacterial shedding in faeces. The natural presence of flagella in *Salmonella enterica* has been shown to be enough to induce pro-inflammatory responses in the gut, resulting in recruitment of polymorphonuclear cells, gut inflammation and, consequently, reducing the severity of systemic infection in chickens. On the other hand, the absence of flagellin in some *Salmonella* strains favours systemic infection as a result of the poor intestinal inflammatory responses elicited. The hypothesis that higher production of flagellin by certain *Salmonella enterica* strains could lead to an even more immunogenic and less pathogenic strain for chickens was here investigated. In the present study, a *Salmonella* Enteritidis mutant strain harbouring deletions in *clpP* and *fliD* genes (SE $\Delta clpPfliD$), which lead to overexpression of flagellin, was generated, and its immunogenicity and pathogenicity were comparatively assessed to the wild type in chickens. Our results showed that SE $\Delta clpPfliD$ elicited more intense immune responses in the gut during early stages of infection than the wild type did, and that this correlated with earlier intestinal and systemic clearance of the bacterium.

Keywords Flagellin · Poultry · Salmonellosis · Mutant · Deletion

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Introduction

Salmonella are Gram-negative, rod-shaped bacteria that belong to the *Enterobacteriaceae* family. Currently, there are about 2659 recognized serovars, most of which are capable of causing illnesses in both humans and animals [1]. Among them, only *Salmonella* Gallinarum and *Salmonella* Pullorum are non-flagellated and non-motile, whereas the majority of serovars are motile by peritrichous flagella.

At the initial stages of gastrointestinal colonization, *Salmonella* uses flagellum-mediated motility to reach the distal ileum and cecum [2]. Flagellin, the main protein of bacterial flagellum, contributes to intestinal inflammation by activating NOD-like and TLR-5 receptors [3]. This activation triggers an immunological cascade that stimulates the innate and adaptive immune responses, contributing to reduce *Salmonella* multiplication in the gut as well as in extraintestinal sites [4, 5]. On the other hand, absence of flagella in some *Salmonella* strains would assist them to be able to escape from

the bird alimentary tract by avoiding triggering the pro-inflammatory response during invasion, thus favouring the development of systemic infection [6, 7].

The synthesis of bacterial flagellum is a complex process regulated by several global transcriptional genes [8], such as *fliD* and *clpP* [9]. *FliD* is a cap protein that temporarily occupies a position in place of nascent flagellin until new copy reaches the growing end of the filament [10], preventing it from leaking out [11]. Therefore, when *fliD* is absent, flagellin monomers are continuously secreted [12]. The ClpXP protease, composed by ClpX and ClpP subunits, is involved in protein quality control and determination of half-life of several flagellar regulators such as RpoS and FlhDC. When *clpP* is deleted, the master flagellum regulator complex FlhD/FlhC is not degraded and large amounts of flagellin are synthesized [13]. Therefore, deletions on *clpP* and *fliD* genes lead to over-expression of flagellin.

Salmonella Enteritidis (SE) is a major zoonotic pathogen mainly associated with the consumption of contaminated eggs and poultry meat products. In chickens, infections are characterized by extensive gut colonization and bacterial shedding in faeces. However, invasive strains can also spread systemically and provoke more severe infections and mortality [14].

Although the role of flagellin in the infection by SE in chicken has been characterized [15], the consequences of flagellin hyperproduction, and hence a more intense stimulation of the chicken innate immune system, to the immunobiology of SE remain to be investigated. In order to further explore this subject, an SE mutant strain with the ability to synthesize higher amounts of flagellin as result of deletion in *clpP* and *fliD* genes (SE $\Delta clpPfliD$) was generated and its immunogenicity and pathogenicity were comparatively assessed to the wild type in chickens in the present study.

Material and methods

Bacterial strains

The experimental trials were performed using a previously reported nalidixic acid-resistant strain (P125109) of *Salmonella enterica* subspecies *enterica* serovar Enteritidis (SE) [15]. The strain was cultured on lysogeny broth (LB – Becton Dickinson, Sparks, MD, USA) at 37 °C for 24 h at 150 revolutions per min (rpm). Experiments were performed at the Department of Animal Pathology, School of Agricultural and Veterinary Sciences (FCAV/Unesp).

