Comparative Immunology, Microbiology and Infectious Diseases 75 (2021) 101612

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Effects of *aroA* deleted *E. coli* vaccine on intestinal microbiota and mucosal immunity

Breno C.B. Beirão [a,b,*], Max Ingberman [a], Dany Mesa [b], Gleidson B.C. Salles [c], Eduardo C. Muniz [c], Luiz Felipe Caron [b]

[a] Imunova Análises Biológicas, Setor de Ciências Biológicas UFPR, Curitiba, PR, 81531-980, Brazil
[b] Universidade Federal do Paraná, Setor de Ciências Biológicas UFPR, Curitiba, 81531-980, Brazil
[c] Zoetis Indústria de Produtos Veterinários Ltda, São Paulo, SP, 04709-111, Brazil

ARTICLE INFO

Keywords:
E.coli
Spray
Vaccine
IgA
Microbiota
Mucosal

ABSTRACT

E. coli infection of broilers can result in systemic diseases and productivity losses. Use of antimicrobials against this condition is common but other approaches, such as vaccination, are gaining ground. Anecdotal field reports indicate that intestinal health is improved unspecifically following *E. coli* live vaccination. We hypothesized that the intestine may be an important site for the functionality of the vaccine. Vaccine effects on the intestine were assessed. Spray vaccination induced marked alterations of the caecum microbiota of broilers within 3 days, and this effect gradually waned. However, T cell activation occurred in the spleen, but not in caecal tonsils, and anti-*E. coli* IgA was concentrated in the respiratory mucosae. Accordingly, IL-6 mRNA was produced in the lungs following immunization. Overall, these data are an initial indication that any vaccine-induced effects on the intestine are greatly associated with the microbiota. However, immunity conferred by vaccination is not primarily induced in gut-associated lymphoid tissues.

1. Introduction

Avian colibacillosis is an infectious bacterial disease caused by *Escherichia coli*. This agent is responsible for large economic losses in poultry production [1], which may be due to mortality, or indirectly, due to delays in growth, lower uniformity, predisposition to other diseases and condemnation of carcasses in the slaughterhouse [2].

Commensal and pathogenic *E. coli* strains show important differences [3]. These variations justify alterations in the ability of the bacteria to spread, for instance. The pathogenic strains are successfully disseminated via aerosolization of faecal contents that are inhaled by the birds, at which point bacteria multiply in the cells of the upper respiratory tract [4]. Primary *E. coli* infection is common since air sacs and lungs do not have an immune protection system with efficient macrophages. Thus, from there, the bacteria can easily infect the blood and spread through the bloodstream to other organs [5].

The use of live bacterial vaccines is one of the tools available for the control of colibacillosis. These products are safe and show efficacy in field conditions [6]. Vaccines against *E. coli* infections in poultry may contribute to the reduction of antibiotic use, which is of great importance in an "One Health" perspective [7].

Anecdotal reports from field poultry veterinarians indicate that aerosol vaccination with live *E. coli* vaccines can improve intestinal health and macroscopic parameters (oral communications). Unpublished results from our group have demonstrated that a live *E. coli* vaccine is able to reduce intestinal permeability in the first few days following vaccination. Considering that live bacterial vaccines are able to target the intestine and that there is an interaction with its commensal microbiota it was hypothesized that vaccine protection resulted at least partially from an intestinal immune component [8]. Therefore, we tested the role of intestinal, respiratory and systemic immune and microbial components following administration of a live *E. coli* vaccine in broilers.

Abbreviations: D, day; OUT, operational taxonomic unit; TCR, T cell receptor.

