Research Note: Analysis of immune responses in broilers after vaccination against *Campylobacter jejuni*

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ABSTRACT *Campylobacter* infections traced mainly to poultry products are major bacterial foodborne zoonoses. Among the many control strategies evaluated at primary poultry level to reduce these infections, vaccination could be a solution, but no effective vaccines are available to date. A better understanding of the immune mechanisms involved in protection against *Campylobacter* would be helpful for designing novel vaccine strategies.

The present study was designed to analyze in more depth the immune responses developed in broilers in order to potentially identify which immune parameters may be important for establishing protection against *Campylobacter* by comparing the immune responses obtained here with those obtained in a previous study performed on vaccinated specific-pathogen-free Leghorn chickens that presented a partial reduction of *Campylobacter* after experimental challenge. The protection against *Campylobacter* colonization was evaluated at different time points over 40 d of rearing, by measuring specific IgY levels in serum and IgA antibodies in bile reflecting the systemic and mucosal humoral responses respectively and the relative expressions of 9 cecal immune marker genes (cytokines and antimicrobial peptides), which reflect the innate and cellular immune responses.

Despite no reduction of *Campylobacter* in the cecum, a systemic immune response over time characterized by the production of specific anti-flagellin IgY was observed, in addition to upregulation of the antimicrobial peptide avian β-defensin (**AvBD**) 12 gene expression in the cecum of vaccinated broilers compared with the placebo group. However, the levels of specific anti-flagellin mucosal IgA antibodies in the bile as well as the relative expression of other cecal cytokines studied was underexpressed in the vaccinated group or similar in both groups.

Key words: flagellin, immunization, innate immunity, systemic immune response, cecum immune response

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INTRODUCTION

Campylobacter is the most common bacterial cause of human gastroenteritis (known as campylobacteriosis) in the European Union, with 127,840 human cases reported in 2021 (EFSA and ECDC, 2022). Poultry are considered to be the major reservoir as they carry it commensally in their intestines (EFSA, 2020). *Campylobacter jejuni* (***C. jejuni***) is the most frequently reported species, and the consumption and handling of poultry meat products contaminated with *C. jejuni* are responsible for the majority of human infections.

To effectively control the rate of incidence of human infections, it is important to reduce the poultry colonization level of *C. jejuni* at the primary production level. Strategies to reduce *Campylobacter* in poultry include biosecurity measures, food additives, and vaccination. Although immunization is a promising method, no vaccine has been commercialized yet (EFSA, 2020). In response to *Campylobacter* colonization, studies report 1) a role of maternal immunity; 2) innate immune system responses with the involvement of Toll-like receptors, chemokines, and antimicrobial β-defensin peptides, and 3) adaptive immune responses with the production of antibodies or cytokines (Awad et al., 2018).

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In a recent study, specific-pathogen-free (**SPF**) Leghorn chickens (hatched and reared at the Avian breeding and experimental department of ANSES's Ploufragan laboratory) were inoculated by a DNA prime/protein boost flagellin-based vaccine and experimentally challenged with a *C. jejuni* infection (Gloanec et al., 2022). This vaccine regimen induced a slight *Campylobacter* load reduction (mean difference of 1.3 $\log_{10}$ cfu/g) on day (**d**) 40. This vaccine regimen was already known to induce protection in these chickens (Meunier et al., 2018). Systemic and mucosal humoral immune responses characterized by the production of IgY in serum and IgA in bile, respectively, and a transient expression of interleukin (**IL**)-10 and AvBD10 in the cecum were observed (Gloanec et al., 2022).

Nevertheless, this vaccine regimen was described as being unable to reduce *Campylobacter* loads in infected broilers even though serum antibodies were induced (Meunier et al., 2018). The present work was designed to study in more depth the immune responses induced by this vaccine regimen in broilers challenged by *Campylobacter* in order to better understand and characterize the type of immune responses following vaccination at different time points in these broilers, and to identify potential ways of improving vaccination against *Campylobacter* in broilers by comparing these results with those obtained for the Leghorns.