Mutant construction

Briefly, two single mutant strains (SE $\Delta clpP$ and SE $\Delta fliD$) of SE were constructed using the Lambda-red method [16]. After that, transduction with the phage P22 was used to transfer the

mutations to a clean genetic background and generate the double-mutant strain (SE $\Delta clpPfliD$). Putative mutants were selected on lysogeny agar (LA – Difco™, Detroit, MI, USA) and confirmed by polymerase chain reaction (PCR). After selection, the resistance genes were eliminated by using a helper plasmid expressing the FLP recombinase (pCP20), which acts directly on the repeated FRT (FLP recognition target) sites flanking the resistance gene. Specific primers were designed through the Primer-BLAST tool [17] and are available in Table 1. The genetically engineered organisms used in this study were constructed under permission issued by the Brazilian National Technical Commission on Biosafety on August 2, 2017 (CTNBIO, permit: 5487/2017).

In vitro experiments

Motility assay and flagellar agglutination assay

SE and SE $\Delta clpPfliD$ swimming motility was detected by propagation on semi-solid agar (SSA). The strains were transferred onto the surface of semi-solid agar consisting of 0.9% heart infusion broth (Oxoid, Basingstoke, Hampshire, UK) and 0.25% nutrient agar (Oxoid, Basingstoke, Hampshire, UK), and their spread through the semi-solid was assessed after incubation at 28 °C for 8 h and the diameters of the swimming halos measured using a digital calliper (Mitutoyo, Aurora, IL, USA). The flagellar synthesis was additionally confirmed by serum agglutination using specific anti-H:g,m antibodies (Remel, Dartford, Kent, UK). The intensity of agglutination was measured by means of a score scale (0 = no agglutination, + = normal agglutination, and ++ = intense agglutination).

SE growth assay

The experiments to assess the growth of the native and the mutant SE strains were performed in biological triplicates on Bioscreen C (Labsystems) at 37 °C for 24 h. Initially, bacteria were grown overnight in LB medium and had their cell concentrations adjusted to the same optical density (OD) ($OD_{600} = 0.05$). Then, 10 μ L of each overnight bacterial suspension was inoculated into 190 μ L LB broth. After gently mixing, 200 μ L aliquots were transferred in triplicate to 96-well microplates as well as pure LB broth as the blank control. At 15-min intervals during incubation under continuous shaking at 37 °C, OD values at 600 nm length wave (OD_{600}) were measured in a microplate spectrophotometer reader; log-transformed OD_{600} values were plotted considering the lag-phase defined as the time necessary for the cells to reach an OD_{600} of 1. The maximum specific growth rate (μ) was calculated according to the logistic equation model using the biological triplicate values of the mutant strain compared to the wild type.

Transmission electron microscopy

Visualization of flagellar filaments was carried out as follows. A 20 µL aliquot of an overnight culture of each strain (SE and SE $\Delta clpPfliD$) cultured in LB broth was spotted onto a carbon-formvar-coated grid (Agar Scientific) and left for 20 s. The grid was washed with excess sterile PBS. Flagellar filaments were stained with 20 µL of 0.5% uranyl acetate for 15 s. The grids were visualized with a JEOL JEM-100S electron microscope.

In vivo trial

Chickens

A total of 126 1-day-old Hy-Line Brown variety chicks were obtained from a commercial hatchery to be used in the experiments. In each experiment, birds were inoculated into the crop using oral gavage needles with 0.2 mL bacterial culture containing approximately 1×10^9 colony-forming units (CFU) of the respective strain (native or mutant SE). The exact

CFU was determined by plating 10-fold dilutions onto suitable agar media as described below. After inoculation, birds were housed in acclimatized rooms and received water and feed ad libitum. On arrival, samples of faeces in the transport cardboard boxes were collected and tested for the presence of *Salmonella* spp. [19]. Experiments were approved by the Ethical Committee on Animal Experimentation from the School of Agricultural and Veterinary Sciences (FCAV/Unesp) on December 4, 2015 (Permit Number: 23042/15).

Experiment 1—mortality, clinical signs, and faecal shedding

Thirty 1-day-old chickens were distributed randomly into two groups of 15 animals each and orally challenged with SE or SE $\Delta clpPfliD$. Birds were observed for 4 weeks on a daily basis, and those showing severe clinical signs such as ruffled feathers, anorexia, and somnolence were humanely euthanized and registered in the mortality records. Bacterial shedding in faeces was monitored by cloacal swabs twice a week.

