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A colonisation-inhibition culture consisting of *Salmonella* Enteritidis and Typhimurium Δ*hilAssrAfliG* strains protects against infection by strains of both serotypes in broilers

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ABSTRACT

Consumption of contaminated poultry meat is still an important cause of *Salmonella* infections in humans and there is a need for control methods that protect broilers from day-of-hatch until slaughter age against infection with *Salmonella*. Colonisation-inhibition, a concept in which a live *Salmonella* strain is orally administered to day-old chickens and protects against subsequent challenge, can potentially be used as control method. In this study, the efficacy of a *Salmonella* Typhimurium Δ*hilAssrAfliG* strain as a colonisation-inhibition strain for protection of broilers against *Salmonella* Typhimurium was evaluated. Administration of a *Salmonella* Typhimurium Δ*hilAssrAfliG* strain to day-old broiler chickens decreased faecal shedding and strongly reduced caecal and internal organ colonisation of a *Salmonella* Typhimurium challenge strain administered one day later using a seeder bird model. In addition, it was verified whether a colonisation-inhibition culture could be developed that protects against both *Salmonella* Enteritidis and Typhimurium. Therefore, the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain was orally administered simultaneously with a *Salmonella* Enteritidis Δ*hilAssrAfliG* strain to day-old broiler chickens, which resulted in a decreased caecal and internal organ colonisation for both a *Salmonella* Enteritidis and a *Salmonella* Typhimurium challenge strain short after hatching, using a seeder bird model. The combined culture was not protective against *Salmonella* Paratyphi B varietas Java challenge, indicating serotype-specific protection mechanisms. The data suggest that colonisation-inhibition can potentially be used as a versatile control method to protect poultry against several *Salmonella* serotypes.

1. Introduction

Despite the implementation of numerous monitoring and control measures in broiler production, *Salmonella* is still an important cause of poultry meat associated human infections [1]. Broilers often become infected with *Salmonella* early after hatching as they are highly susceptible to infection during these first days of life [2]. This is mainly due to the absence of normal gut microbiota in young chickens and the immaturity of their immune system [3–7]. Infection during this period, even with low numbers of *Salmonella*, can lead to persistent carriers [8,9]. These broilers are often still infected at slaughter age, which may result in introduction of *Salmonella* in the slaughter house and food chain [10]. Consequently, prevention of infection during this period in which the chick is highly susceptible to infection could strongly reduce the introduction of *Salmonella* in the food chain.

Colonisation-inhibition (CI) is a phenomenon in which chickens are administered a live *Salmonella* strain that protects against subsequent challenge with another *Salmonella* strain [11]. By administering a CI strain that colonises the gut rapidly and extensively, it is possible to increase resistance to *Salmonella* strains quickly after hatching [12]. This concept has been recognised for a long time, and a great deal of effort has been put in developing strains that are appropriate for use as CI strains [13,14]. Earlier research demonstrated that deletion of the *hilA*, *ssrA* and *fliG* genes in a *Salmonella* Enteritidis strain resulted in a CI strain that was safe and effective in protecting broilers against challenge with a *Salmonella* Enteritidis wild-type strain [15]. Because there is greater inhibition within a serovar than between serovars [16], the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain can be expected to mainly protect against *Salmonella* Enteritidis infection, and not or to a lesser extent against *e.g.*, *Salmonella* Typhimurium infection. In 2011, 0.3% of all broiler flocks were positive for *Salmonella* Enteritidis and *Salmonella* Typhimurium in Europe, while 3% were positive for other *Salmonella* serotypes [1]. Consequently, if CI strains are needed that protect against these other serovars, new

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CI strains need to be developed. It is however unknown whether introduction of the *hilA*, *ssrA* and *fliG* mutations in a *Salmonella* strain belonging to another serovar yields a CI strain displaying the same degree of attenuation and similar protective properties as the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain. Additionally, it is not known whether a combination of two or more CI strains, belonging to different serovars, is able to protect against infection by different *Salmonella* serovars.