MATERIALS AND METHODS

Bacterial Strain and Growth

Campylobacter jejuni strain C97Anses640, isolated from broiler feces and belonging to the sequence typing (**ST**)-45 complex, was used for the in vivo oral challenge. The strain was grown under microaerophilic conditions (85% N_2, 10% CO_2, and 5% O_2) at 41.5°C on selective modified charcoal cefoperazone deoxycholate agar (**mCCDA**; Thermo Fisher Diagnostics, Dardilly, France) for 48 h or in Brucella broth (Becton Dickinson, Le Pont de Claix, France) for 24 h.

Animals and Experimental Design

All experimental procedures were performed in accordance with French national guidelines on the care and use of animals for research purposes, approved by the ComEth Anses/ENVA/UPEC ethics committee and authorized by the French Ministry of Higher Education, Research and Innovation (referenced as APAFIS#24442-2020022816285714 v2). The trial was carried out at the Animal Biosafety Level 2 facilities of ANSES's Ploufragan Laboratory in northwestern France. Housing, environmental, and feeding conditions were the same as previously described (Gloanec et al., 2022). At the beginning of the experiment, the absence of *Campylobacter* spp. was confirmed in the experiment rooms (including the feeding and drinking systems) and in 5 chicks according to NF EN ISO 10272-1 (2017). This study was conducted on 64 conventional Ross 308 broilers, which were purchased from a local hatchery. On the day of hatching (d 1), the animals were randomly divided into 2 groups, with 32 chickens in the vaccinated group and 32 in the placebo group (adjuvant alone). However, one chick in the placebo group died before d 19. A DNA prime vaccine was inoculated by intramuscular route in the thigh on d 5 (150 μg of pcDNA3-flagellin for the vaccinated group or pcDNA3 for the placebo group with 25 μg of unmethylated ODN2007 CpG (TCGTCGTTGTCGTTGTCGTT, with a phosphorothioate backbone [Sigma-Aldrich, Saint Quentin Fallavier, France] used as an adjuvant). In the same way, a protein vaccine booster was administered on d 12 (100 μg recombinant flagellin protein or PBS emulsified in MONTANIDE ISA71 (30/70, wt/wt) (Seppic, La Garenne-Colombes, France)). All the chickens were challenged orally on d 19 with 10^5 cfu of *C. jejuni* strain C97Anses640. Blood samples were collected from the occipital sinus of live animals using a Terumo 1 mL syringe with a G26 needle on d 19 (5 chickens/group), d 22 (4 chickens/group), d 27 (23 and 22 chickens from the vaccinated and placebo groups, respectively), d 34 (18 and 17 chickens from the vaccinated and placebo groups, respectively), and d 9 (13 and 12 chickens from the vaccinated and placebo groups respectively). The sera were recovered after coagulation and centrifugation (2,000 × *g*, 10 min, room temperature) and stored at −20°C until the systemic humoral immune response was determined. Bile and ceca samples were taken after necropsy on d19 (5 chickens/group), d 22 (4 chickens/group), d 28 (5 chickens/group), d 35 (5 chickens/group), and d 40 (13 and 12 chickens from the vaccinated and placebo groups respectively). Bile samples were stored at −20°C until the mucosal humoral immune response was evaluated. All cecal contents were collected for *Campylobacter* spp. enumeration. A portion (around 0.5 cm long) of cecal wall was immediately placed in 1 mL of RNAlater (ThermoFisher Scientific, Villebon sur Yvette, France), incubated for 1 d at 4°C then stored at −80°C until gene expression was assessed. Both the vaccinated and placebo groups received a starter-grower diet from d 1 to d 19 and then a grower-finisher diet until d 40. On d 5, d 19 and on each slaughter day (d 22, d 28, d 35, and d 40), each bird was individually weighed. Moreover, the chickens were observed daily to ensure that no detrimental effects of vaccinations and challenge reactions occurred.