Table 1 Plasmids and oligonucleotide sequences for PCR-based amplification

Oligonucleotides and plasmids	Sequence 5'-3'	Reference
Oligonucleotides		
C1 F	ttatacgcaaggcgacaagg	[16]
C2 R	gatcttcggtcacaggtagg	[16]
K1 F	cagtcatagccgaatagcct	[16]
K2 R	cggcgccctgaatgaactgc	[16]
fliD50 F	Atggctcaattcatcattaggttagggctcaaa cttacctctggattcgttaggctggagctgttc*	Designed for this study
fliD50 R	Tcaggacttggtcatagcattaaattgctgggtc aaataagtactggtgcatatgaatatcctccttag*	Designed for this study
fliD F	cccacggtttctcaccgtaa	Designed for this study
fliD R	tcaatcaactgatgcgggct	Designed for this study
clpP50 F	Atgtcatacagcggagacgagataattggccc ctcatatggcgtggtgttaggctggagctgttc*	Designed for this study
clpP50 R	Tcaattacgatgggtcaaaattgagtcaccaaacc gtactctaccgcttcataatcctccttag*	Designed for this study
clpP F	cgaaaaccgcgtttcagtg	Designed for this study
clpP R	ttgtccgccttcattag	Designed for this study
Plasmids		
pKD46	Plasmid with λ -red recombinase expressed from arabinose inducible promoter.	[16]
pKD3	Temperature-sensitive replication. Amp ^R	
pKD4	Vector carrying an FRT-Chl-FRT cassette, Chl ^R , Amp ^R ,	[16]
pCP20	Vector carrying an FRT-Kan-FRT cassette, Kan ^R , Amp ^R	[16]
	Carries genes encoding FLP recombinase, temperature-sensitive replication, Amp ^R Chl ^R	[18]

Amp^R ampicillin, Chl^R chloramphenicol, Kan^R kanamycin

*Long primers were used for amplifying antibiotic cassettes. Shorter primers were used for verifying cassette insertion

Experiment 2—caecal colonization, systemic infection, and immunological characterization

Eighty-four 1-day-old chickens were distributed randomly into two groups. They were orally inoculated with SE or SE $\Delta clpPfliD$. A third group comprised by twelve 1-day-old uninoculated birds was kept as the negative control.

Bacterial count in organs

At 2, 5, 7, 14, 21, and 28 days post-infection (dpi), five birds of each inoculated group were euthanized by cervical dislocation and samples of spleen, liver, and caecal contents were collected for bacterial enumeration following methodology described by Berchieri et al. [20].

Immunohistochemistry

Immunohistochemistry was used to determine the influx of macrophages and CD8⁺ and CD4⁺ T cells. Portions of the liver, spleen, and caecal tonsils were collected from three animals in the infected and uninfected groups at 1, 3, 7, and 14 dpi. All samples were fixed in formalin for 24 h, transferred to ethanol 70%, and embedded in paraffin. Then, tissue sections of liver

and caecal tonsil samples were deparaffinized and rehydrated. Antigen recuperation was achieved by heat treatment in 10 mM of sodium citrate buffer (pH 6.0) or Tris-EDTA buffer (pH 9.0) for 30 min. Slides were incubated with endogenous peroxidase blocking solution (3% hydrogen peroxide) for 30 min at room temperature (RT). After washing slides for 10 min with Tris-HCl, sections were incubated for 30 min at RT with Protein Block (Dako, USA) to minimize unspecific binding, and then incubated overnight at 4 °C with anti-chicken CD8 α ⁺ antibody (1:100, SouthernBiotech, USA), anti-chicken CD4⁺ antibody (1:250, SouthernBiotech, USA), and anti-chicken KUL01 (1:100, SouthernBiotech, USA). The reaction was detected by means of a commercial biotin-free polyvalent DAB kit (Reveal, SPD-125, Spring Bioscience, Pleasanton, CA, USA) and 3,3'-diaminobenzidine (DAB, Dako, USA). Tissue sections were randomly photographed in a light microscope (Eclipse Moticam, Nikon, Japan). The percentage of positively stained areas was analyzed using the Image-Pro Plus Software (Media Cybernetics, USA).