In the present study, the efficacy of a *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant as a CI strain was evaluated. Secondly, the protective effect of a CI culture consisting of both a *Salmonella* Enteritidis and Typhimurium *hilAssrAfliG* deletion mutant against *Salmonella* Enteritidis, Typhimurium and Paratyphi B var. Java infection was evaluated.

2. Materials and methods

2.1. Chickens

One-day-old Ross 308 broiler chickens were obtained from a local hatchery and housed in separate rooms in containers on wood shavings. Commercial feed and drinking water were provided *ad libitum*. Experiments were performed with the permission of the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (experiment authorisation number: EC2012/96).

2.2. Bacterial strains and deletion mutants

A spontaneous nalidixic acid-resistant mutant of *Salmonella* Typhimurium strain 112910a, originally isolated from a pig stool sample [17], was used for the production of isogenic mutants. This resistance has previously been shown to have no impact on the *in vivo* results [13]. Deletion of *hilA*, *ssrA* and *fliG* genes in this strain was done using the one-step inactivation method described by Datsenko and Wanner [18]. *Salmonella* Typhimurium MB2136, a streptomycin resistant wild-type strain originally isolated from swine was used as a challenge strain. A nalidixic acid-resistant *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant, which has been described earlier [15], was also used in this study. The original *Salmonella* Enteritidis 76Sa88 strain, from which the *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant is derived, was originally isolated from a poultry farm [2,19]. *Salmonella* Enteritidis 147 (streptomycin resistant), a strain originally isolated from chicken egg white and which is known to colonise the gut and internal organs of chickens to a high level [12,20,21], was used as a challenge strain. Additionally, a wild-type *Salmonella* Paratyphi B. var. Java strain (carbenicillin resistant) originally isolated from poultry, was also used as a challenge strain.

3. Experimental design

3.1. Experiment 1: efficacy of a Salmonella Typhimurium hilAssrAfliG deletion mutant against experimental Salmonella Typhimurium infection

In order to evaluate the persistence of a *Salmonella* Typhimurium Δ*hilAssrAfliG* strain in chickens and its efficacy against colonisation by a wild type *Salmonella* Typhimurium strain, 225 one-day-old chicks were divided into three groups of 75 animals and each group was housed in a container of 2 m^2. Two groups (Group V and C) were given 10^9 CFU of the *Salmonella* Typhimurium *ΔhilAssrAfliG* strain by oral gavage while the third group was given sterile HBSS (Hank's Balanced Salt Solution, Invitrogen, Paisley, England) as a control (Group I). Twenty-four hours later, 25 randomly selected chickens in Group I and Group C were given 10^5 CFU *Salmonella* Typhimurium MB2136 by oral gavage. These seeder birds were housed together with the other chickens of their group. Bacterial counts in caecum and spleen were determined for one third of the original number of chickens by bacteriological analysis at 7, 21 and 42 days old. At each time point, one in three sampled animals were seeder birds. Shedding of both strains was monitored by cloacal swabbing on days 2, 3, 9, 16, 23, 30 and 37.

3.2. Experiment 2: efficacy of simultaneous administration of a Salmonella Typhimurium hilAssrAfliG and a Salmonella Enteritidis hilAssrAfliG deletion mutant against infection with several Salmonella serotypes

In order to evaluate the efficacy of a CI culture containing a *Salmonella* Enteritidis Δ*hilAssrAfliG* and *Salmonella* Typhimurium Δ*hilAssrAfliG* strain against infection by several *Salmonella* serotypes, 60 one-day-old chickens were divided into 6 groups of 10 animals. Three of these groups (Group VSE, VST and VSJ) were given 0.5 ml of a mixture containing 2×10^8 CFU/ml of the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain and 2×10^8 CFU/ml of the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain by oral gavage. The three remaining groups (CSE, CST and CSJ) were given sterile HBSS as a control. Twenty-four hours later, 2 randomly selected chickens in each group were given 10^5 CFU of a challenge strain by oral gavage. These seeder birds were then housed together again with the other chickens of their group. Groups VSE and CSE were challenged with *Salmonella* Enteritidis strain 147, Groups VST and CST with *Salmonella* Typhimurium strain MB2136 and Groups VSJ and CSJ with the wild-type *Salmonella* Paratyphi B var. Java strain. Bacterial counts of CI strains and challenge strains in caecum and spleen were determined by bacteriological analysis on day 7.