Campylobacter Cecal Enumeration

Cecal samples were homogenized and serially diluted 10 fold to 10^{-6} in tryptone-salt broth (Biomerieux, Craponne, France). Dilutions were plated on mCCDA using the easySpiral automatic plater (Interscience, Saint-Nom-la-Bretèche, France). After 48 h of incubation under microaerobic conditions at 41.5°C, typical *Campylobacter* colonies were counted manually, according to the manufacturer's instructions.

Antibody Production by a Specific ELISA

The level of antibodies against flagellin protein in serum and bile was measured by an ELISA previously developed in the laboratory and described (Gloanec et al., 2022). Briefly, plates containing 2 μg/mL flagellin protein per well were successively incubated with 1:4,800 dilutions of serum or 1:100 dilutions of bile, followed by 1:35,000 diluted goat anti-chicken IgY-HRP (horseradish peroxidase) (Abcam, Paris, France) or 1:5,000 diluted goat anti-chicken IgA-HRP antibodies. Each plate contained one serum internal control and one bile internal control to allow standardization between experiments, and each sample was measured in duplicate.

Gene Expression Analyses by RT-qPCR

The total mRNA from ceca samples preserved in RNAlater was extracted using the Agencourt RNAdvanceTM Tissue kit (Beckman Coulter, Brea, CA) with the following modifications described by Gloanec et al. (2022) and according to the manufacturer's recommendations, then processed with the Turbo DNA-free kit (Thermofisher Scientific, Vilnius, Lithuania). Quantification was based on fluorimetry using the Qubit RNA high sensitivity assay kit (Life Technologies Corporation, Eugene, OR) and the Qubit Fluorimeter 2.0 (Life Technologies, Saint-Aubin, France).

The cDNAs were obtained from 320 ng of total RNA using the High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Villebon sur Yvette, France) according to the manufacturer's recommendations.

As previously described (Gloanec et al., 2022), the relative expression of the following cytokine and antimicrobial peptide genes were determined by qPCR using the 7500 real-time PCR system (Applied Biosystems): Interferon (**IFN**)-γ, IL-1β, IL-4, IL8like(**L**)1, IL8L2, IL-17A, IL-10, and avian beta defensin AvBD10 and AvBD12. The qPCR reaction was performed with 4 ng of cDNA in duplicate using the SYBRGreen Master mix (Applied Biosystems) following 40 amplification cycles (95°C/15 s, 60°C/1 min). The relative amount of target gene expression was determined by the $2^{-\Delta\Delta Ct}$ method with beta-actin as the reference gene (Livak and Schmittgen, 2001). The absence of any genomic DNA contamination was checked by a qPCR on the total RNA. PCR efficiency was between 90 and 110%. Statistical tests were performed using duplicates of the $2^{-\Delta Ct}$ data for each gene in the vaccinated and placebo groups.

Statistical Analyses

R software (version 4.0.3) was used for statistical analyses. Student's parametric test was used when the normality and homogeneity criteria of the variances were validated (checked by the Shapiro−Wilk normality test and Bartlett's test, respectively); otherwise, the nonparametric Mann−Whitney test was used. A P-value lower or equal to 0.05 ($P \leq 0.05$) was considered statistically significant.

RESULTS AND DISCUSSION

The vaccination did not affect broiler growth, as there was no significant difference in mean body weight between the placebo and vaccinated groups throughout the whole rearing period (data not shown). No detrimental effect was observed in either group.

On d 22, three days after the challenge and throughout the *Campylobacter* colonization period in the 2 groups, both vaccinated and placebo groups were colonized by high levels of *Campylobacter* cecal contents even though a significant mean difference ($P \leq 0.05$) of 1.6 $\log_{10}$ cfu/g (6.4 $\log_{10}$ cfu/g in the placebo group vs. 8.0 $\log_{10}$ cfu/g in the vaccinated one) was observed between the 2 groups (Figure 1). Afterward in the trial, from d 28 to d 40 that is to say at around the slaughter ages on chicken farms, high levels of approximately 8 $\log_{10}$ cfu/g of *Campylobacter* were measured in both vaccinated and placebo groups with no significant difference between the 5 (Figure 1).