Gene expression by qPCR

The minimum information for publication of quantitative real-time PCR experiment (MIQE) principle was adopted in this

Table 2 List of primers used in the SYBR Green-based qPCR analysis

Gene*	Accession number	Sequence (5'→3')	Amplicon (bp)	Reference
UBB	M11100	F: GGGATGCAGATCTTCGTGAAA R: CTTGCCAGCAAAGATCAACCTT	147	[23]
GAPDH	K01458	F: GGCACGCCATCACTATC R: CCTGCATCTGCCCATTT	61	[24]
HPRT	AJ132697	F: CCCAAACATTATGCAGACGA R: TGTCCTGTCCATGATGAGC	66	[24]
ACTB	L08165	F: CACAGATCATGTTTGAGACCTT R: CATCACAATACCAGTGGTACG	101	[24]
28S	XR_003078040.1	F: GCGGAAGCCAGAGGAAACT R: GACGACCGATTTGCACGTC	61	[25]
CCL4	NM_204720	F: CCCCTTGTCATCGGTCAC R: AGAGGCAGGAGCAGAGCA	166	[26]
IL22	NM_001199614.1	F: CAGACTCATCGGTCAGCAAA R: GGTACCTCTCCTTGGCCTCT	217	[27]
IL18	NM_204608.1	F: ACGTGGCAGCTTTTGAAGAT R: GCGGTGGTTTTGTAACAGTG	88	[27]
CXCLi2	NM_205498.1	F: GCCCTCCTCTGGTTTCAG R: TGGCACCGCAGCTCATT	74	[25]
IL6	NM_204628.1	F: GCTCGCCGGCTTCGA R: GGTAAGTCTGAAAGGCGAACAG	71	[25]
IL17	NM_204460.1	F: TATCAGCAAACGCTCACTGG R: AGTTCACGCACCTGGAATG	110	[27]

*UBB, polyubiquitin-C-like; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyltransferase 1; ACTB, actin, beta; 28S, 28S ribosomal RNA; CCL4, C-C motif chemokine ligand 4; IL22, interleukin 22; IL18, interleukin 18; CXCLi2, CXC-like chemokine; IL6, interleukin 8; IL17, interleukin 17

study to determine gene expression [21]. The spleen and caecal tonsils were collected at 1, 3, 7, and 14 dpi from three chickens of each group, including the uninoculated control. Total RNA was purified (RNeasy Mini Kit, Qiagen, GE), and transcription to cDNA was performed using the QuantiTect Reverse Transcription Kit (Qiagen, GE) following the manufacturer's instructions. RNA integrity was assessed by agarose gel electrophoresis and concentration was determined by a spectrophotometer (DeNovix DS11+, USA). RNA integrity and purity were further assessed in an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) as per the manufacturer's instructions for an Agilent RNA 6000 Nano Kit. All RNA samples showed distinct 18S and 28S bands with an average RNA integrity number (RIN) of > 9.0. The cDNA was stored at -20°C until use.

Real-time PCR was carried out in triplicates in a 12.5 μL final volume containing 6.25 μL SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, USA), 0.6 μM of each primer (Sigma-Aldrich, USA), about 50 ng template (cDNA), and ultra-pure water (Sigma-Aldrich, USA). The cycling conditions were 94°C for 2 min, followed by 40 cycles at 94°C for 15 s and 58°C for 30 s. Melting curves were generated after the amplification cycles by gradually increasing the temperature from 65 to 95°C while the signal was taken at each 0.5°C of temperature enhancement. Selection of suitable reference genes for this study was done by the NormFinder software [22]. Transcription stability of ubiquitin (UBB), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyltransferase (HPRT), β -actin (ACTB), and 28S rRNA (28S) were all tested in a representative set of samples

and the cycles of quantification (Cq) submitted to analysis (information obtained from the efficiency curves are displayed in Table S1). Levels of CCL4, CXCLi2, IL6, IL22, IL17, and IL18 mRNA expression were measured in the caecal tonsils at 1, 3, 7, and 14 dpi. Additionally, levels of CCL4, CXCLi2, and IL6 mRNA expression were quantified in the spleen at the same time points. The efficiency curves were generated with tenfold dilution of pooled samples using equal volumes of each cDNA sample, including the negative controls, and new curves were performed for each tested gene every time that a new plate was analyzed. Target samples and non-template controls and efficiency curves were run in triplicates. Primer sequences are listed in Table 2.