3.3. Bacteriological analysis

Cloacal swabs were directly inoculated on Xylose Lysine Deoxycholate agar (XLD, Oxoid, Basingstoke, England) plates with 20 μg/ml nalidixic acid (Sigma–Aldrich, St. Louis, MO, USA) or 100 μg/ml streptomycin (Sigma–Aldrich, St. Louis, MO, USA). Samples negative after direct inoculation were pre-enriched in buffered peptone water (BPW, Oxoid, Basingstoke, England) and incubated overnight at 37 °C. One ml of this suspension was further enriched by adding 9 ml tetrathionate-brilliant green broth (Merck, Darmstadt, Germany). After overnight incubation at 37 °C, this suspension was plated on XLD plates supplemented with the appropriate antibiotic. Samples of caecum and spleen were homogenised in BPW and 10-fold dilutions were made in HBSS. Six droplets of 20 μl of each dilution were plated on XLD plates supplemented with 20 μg/ml nalidixic acid, 100 μg/ml streptomycin or 100 μg/ml carbenicillin (Sigma–Aldrich, St. Louis, MO, USA). After overnight incubation at 37 °C, the number of CFU/g tissue was determined by counting the number of bacterial colonies on the plates.

3.4. Statistical analysis

GraphPad Prism 5 software was used for statistical analysis. A Fisher's exact test (one-sided) was used to analyse differences in mortality between groups. A Kruskal–Wallis test (one-way ANOVA) was used to determine statistical differences of the number of *Salmonella* positive cloaca swabs among groups. Bacterial counts in caecum and spleen were converted into logarithmic form for statistical analysis. Samples of caecum and spleen negative after direct plating were rated as $\log_{10} = 0$. The mean CFU/g tissue was calculated for each group on every time point and differences

Table 1
The number of cloacal swabs positive for a *Salmonella* Typhimurium Δ*hilAssrAfliG* strain or a *Salmonella* Typhimurium challenge strain. Groups V and C were orally inoculated with 10^9 CFU of a *Salmonella* Typhimurium Δ*hilAssrAfliG* strain at day 1 of the experiment (day-of-hatch). Groups I and C were challenged with 10^5 CFU of a *Salmonella* Typhimurium challenge strain on day 2 of the experiment. Samples were taken at days 2, 3, 9, 16, 23, 30 and 37 of the experiment.

Strain	Group	Day 2	Day 3	Day 9	Day 16	Day 23	Day 30	Day 37
Salmonella Typhimurium Δ*hilAssrAfliG* strain	V	75/75[a] (75[b])	75/75 (72*)	49/49 (47)	47/47 (30*)	24/24 (20*)	24*/24 (20*)	22*/23 (18*)
	C	75/75 (67)	NA/75 (17*)	NA/50 (NA)	NA/48 (10*)	0/23 (0*)	0*/22 (0*)	0*/22 (0*)
Salmonella Typhimurium challenge strain	I	NA	18/75 (8)	45*/50 (41*)	35*/49 (12)	5/25 (5)	10/25 (7)	10/25 (7)
	C	NA	12/75 (0)	10*/50 (0*)	0*/48 (0)	0/23 (0)	0/22 (0)	0/22 (0)

[a] Number of positive samples after enrichment/total number of samples.
[b] Number of positive samples after direct plating.
* Significant difference in positive samples between both groups (p-value < 0.05). NA = Not available.

between groups were analysed using a Kruskal–Wallis test (one-way ANOVA) or a Mann–Whitney test. Differences with p-values lower than 0.05 were considered to be significant.

4. Results

4.1. Experiment 1: efficacy of a Salmonella Typhimurium hilAssrAfliG deletion mutant against experimental Salmonella Typhimurium infection

During the experiment, four chickens died in Group V, five in Group I and four in Group C. The observed differences in mortality were not statistically significant.