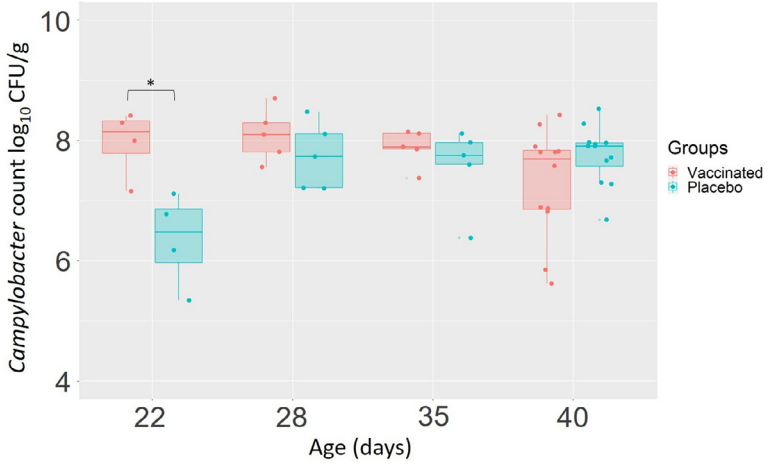

Figure 1. Effect of vaccination on *Campylobacter* colonization of chicken ceca according to the age of the chickens (corresponding to the days of the experiment). Significant differences between the two groups are indicated by asterisks (*:$P \leq 0.05$; Wilcoxon test).

Similarly, a previous study demonstrated no reduction of *Campylobacter* on D 42 in the ceca of broilers using a bacterial strain isolated from humans (*C. jejuni* 81-176) (Meunier et al., 2018) different from that used in this study (*C. jejuni* C97ANSES640). The flagellin sequences of the 2 *Campylobacter* strains are identical, so the same vaccine could be used in both studies. Consequently, unlike the assumption made for the SPF Leghorn chickens (Gloanec et al., 2022), the response in *Campylobacter* cecal load to flagellin vaccination among broilers does not appear to be influenced by the challenging strain.

The absence of *Campylobacter* reduction following vaccination was not correlated to the immune responses induced. In fact, the production of IgY was significantly higher in the serum of the vaccinated group than in that of the placebo group from d 22 after *Campylobacter* challenge to d 39, characterizing a specific systemic immune response. Increases in anti-flagellin IgY antibody levels and high interindividual variabilities were observed at different time points in the vaccinated group contrary to the placebo group (Figure 2A). The production of anti-flagellin IgY in SPF chickens had already been observed in the vaccinated group compared with the placebo group using the same vaccine regimen (Gloanec et al., 2022). Thus, these antibodies do not appear to be involved in the protective response or to be sufficient on their own to induce protection against *Campylobacter* in broilers.

In this work, the levels of anti-flagellin IgA were measured in bile from d 19 to d 40 (Figure 2B). No significant difference was observed between the 2 groups at any time. Contrasting results have been previously reported in SPF chickens, a significant increase in IgA being observed in the vaccinated group compared with the placebo group. However, the IgA level in vaccinated chickens was not correlated with *Campylobacter* cecal load (Gloanec et al., 2022). These results showed that vaccination primed the immune response but it is impossible to discriminate between responses due to the vaccination alone or due to both vaccination and *Campylobacter* colonization. Consequently, the protection induced by the flagellin against *Campylobacter* may not be strictly antibody mediated, so the selection of vaccine candidates should not be based only on antibody response.

This absence of mucosal response to vaccination in conventional broilers could be due to the interference of maternal antibodies as previously suggested by (Meunier et al., 2018). Indeed, specific maternal antibodies would be able to recognize and then neutralize the action of the flagellin-based vaccine (Shoaf-Sweeney et al., 2008). On the contrary, humoral immunity could be involved in the reduction of *Campylobacter* in chickens and more specifically the intestinal mucosal immune response (Rice et al., 1997).