The fold change in the mRNA expression of each cytokine-encoding gene was calculated in relation to the reference gene using the $2^{-\Delta\Delta\text{Cp}}$ method [28].

Statistical analysis

Data on mortality and faecal shedding were compared by the chi-square test. Statistical differences among the bacterial counts recovered from the livers, spleens, and caecal tonsils, besides population of macrophages, TCD4+ and TCD8+ lymphocytes by immunohistochemistry, and survival of strains in chicken macrophages, were determined using two-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. Bonferroni's test was used to compare the mean growth rate between the two SE strains.

qPCR data were according to Kolmogorov-Smirnov (KS), D'Agostino-Pearson (DAP), and Shapiro-Wilk (SW) tests.

Fig. 1 Swimming motility exhibited by the wild-type SE and SE $\Delta\text{clpPfliD}$ mutant strain after 8 h of incubation and slide agglutination test with serum against flagellar antigens (a). Growth curves of both strains in LB broth (b). *Statistical difference ($P < 0.01$) between SE $\Delta\text{clpPfliD}$ and SE cell concentrations measured in mean optical density (OD). Transmission electron microscopy of SE $\Delta\text{clpPfliD}$ and SE (c). Black arrows indicate the flagellar filament formation. Scale bar = 2 μm

For parameters showing a non-Gaussian distribution, the non-parametric Mann-Whitney *U* test and Wilcoxon signed-rank test were used. *P* values with *P* < 0.05 were considered statistically significant.

Normalization of cytokine gene expression and all statistical analysis and graph plotting were performed using the GraphPad Prism 7 for Windows, version 7.01.

Results

In vitro studies

After 8 h of incubation in semi-solid agar, diameters of the swimming halos were 39 mm for SE and 31 mm for SE $\Delta clpPfliD$ (Fig. 1a). The serum agglutination test targeting the flagellar antigens (H:g,m) was positive for both strains; however, reaction was more intense for SE $\Delta clpPfliD$ (++) than for SE (+). Both strains were grown on LB broth (Fig. 1a). Bacterial culture reached the logarithmic growth phase at 3 h of inoculation and reached the stationary growth phase at 10 h. An increased growth rate (*P* < 0.05) of the wild-type SE strain was observed from 3 h of incubation (Fig. 1b).

Transmission electron microscopy

Transmission electron microscopy showed an augmented flagellar structure formation on the bacterial surface of the

$\Delta clpPfliD$ mutant compared to that of the wild-type SE strain (Fig. 1c).

In vivo experiments

Experiment 1

Mortality, clinical signs, and faecal shedding At 4 dpi, chickens inoculated with the wild-type SE showed signs of apathy, whereas no clinical signs were detected in birds challenged with SE $\Delta clpPfliD$. Same mortality rates (13.3%, *n* = 2/15) were observed in birds of the two groups. No differences (*P* > 0.05) between the numbers of positive cloacal swabs for SE $\Delta clpPfliD$ (50.9%) and SE (62.7%) were observed.

Experiment 2

Caecal colonization and systemic infection Enumeration of *Salmonella* strains in samples of the liver, spleen, and caecal tonsil is shown in Fig. 2. Both strains were able to invade from the intestine to systemic sites (liver and spleen). However, lower counts of SE $\Delta clpPfliD$ were observed in the spleen at 2 and 14 dpi (*P* < 0.05) and in the liver at 14 dpi compared to SE. Furthermore, the SE was able to colonize the liver and spleen up to 21 dpi, while infection caused by the SE $\Delta clpPfliD$ mutant cleared earlier. It was noticed that the counts of SE $\Delta clpPfliD$ were also lower than SE at 14 dpi.