Shedding of the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain remained high during the entire experiment in Group V (Table 1). Shedding of this strain decreased quickly in Group C. The *Salmonella* Typhimurium Δ*hilAssrAfliG* strain was excreted until day 16 by a limited number of chickens, after which it could no longer be detected. Shedding of the *Salmonella* Typhimurium challenge strain was lower in the group treated with a *Salmonella* Typhimurium Δ*hilAssrAfliG* strain during the entire experiment when compared to the sham-treated control. Statistical differences are shown in Table 1. Data on shedding of the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain on days 3, 9 and 16 of the experiment are not available due to overgrowth of other bacteria on the culture media.

Bacteriological analysis of the caecum samples showed that the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain colonised the caecum

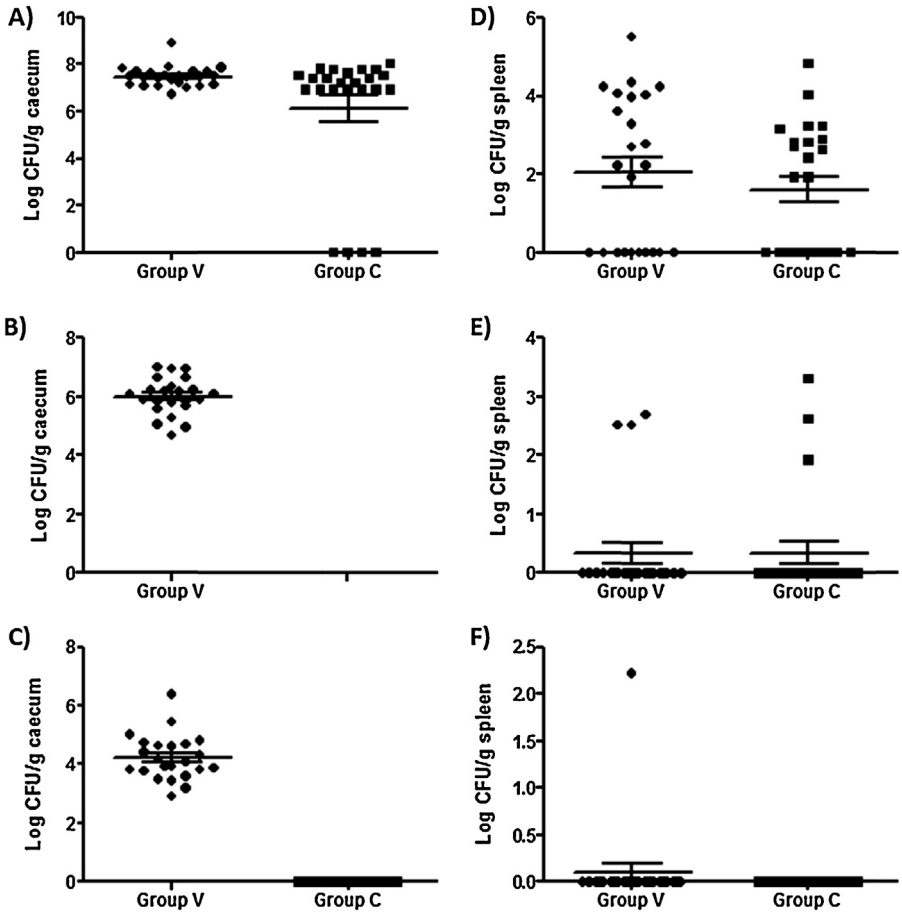

Fig. 1. Caecal (A–C) and spleen (D–F) colonisation by a *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant strain. Chickens in Group V and C were given 10^9 CFU of a *Salmonella* Typhimurium Δ*hilAssrAfliG* deletion mutant strain at day-of-hatch. Additionally, chickens in Group C were given 10^5 CFU of a *Salmonella* Typhimurium challenge strain on day 2 of the experiment. Subfigures A and D show colonisation on day 7, B and E on day 21 and C and F on day 42. Values shown are $\log_{10}$ of CFU/g sample. The horizontal lines represent the mean, the error bars represent the standard error of mean (SEM). The number of samples equals 25, 23 and 23 in Group V and 25, 23 and 22 in Group C on day 7, 21 and 42 respectively. No data are available for the caeca of Group C on day 21.