* Corresponding author at: Av. Cel. Francisco H. dos Santos, s/n, Setor de Ciências Biológicas UFPR, Curitiba, CEP 81531-980, Brazil.
E-mail address: breno.beirao@ufpr.br (B.C.B. Beirão).

https://doi.org/10.1016/j.cimid.2021.101612
Received 20 August 2020; Received in revised form 10 January 2021; Accepted 10 January 2021
Available online 14 January 2021

2. Materials and methods

2.1. Animals and procedures

For experiment 1, 160 1-day-old Cobb broilers were housed in isolators with HEPA-filtered air flow; contact between the animal handlers and the chickens was made exclusively from outside the isolator after housing, to avoid spread of the live vaccine between the groups. Animals were divided in two groups: 1) Negative control (no treatment); 2) Vaccine (animals spray-vaccinated with Poulvac E.coli®, Zoetis, Kalamazoo, AL).

Following vaccination at arrival (1 day of age), samples were collected for cytokine measurements on days 1 [2 h post-vaccination] *(number of samples/group = 7)*, 3 *(20)* and 14 *(10)*. For determination of the peripheral immunophenotype, blood was drawn on days 1 *(10 samples/group)*, 3 *(30)*, 7 *(10)*, 14 *(10)*, 21 *(10)* and 25 *(10)*. For spectratyping analysis of the TCR, organs were collected on days 3, 7 and 14 (see specific section below). For the determination of the caecal microbiota, samples were collected on days 3, 14 and 25 *(8 samples/date/group)*.

For experiment 2, 48 1-day-old Cobb broilers were housed in isolators and were divided in two groups as in experiment 1. However, in this instance both groups were challenged at D7 with a 100-fold higher dose of IBV vaccine (Mass I, Zoetis, Kalamazoo, AL) followed by nasal administration of 5×10^8 CFU of *E. coli* on day 11 (field strain from air sac infections from the State of Paraná, Brazil). Caecal contents, tracheal washes and tears were collected for anti-*E. coli* IgA measurements on days 3*(7 samples/group)*, 7 *(8)* and 17 *(7)*. Tears were collected by provoked production of lacrimal fluid following administration of glycerol to the eyes. Tracheal washes were obtained from culled animals with instilled PBS. The animal experiment for ELISA measurements of IgA was conducted in independent duplicates. Organs were collected after euthanasia. Liver, myocardium, and lung fragments were collected on day 17 *(7)*.

Animals were fed *ad libitum* with a mash diet designed to meet their nutritional requirements [9]. Animal procedures were approved by the Animal Experimentation Ethics Committee of the Federal University of Paraná, process CEUA SCA UFPR 060/2016.

2.2. Peripheral blood immunophenotype

A no-lysis-no-wash protocol was used for flow cytometry. Briefly, 50 µL of whole blood was stained with 1 µL (concentration was fluorophore-dependant) of primary antibodies (diluted in 10 µL) for 30 min at room temperature (antibodies shown on Table 3). Paraformaldehyde at 2.3 % was used to fix the sample; it was added at 30 µL/sample and was kept for 30 min at room temperature. Following fixation, samples were diluted to 2 mL in PBS/1% BSA. The samples were then read in a FACSCalibur (BD). All samples were stained for CD45 (pan-leucocyte marker), which is SPRD-coupled (red, read at FL-3). $CD45^{bright+}$ cells were gated and then analysed for expression of other markers. Small debris were excluded from analysis. Anti-CD4 and anti-Kul-1 were FITC-coupled (green, read at FL-1); anti-TCR Vβ1, Bu-1 and anti-MHC II were PE-labelled (yellow, read at FL-2). Absolute cell counts were obtained with BrightCount (Thermo Scientific); at least 400 beads were counted in FL2 vs FL3 dot blots of Bu-1-stained cells. For all other cell types, at least 10,000 leucocytes were counted. Single-colour controls were used for diminishing spectral overlaps. All fluorophores were excited with an argon laser. Antibody combinations were: CD4/TCR Vβ1/CD45; CD8α/CD28/CD45; Kul-1/MHCII/CD45; Bu-1/CD45. All antibodies by Southern Biotechnologies (Birmingham, AL).