Campylobacter has been reported to induce the release of pro- and anti-inflammatory cytokines and antimicrobial peptides as beta-defensins (Li et al., 2010; Reid et al., 2016; Connerton et al., 2018; Garcia et al., 2018), reflecting the activation of different innate and adaptive immune pathways. The relative expression of different cytokines and antimicrobial peptides was studied on d 19 before the challenge to observe the impact of vaccination, and on the final timepoint (d 40), when the antibody responses were higher (Figure 2C). An overexpression of AvBD12 reflecting an innate immune response was observed only in the vaccinated broilers on d 19. Upregulation of AvBD12 has already been observed in the cecum in the presence of *Campylobacter* (Li et al., 2010; Garcia et al., 2018). AvBDs are not generally investigated in vaccination studies, but an underexpression of AvBD10 was previously observed in the vaccinated group on d 40 (Gloanec et al., 2022). Thus, we could suggest that the flagellin vaccine may have stimulated the innate immune response differently depending on the avian breed used before the challenge by *Campylobacter*. Moreover, the overexpression of AvBD12 was probably not involved, or was insufficient to induce a reduction in *Campylobacter*.

The underexpression of pro-inflammatory targets such as IL-17A, IL8L2, Il-1β (Th17 pathway), IFN-γ (Th1 pathway), and anti-inflammatory targets such as IL-10 (Treg pathway) was observed on d 19 and/or d 40 in the vaccinated group (Figure 2C). It has already been suggested that T regulatory cells (Treg pathway), producing the anti-inflammatory cytokine IL-10, could reduce colonization of *Campylobacter* (Humphrey et al., 2014). Moreover, another study indicated that *Campylobacter* reduction was linked to the Th17 pathway (Reid et al., 2016). This balance between pro- and anti-inflammatory responses has been previously reported after a *Campylobacter* challenge in conventional broilers (Reid et al., 2016; Connerton et al., 2018), and is important for maintaining immune homeostasis in the gut and could explain the nonpathogenic effect of *Campylobacter* in chickens. In our previous study, only an overexpression of IL-10 associated with a slight reduction in *Campylobacter* was observed on d 28, suggesting the Treg pathway could be involved in the protection against *Campylobacter* of Leghorn chickens, unlike the broilers (Gloanec et al., 2022). In the present study, none of these pathways (including the Treg pathway) were activated. Several hypotheses could explain these results 1) these pathways were not involved in the response to *Campylobacter* colonization, 2) the immune system (innate and adaptive) was stimulated differently in the 2 avian breeds, 3) the vaccination protocol (vaccine candidate, dose, frequency, nature of vaccine, adjuvant, and route of inoculation) was not suitable for stimulating these pathways or 4) the immune responses were influenced by the microbiota, which was different in the 2 avian breeds. Moreover, it could be beneficial to investigate immune cells or Toll-like receptors (**TLRs**) that may stimulate cytokine production.

Thus, the vaccination did not reduce *Campylobacter* colonization in broilers despite the stimulation of a specific systemic humoral immune response and the production of AvBD12. According to this study with the use of flagellin, AvBD12, and the systemic humoral immune