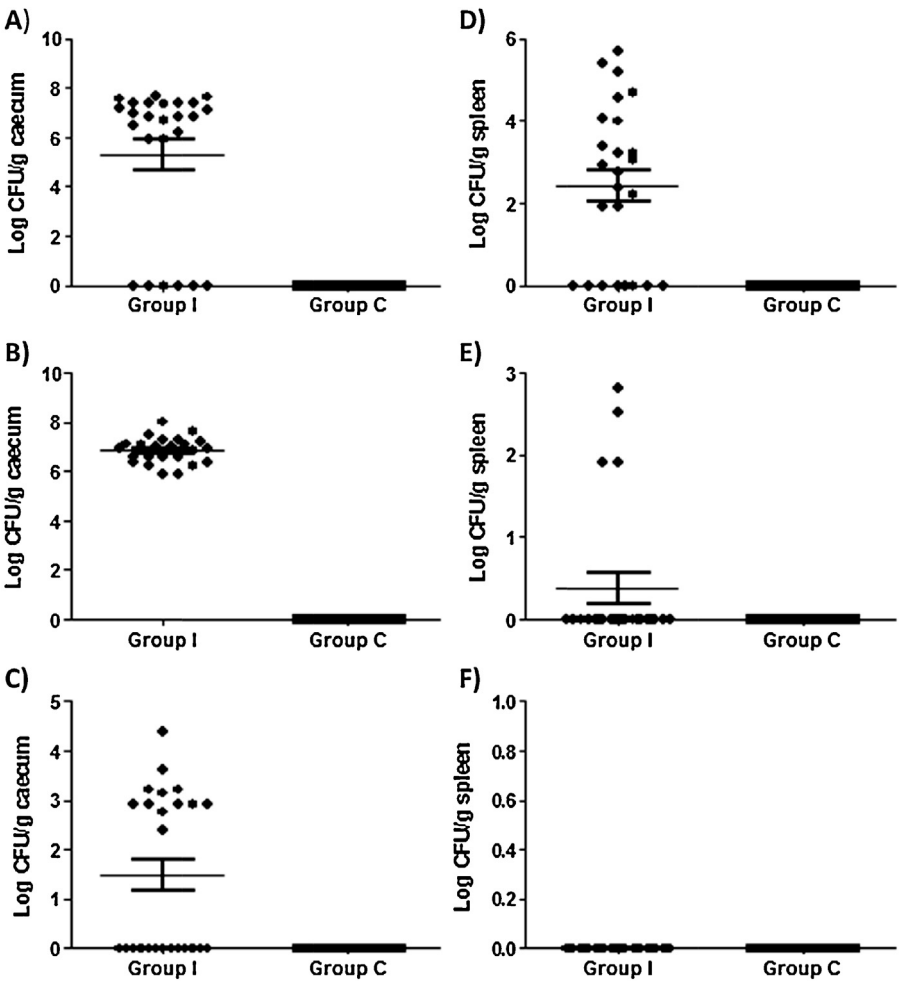

Fig. 2. Caecal (A–C) and spleen (D–F) colonisation by a *Salmonella* Typhimurium challenge strain. Chickens in Group C were given 10^9 CFU of a *Salmonella* Typhimurium *hilAssrAfliG* deletion mutant strain at day-of-hatch. Chickens in Group I and C were given 10^5 CFU of a *Salmonella* Typhimurium challenge strain on day 2 of the experiment. Subfigures A and D show colonisation on day 7, B and E on day 21 and C and F on day 42. Values shown are $\log_{10}$ of CFU/g sample. The horizontal lines represent the mean, the error bars represent the standard error of mean (SEM). The number of samples equals 25, 24 and 25 in Group I and 25, 23 and 22 in Group C on day 7, 21 and 42 respectively.