Fig. 2 The number of viable colony-forming units (CFU) per gram (g) of liver, spleen, and caecal contents of chickens inoculated with SE or SE $\Delta clpPfliD$. Results are expressed as mean $\pm$ standard deviation. Different letters on the plots indicate statistical difference (*P* < 0.05) by Tukey's test at respective time point

Immunohistochemistry After inoculation, changes in the macrophages, and CD4+ and CD8+ T cell populations were measured in the liver, spleen, and caecal tonsils of chickens from the inoculated (SE and SE $\Delta clpPfliD$) and control groups (Fig. 3). Differences on the population of lymphocytes (CD4+ and CD8+) and macrophages in caecal tonsils were observed between the inoculated and control groups at 1, 3, and 7 dpi. At the liver and spleen, these populations differed between inoculated and uninoculated groups mainly at 14 dpi.

Gene expression The analysis using the NormFinder software indicated HPRT as the most stable reference gene (supplementary Table S1). Results of mRNA gene expression are shown in Figs. 4 and 5.

SE $\Delta clpPfliD$ elicited higher levels of CCL4, IL22, and IL18 mRNA expression in the caecal tonsils than SE at 1 dpi and of IL17 at 1 and 3 dpi. No statistical difference was found for IL6 in the spleen and caecal tonsils.

In the spleen, higher levels of Cxcli2, CCL4, and IL6 mRNA were observed in chickens challenged with SE $\Delta clpPfliD$ or SE at 3, 7, and 14 dpi.

Discussion

The findings of the present study indicated that SE $\Delta clpPfliD$ mutant led to increased immune responses in chickens compared with the wild-type SE. These results corroborate a previous study on mouse model showing that the hyperexpression of

Fig. 3 Percentage of populations of macrophages, and CD4+ and CD8+ T lymphocyte cells in the caecal tonsils, spleens, and livers of uninfected chickens and chickens infected with SE or SE $\Delta clpPfliD$ strains at

different days post-infection. Different letters indicate a statistical difference by Tukey's test ($P < 0.05$) among the experimental groups at the respective day post inoculation (dpi)

flagellin due to *clpP* or *fliD* gene deletions could lead to more immunogenic mutant *Salmonella* strains [12].

In spite of the generally accepted concept that flagellar activity in vitro is associated with the stationary phase of the bacterial growth curve [29], it seems that increased levels of transcription of genes encoding the flagellar structure occur during the exponential growth phase [30]. Our data showed a decreased growth rate of the SE $\Delta clpPfliD$ mutant compared to the wild-type SE strain from the beginning of the exponential phase onwards. This was further observed by the smaller

motility halo produced by SE $\Delta clpPfliD$. This is probably attributed to the heavy metabolic burden for the bacterial cell imposed by the overexpression of flagellin, resulting in poor growth rate [31].

Abundant filamentary structure formation resulted from the interaction between *clpP* and *fliD* genes was similar to observed previously in single knockout of *clpP* in *Bacillus subtilis* and *S. Typhimurium* [32, 33]. It seems that deletion of *clpP* leads to misregulation or high levels of *RpoS* and downregulation of the type III secretion system encoded by

Fig. 4 Levels of CCL4, CXCLi2, IL6, IL22, IL17, and IL18 mRNA expression in the caecal tonsils of chicks inoculated with SE or SE $\Delta clpPfliD$ in comparison to uninfected birds at 1, 3, 7, and 14 days

post-infection (dpi). Different letters indicate statistical difference by Tukey's test ($P < 0.05$) among groups at respective time point

Salmonella pathogenicity island 1 (SPI1). Mutations within *rpoS* have been associated with attenuation of virulence of both *S. Typhimurium* and *S. Typhi* in mouse model and humans, respectively [34]. Moreover, *clpP* also regulates the expression of the tubulin-like protein FtsZ, affecting the cell division and resulting in the elongation of the flagellar filament structure [33].

It has been previously demonstrated that the absence of flagellin in SE is critically detrimental to the colonization of the chicken gut [15, 35]. In the present study, data on faecal shedding and caecal colonization indicated that both the mutant SE $\Delta clpPfliD$ and its wild type are similarly capable ($P > 0.05$) to colonize the chicken gut. Our findings corroborate a previous report showing that the faecal shedding of a *S. Typhimurium* mutant containing single gene knockout (*clpP* or *fliD*) has not been abolished in mouse infection [12].