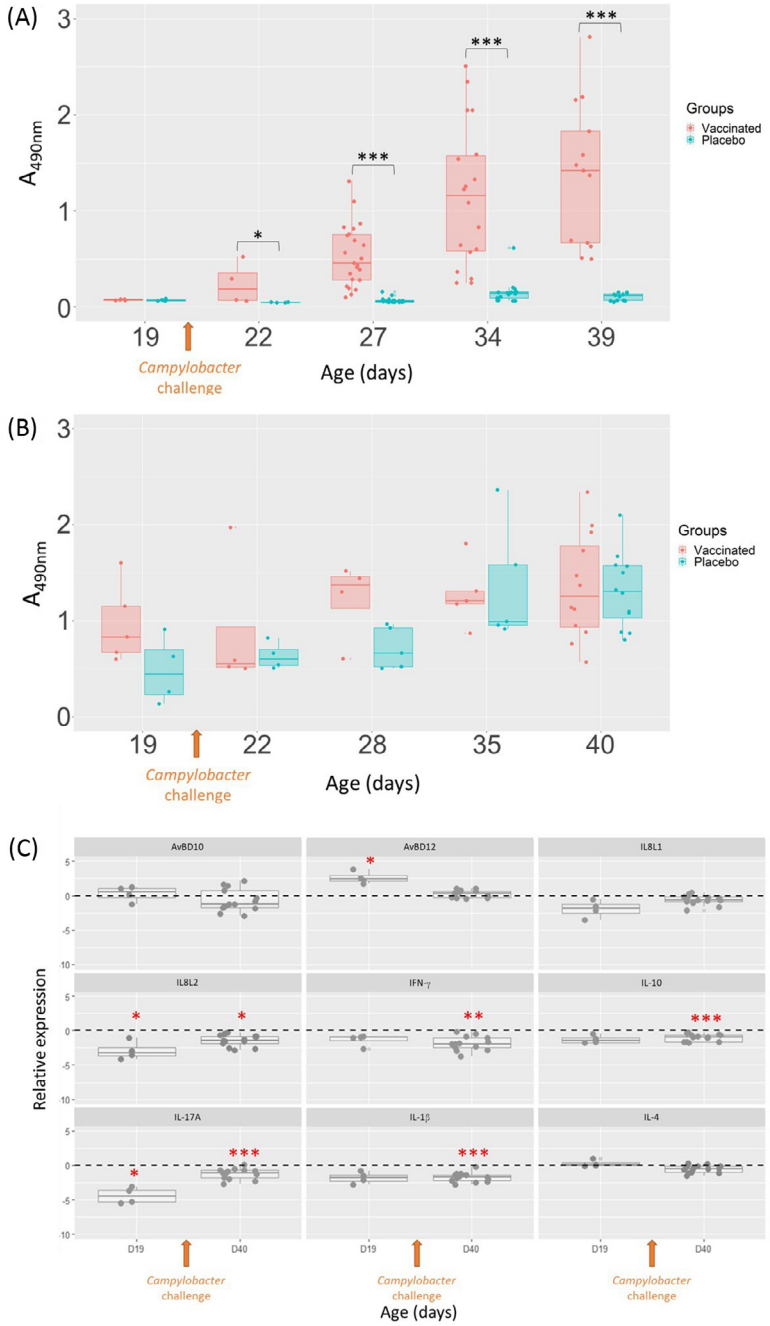

Figure 2. Effect of vaccination against *Campylobacter* on immune responses. (A) Levels of anti-flagellin IgY antibodies in serum. (B) Levels of anti-flagellin IgA antibodies in bile. Significant differences between the two groups are indicated by asterisks (*:$P \leq 0.05$, ***:$P \leq 0.001$; Wilcoxon rank sum test). (C) Relative gene expressions of cytokines and AMP in cecum on d 19 and d 40. Relative gene expression represents $\log_2$ ratio vaccinated/placebo. Values >0 (above the dotted black line) represent relative overexpressions of cytokine or AMP genes in the cecum of vaccinated chickens compared with the placebo group while values <0 (below the dotted black line) represent relative subexpressions of cytokine or AMP genes in the cecum of vaccinated chickens compared with the placebo group. Significant differences between $2^{-\Delta Ct}$ values of the vaccinated and placebo groups are indicated by red asterisks (*:$P \leq 0.05$, **: $P \leq 0.01$, ***:$P \leq 0.001$) for the expression of each gene at the corresponding time points when the fold change ratio between the vaccinated and placebo groups was either higher than 2 or lower than 0.5.

response do not appear to be involved, or are insufficient to induce protection against *Campylobacter* in broilers. The role played by the tested immune parameters remains to be explained in the response to anti-*Campylobacter* vaccination.

Furthermore, as suggested previously, the vaccination against *Campylobacter* could impact microbiota (Gloanec et al., 2022) and it is not impossible that microbiota could influence the immune system or vice versa. Thus, additional studies on the structure and composition of microbiota in relation to the immune responses after vaccination against *Campylobacter* could be advantageous.

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DISCLOSURES

The authors declare no conflicts of interest.

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