to similar high levels in Group V and Group C on day 7 (Fig. 1). The bacterial load of this strain was mean $\log_{10} = 7.48$ CFU/g in Group V and mean $\log_{10} = 6.12$ CFU/g in Group C. This reduced to mean $\log_{10} = 6.01$ CFU/g on day 21 and mean $\log_{10} = 4.23$ CFU/g on day 42 in Group V. The strain could no longer be detected on day 42 in Group C. No data were available for the caecum of Group C on day 21 due to overgrowth of other bacteria on the culture media. In the spleen, the bacterial load of the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain amounted on day 7 to mean $\log_{10} = 2.05$ CFU/g in Group V and $\log_{10} = 1.61$ CFU/g in Group C. Bacterial numbers reduced as the experiment proceeded, as the load amounted to mean $\log_{10} = 0.337$ CFU/g in Group V and mean $\log_{10} = 0.341$ CFU/g in Group C on day 21. This reduced further to mean $\log_{10} = 0.097$ CFU/g in Group V on day 42. By then, the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain could no longer be detected in Group C.

Bacteriological analysis of the caecum and spleen showed that the *Salmonella* Typhimurium challenge strain colonised the caecum of the chickens in Group I to high levels, while it could not be detected in any of the caeca of the chickens in Group C at any time point (Fig. 2). The bacterial load and number of spleens positive for the *Salmonella* Typhimurium challenge strain in Group I was initially high, but declined as the experiment proceeded. The *Salmonella* Typhimurium challenge strain could not be detected in any of the spleens on day 42 in Group I, and in any of the spleens of the chickens belonging to Group C at any time point.

4.2. Experiment 2: efficacy of simultaneous administration of a Salmonella Typhimurium hilAssrAfliG and a Salmonella Enteritidis hilAssrAfliG deletion mutant against infection with several Salmonella serotypes

None of the chickens died during the experiment. Bacteriological analysis of the samples showed that the *Salmonella* Enteritidis and Typhimurium Δ*hilAssrAfliG* strains colonised the caecumand spleen to a similar level in all treated groups. Mean colonisation was $\log_{10} = 6.87 \pm 0.12$ and 6.44 ± 0.76 CFU/g in the caeca, and $\log_{10} = 0.74 \pm 0.50$ and 2.02 ± 0.77 CFU/g in the spleens of Groups VSE and VST, respectively. Data on colonisation by the CI culture in Group VSJ is not available because the *Salmonella* Paratyphi B var. Java strain is, like the CI strains, nalidixic acid resistant. Consequently, the CI strains could not be distinguished from the challenge strain. Additionally, bacteriological analysis of caecum and spleen showed that colonisation by the *Salmonella* Enteritidis challenge strain was significantly lower in the caecum of the group treated with CI culture (Fig. 3). No differences could be observed in spleen colonisation. Similarly, colonisation by the *Salmonella* Typhimurium strain was significantly lower in the caecum of the treated group, while no significant difference could be observed in the spleen. Colonisation by the *Salmonella* Paratyphi B var. Java strain did not differ significantly between the treated and untreated group.

