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

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**A B S T R A C T**

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 ΔhilAsrAfliG strain as a colonisation-inhibition strain for protection of broilers against *Salmonella* Typhimurium was evaluated. Administration of a *Salmonella* Typhimurium ΔhilAsrAfliG 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 ΔhilAsrAfliG strain was orally administered simultaneously with a *Salmonella* Enteritidis ΔhilAsrAfliG 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.

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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 ΔhilAsrAfliG 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* serovars [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 \textit{hilA}, \textit{ssrA} and \textit{flg} mutations in a \textit{Salmonella} strain belonging to another serovar yields a CI strain displaying the same degree of attenuation and similar protective properties as the \textit{Salmonella} Enteritidis \textit{ΔhilAssrAflg} 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 \textit{Salmonella} serovars.

In the present study, the efficacy of a \textit{Salmonella} Typhimurium \textit{hilAssrAflg} deletion mutant as a CI strain was evaluated. Secondly, the protective effect of a CI culture consisting of both a \textit{Salmonella} Enteritidis and \textit{Salmonella} Typhimurium \textit{hilAssrAflg} deletion mutant against \textit{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 \textit{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 \textit{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 \textit{in vivo} results [13]. Deletion of \textit{hilA}, \textit{ssrA} and \textit{flg} genes in this strain was done using the one-step inactivation method described by Datsenko and Wanner [18]. \textit{Salmonella} Typhimurium MB2136, a streptomycin resistant wild-type strain originally isolated from swine was used as a challenge strain. A nalidixic acid-resistant \textit{Salmonella} Enteritidis \textit{hilAssrAflg} deletion mutant, which has been described earlier [13], was also used in this study. The original \textit{Salmonella} Enteritidis 76Sa88 strain, from which the \textit{Salmonella} Enteritidis \textit{hilAssrAflg} deletion mutant is derived, was originally isolated from a poultry farm [2,19]. \textit{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 \textit{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 \textit{Salmonella} Typhimurium \textit{hilAssrAflg} deletion mutant against experimental \textit{Salmonella} Typhimurium infection

In order to evaluate the persistence of a \textit{Salmonella} Typhimurium \textit{ΔhilAssrAflg} strain in chickens and its efficacy against colonisation by a wild type \textit{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 m². Two groups (Group V and C) were given 10⁹ CFU of the \textit{Salmonella} Typhimurium \textit{ΔhilAssrAflg} 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 10⁵ CFU \textit{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 \textit{Salmonella} Typhimurium \textit{hilAssrAflg} and a \textit{Salmonella} Enteritidis \textit{hilAssrAflg} deletion mutant against infection with several \textit{Salmonella} serotypes

In order to evaluate the efficacy of a CI culture containing a \textit{Salmonella} Enteritidis \textit{ΔhilAssrAflg} and \textit{Salmonella} Typhimurium \textit{ΔhilAssrAflg} strain against infection by several \textit{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 x 10⁶ CFU/ml of the \textit{Salmonella} Enteritidis \textit{ΔhilAssrAflg} strain and 2 x 10⁶ CFU/ml of the \textit{Salmonella} Typhimurium \textit{ΔhilAssrAflg} 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 10⁵ 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 \textit{Salmonella} Enteritidis strain 147, Groups VST and CST with \textit{Salmonella} Typhimurium strain MB2136 and Groups VSJ and CSJ with the wild-type \textit{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 tetraphionate-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 \textit{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 \(\Delta hilA\) strain or a Salmonella Typhimurium challenge strain. Groups V and C were orally inoculated with \(10^9\) CFU of a Salmonella Typhimurium \(\Delta hilA\) strain at day 1 of the experiment (day-of-hatch). Groups I and C were challenged with \(10^9\) 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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 9</th>
<th>Day 16</th>
<th>Day 23</th>
<th>Day 30</th>
<th>Day 37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella Typhimurium</td>
<td>V</td>
<td>75/75</td>
<td>75/75</td>
<td>49/49</td>
<td>47/47</td>
<td>24/24</td>
<td>24/24</td>
<td>22/23</td>
</tr>
<tr>
<td>(\Delta hilA) strain</td>
<td>C</td>
<td>75/75</td>
<td>75/75</td>
<td>49/49</td>
<td>47/47</td>
<td>24/24</td>
<td>24/24</td>
<td>22/23</td>
</tr>
<tr>
<td>Salmonella Typhimurium</td>
<td>I</td>
<td>NA/18</td>
<td>/50</td>
<td>45/50</td>
<td>35/49</td>
<td>5/25</td>
<td>10/25</td>
<td>10/25</td>
</tr>
<tr>
<td>challenge strain</td>
<td>C</td>
<td>NA/12</td>
<td>10/50</td>
<td>0/48</td>
<td>0/23</td>
<td>0/22</td>
<td>0/22</td>
<td></td>
</tr>
</tbody>
</table>

\(a\) Number of positive samples after enrichment/total number of samples.
\(b\) Number of positive samples after direct plating.
\(\dagger\) 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 \(hilA\) 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 \(\Delta hilA\) strain remained high during the entire experiment in Group V (Table 1). Shedding of this strain decreased quickly in Group C. The Salmonella Typhimurium \(\Delta hilA\) 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 \(\Delta hilA\) 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 \(\Delta hilA\) 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 caecal samples showed that the Salmonella Typhimurium \(\Delta hilA\) strain colonised the caecum.
Fig. 2. Caecal (A–C) and spleen (D–F) colonisation by a Salmonella Typhimurium challenge strain. Chickens in Group C were given $10^5$ CFU of a Salmonella Typhimurium $\Delta hilA\Delta AfiG$ deletion mutant strain at day-of-hatch. Chickens in Group I and C were given $10^6$ 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 23, 23 and 22 in Group C on day 7, 21 and 42 respectively.

4.2. Experiment 2: efficacy of simultaneous administration of a Salmonella Typhimurium $\Delta hilA\Delta AfiG$ and a Salmonella Enteritidis $\Delta hilA\Delta AfiG$ 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 $\Delta hilA\Delta AfiG$ strains colonised the caecum and spleen to a similar level in all treated groups. Mean colonisation was log$_{10} = 6.87 \pm 0.12$ and $6.44 \pm 0.76$ CFU/g in the caeca, and log$_{10} = 0.74 \pm 0.50$ and $2.02 \pm 0.77$ 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.

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 $\Delta hilA\Delta AfiG$ 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 $\Delta hilA\Delta AfiG$ 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.
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, probiotics, or feed supplements can help 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 chicks early post-hatch might be a valuable addition to these strategies.

The Salmonella Typhimurium ΔhilA ΔsrAfliG strain used in the present study was very effective at protecting against Salmonella Typhimurium challenge. Unfortunately, the Salmonella Typhimurium ΔhilA ΔsrAfliG 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 ΔhilA ΔsrAfliG 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 ΔhilA ΔsrAfliG strain is due to its high colonising capacity. A Salmonella Enteritidis ΔhilA ΔsrAfliG 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 \( \Delta hiAI\text{aR}A^{\text{AIG}} \) 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 \( \Delta hiAI\text{aR}A^{\text{AIG}} \) 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 \( \Delta hiAI\text{aR}A^{\text{AIG}} \) strain and Salmonella Typhimurium \( \Delta hiAI\text{aR}A^{\text{AIG}} \) 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 \( \Delta hiAI\text{aR}A^{\text{AIG}} \) mutant strain to one day old broiler chickens. Additionally, when this strain was administered simultaneously with a Salmonella Enteritidis \( hiAI\text{aR}A^{\text{AIG}} \) 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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