Salmonella colonizes the intestinal tract and breaks through the epithelial barrier in order to trigger a systemic infection. During this process, the innate immunity system is stimulated and macrophages, granulocytes, and dendritic cells are recruited to the sites of infection in order to control the bacterial replication [36, 37]. Flagellin is a key antigen involved in this process acting by stimulating the innate immune system through TLR-5 activation which in turn helps to start and modulate adaptive immune responses [6, 7, 38]. In the present study, the recovery of SE $\Delta clpPfliD$ was lower in caecal contents at 14 dpi compared to that of the SE wild type. We hypothesize that the increased amounts of flagellin synthesized by SE $\Delta clpPfliD$ led to an improved humoral and cellular adaptive immune responses resulting in earlier reductions of the

intestinal colonization by the mutant strain [39, 40]; however, we cannot rule out that slightly lower growth rate may also affect this result.

Moreover, the counts of the mutant strain were lower in the spleen (at 2 dpi) and in the liver (at 14 dpi) and SE $\Delta clpPfliD$ was no longer detected in chickens by 21 dpi. Our results corroborate with those of Kremer et al. [40] on a study in turkeys, who observed lower invasiveness of a SE mutant strain able to overproduce flagellin, which was probably attributed to an increased TLR5 activation.

Interleukins and chemokines are regulatory molecules that act as extracellular signals between cells during the course of immune responses. They can elicit and regulate immune responses and can be produced by every cell type, having pleiotropic effects on cells of the immune system, as well as modulating inflammatory responses. There are different families of interleukins, some of them with pro-inflammatory (CXCLi2, IL6, IL22, and IL18) or anti-inflammatory (IL10) properties. Others are typically produced by certain types of cells; for instance, Th1 lymphocytes produce IFN- γ , and meanwhile, IL4 and IL13 are produced by Th-2, IL-17 by Th17 cells, and CCL4 by macrophages after stimulation with bacterial endotoxins [41].

The mRNA gene quantification of key interleukins and chemokines indicated that SE $\Delta clpPfliD$ induced a more intense host immune stimulation compared to the wild type, mainly at the early stages of infection. At 1 dpi, pro-inflammatory chemokines (CXCLi2 and CCL4) and interleukins (IL22, IL17, and IL18) were expressed at higher levels in the caecal tonsils of SE $\Delta clpPfliD$ -inoculated birds, demonstrating the early

Fig. 5 Levels of CCL4, CXCLi2, and IL6 mRNA expression in spleens of chickens infected with SE or SE $\Delta clpPfliD$ in comparison to uninfected birds at 1, 3, 7, and 14 days post-infection (dpi). Different letters indicate statistical difference by Tukey's test ($P < 0.05$) among groups at respective time point

activation of the innate immunity components in the caecum. It is known that the synthesis of flagellin by *Salmonella* is abolished during systemic infection, right after crossing the intestinal barrier lumen, indicating that either flagellin does not play a role in the systemic phase of infection [2] or it is detrimental to the bacterium to express the flagella at systemic sites. This fact could explain the similar patterns of mRNA expression elicited by both strains in the spleen observed in our study.

The triggering of the adaptive immune response depends on the antigen-presenting dendritic cells that activates CD4+ or CD8+ T cells by presenting antigens via MHC class II or class I molecules, respectively. Changes in the organ populations of CD4+ or CD8+ T cells and macrophages were similar between SE $\Delta clpPfliD$ - and SE-inoculated chickens. Significant differences were observed only in uninoculated birds. These results corroborate with those of Lee et al. [42] who reported a slight boost of T cell-mediated response in mice immunized with flagellin. Although the overexpression of flagellin did not lead to higher population of lymphocytes and macrophages in the SE $\Delta clpPfliD$ -inoculated birds, the immune response elicited by this mutant strain was more efficient to clear systemic infection, as indicated by the bacterial counts in the liver and spleen at 14 and 21 dpi. Therefore, our results suggest a Th1-mediated modulation of the adaptive immune response associated with a more efficient tissue clearance in SE $\Delta clpPfliD$ -inoculated chickens.

Conclusion

The hyperproduction of flagellin by a mutant *Salmonella* Enteritidis lacking both *clpP* and *fliD* genes modulates the T cell immune response in the chicken gut at early stages of infection affecting bacterial clearance and invasiveness, although no changes in pathogenicity occur.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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