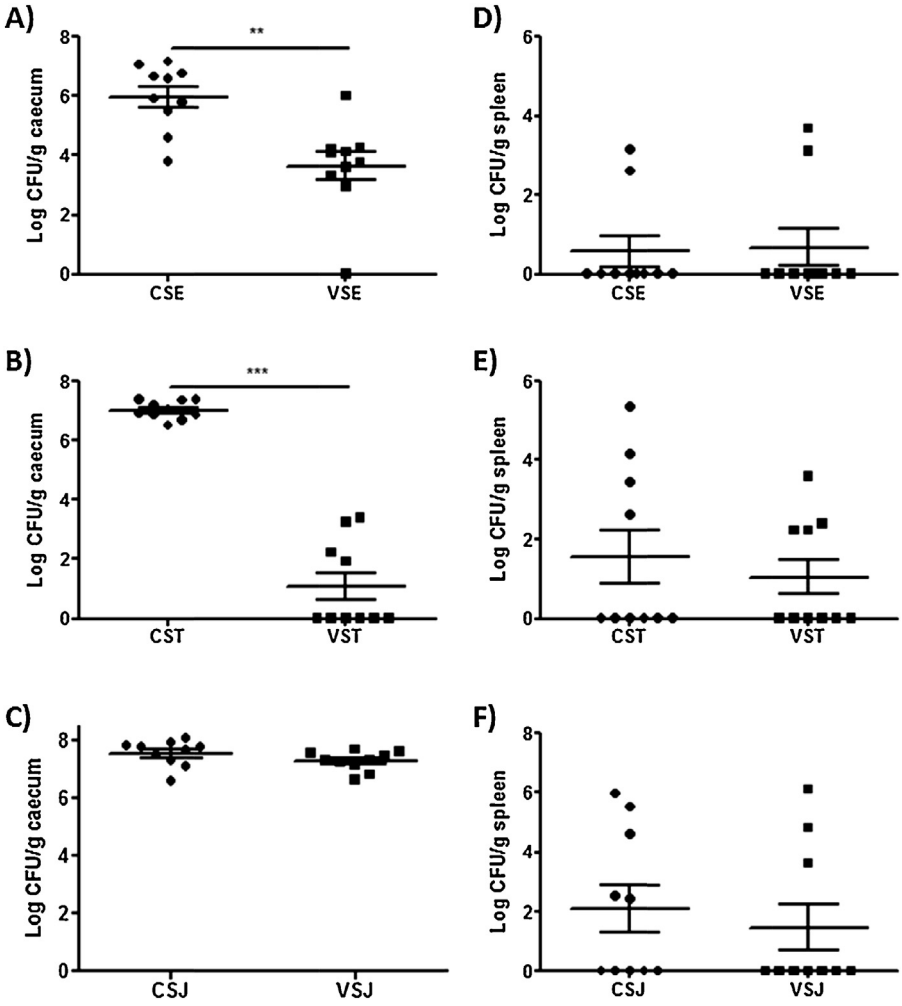

Fig. 3. Caecal (A–C) and spleen (D–F) colonisation by a *Salmonella* Enteritidis, *Salmonella* Typhimurium or *Salmonella* Paratyphi B var. Java challenge strain. Animals from groups VSE, VST and VSJ were orally inoculated with a combination of 10^8 CFU of a *Salmonella* Enteritidis Δ*hilAssrAfliG* strain and 10^8 CFU of a *Salmonella* Typhimurium Δ*hilAssrAfliG* strain at day 1 of the experiment (day-of-hatch). All groups were infected with 10^5 CFU of a challenge strain on day 2 of the experiment. Groups CSE and VSE were infected with a *Salmonella* Enteritidis challenge strain (A and D), groups CST and VST with a *Salmonella* Typhimurium strain (B and E) and groups CSJ and VSJ with a *Salmonella* Paratyphi B var. Java strain (C and F). Samples were taken at day 7 of the experiment.

5. Discussion

Newly hatched chicks are highly susceptible to *Salmonella* infections during the first days of life and inoculation with very low doses can result in persistent infections [2,8,9]. This high susceptibility has been associated with the absence of normal gut microbiota [3,4] and the immature immune system of young chickens [5–7]. As a consequence, classical vaccination is not an effective means to achieve protection against *Salmonella* infection during the first days of life [14]. Alternatively, the use of organic acids, essential oils, pro- and prebiotics as feed supplements can help to control *Salmonella* infections in broiler chickens, but the protective responses elicited by these compounds only start several days post-hatch [22–24]. Since a rapid colonisation-inhibiting effect has been described in birds inoculated with a live *Salmonella* strain that protected the animals against subsequent challenge with another *Salmonella* strain, administration of CI strains to chickens early post-hatch might be a valuable addition to these strategies.

The *Salmonella* Typhimurium Δ*hilAssrAfliG* strain used in the present study was very effective at protecting against *Salmonella* Typhimurium challenge. Unfortunately, the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain was highly colonising and persisted in the caecum until slaughter age when the chickens were not challenged with a wild-type strain. However, when challenging the chickens with a wild-type strain, the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain was cleared rapidly from the chickens. This suggests an interaction between both strains that influences persistence and clearance of the CI strain from the chickens. Still, because this might result in the introduction of the deletion mutant strain in the food chain when applied in the field, the developed CI strain might not be an appropriate candidate for use in broiler production. As the ability of *Salmonella* strains to colonise represents an important prerequisite for effective colonisation inhibition of wild-type strains, persistence of a CI strain and protection offered by a CI strain are probably related to each other. Furthermore, it has been suggested that a CI strain is more protective against challenge when it is highly colonising [16,25]. It is therefore not improbable that the observed strong protective effect of the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain is due to its high colonising capacity. A *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant has been shown to colonise spleen and caecum to a lesser extent and was cleared rapidly from poultry, but also offered relatively less protection against *Salmonella* Enteritidis infection [15]. These and earlier observations suggest thus that there will be a trade-off between persistence and protection, as a highly colonising and thus protective strain will probably not be eliminated by slaughter age

[26]. In contrast, a strain that is poorly colonising will be eliminated by slaughter age, but will probably not offer a long lasting protection. Obviously, these aspects should be taken into account when developing a CI strain.

Earlier research showed that the colonisation-inhibition effect is more pronounced between isogenic strains and that there is greater inhibition within a serovar than between serovars [14,16,27]. Consequently, it is likely that the *Salmonella* Typhimurium Δ*hilAssrAfliG* strain is not able to inhibit strains belonging to other serovars than Typhimurium. It has however been suggested that a mixture of *Salmonella* strains belonging to several serovars would be able to inhibit a broad spectrum of virulent wild-type strains [16]. Therefore, we investigated the protective properties of a mixed culture consisting of both the *Salmonella* Enteritidis and Typhimurium Δ*hilAssrAfliG* strain against infection by 3 different *Salmonella* serovars. The results obtained in this study showed that the combined CI culture confers protection against a non-isogenic *Salmonella* Enteritidis and *Salmonella* Typhimurium challenge strain quickly after hatching. This suggests that both CI strains do not inhibit each other, or if they do it is to such a limited extent that they do not impede each other's protective properties. Earlier research showed that administering a mixture consisting of a wild-type *Salmonella* Enteritidis and *Salmonella* Typhimurium strain resulted in a pronounced protection against their isogenic challenge strains, but also against *Salmonella* Hadar and *Salmonella* Infantis challenge strains [16]. This suggests a synergistic protective effect when administering multiple CI strains simultaneously. In the present study, the CI mixture consisting of the *Salmonella* Enteritidis Δ*hilAssrAfliG* strain and *Salmonella* Typhimurium Δ*hilAssrAfliG* strain did, however, not offer protection against challenge with a *Salmonella* Paratyphi B. var. Java strain. Consequently, this suggests that a *Salmonella* Paratyphi B. var. Java CI strain needs to be developed and added to the CI culture in order to achieve simultaneous protection against *Salmonella* Enteritidis, Typhimurium and Paratyphi B. var. Java infection.

In conclusion, a significant reduction in faecal shedding and caecal and internal organ colonisation by a virulent *Salmonella* Typhimurium challenge strain could be obtained by administering a *Salmonella* Typhimurium Δ*hilAssrAfliG* mutant strain to one day old broiler chickens. Additionally, when this strain was administered simultaneously with a *Salmonella* Enteritidis *hilAssrAfliG* deletion mutant protection against infection by both a *Salmonella* Enteritidis and Typhimurium challenge strain could be obtained. These data demonstrate that colonisation-inhibition represents a promising tool to protect broilers early after hatching against multiple *Salmonella* serotypes. They pave the way for developing new CI strains and CI cultures that are cleared at slaughter age and protect against a wide variety of *Salmonella* serovars that are of importance for broiler production.

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