2.3. TCR spectratyping

Clonal expansion of lymphocytes was determined in spleen (days 3 and 14) and caecal tonsils (day 7) by TCR Vβ1 spectratyping, following a published protocol [10]. Number of samples varied between 4 and 14/date/group. The variation is due to the technical impossibility of obtaining data from some samples Data was analysed by comparison of gaussian curve fit between control and vaccinated animals. The number of animals in each group deviating from the gaussian was also assessed, by one-tailed χ^2.

2.4. Microbiome analysis

Microbiome was assessed in animals from an independent replicate of the experiment. DNA was extracted from faeces using the QIAamp DNA Stool Mini Kit (Qiagen). DNA was quantified with a Nanodrop spectrophotometer (Thermo Scientific). The variable V4 region of 16S rRNA was amplified using the universal primers 515 F and 806R [11]. PCR conditions were as follows: 94 °C, 3 min; 18 cycles of 94 °C, 45 s, 50 °C, 30 s e 68 °C, 60 s; followed by 72 °C, 10 min. These amplicons were then sequenced (MiSeq, Illumina) [12]. Sequencing reads were analysed with the QIIME (Quantitative Insights Into Microbial Ecology) platform [11,13]. Sequences were classified into bacterial genera through the recognition of operational taxonomic units (OTUs) based on the homology of the sequences when compared to the SILVA 128 ribosomal sequence database (2017 release) [14]. OTU recognition was based on 13,100 reads/sample. Basic diversity analysis (OTU number and Unifrac-distances) was conducted using QIIME, and the OTU table exported to R for further analyses. Sequence data was submitted to the BioSample database under accession number SAMN10252308.

2.5. Anti-E. coli IgA titres

Plates were sensitized with LPS derived from *E. coli* (the challenge strain). Antibodies were detected following a standard indirect ELISA protocol. The detection antibody was an anti-IgA (Bio Rad).

2.6. Cytokine measurements

Cytokines were measured by quantitative real-time PCR in lung. Samples were collected and stored in RNAlater solution in -20OC. RNA was extracted with the use of TRIzol reagent (1 mL) using a TissueLyser equipment (3 min at 25 Hz). RNA was purified according to the instructions of the manufacturer. Sample DNA was removed with TurboDNAse (Thermo). cDNA was prepared using 1 µg of RNA in a RT-PCR reaction using a High-Capacity cDNA Synthesis kit following the instructions of the manufacturer. cDNA was diluted 10-fold and was quantified using Power SYBR-Green PCR Master Mix in a StepOne Plus Real-Time PCR System. Primer3 was used for primer design. Samples were analysed against both normalizer genes (β-actin and GAPDH) using the 2-ΔΔCt method, normalized with β-actin and GAPDH housekeeping genes (Livak and Schmittgen, 2001). Primer sequences were as follows: β-actin F – TATTGCTGCGCTCGTTGT; β-actin R – ACCAACCATCACACCCTG; GAPDH F – TCTGGAGAAACCAGCCAAGT; GAPDH R – GAGACAACCTGGTCCTCTGTG; IFNγ F –CCCGATGAACGACTTGAG; IFNγ R – TGCATCTCCTCTGAGACTG; IL-6 For – GAACGTCGAGTCTCTGTGCT; IL-6 Rev – AGTCTCGGAGGATGAGGTG.

2.7. Statistical analysis

Statistical tests are described under each figure. $P < 0.10$ was considered significant. Graphs and statistics for immune parameters were performed using GraphPad Prism 6. For genus analysis of the microbiome the most frequent genera were selected. Phylum and diversity analysis were performed using the entire dataset. Data were analysed with the R platform (The R Project). Data distribution was analysed by Shapiro-Wilk, and groups were compared by ANOVA with a Tukey *post hoc* test assessing both mean treatment effects and differences in each time point. Pairwise Pearson's correlation was performed using R package, 'psych' version.

3. Results

3.1. The microbiota is altered by vaccination

In a previous report, our group had demonstrated that mucosal immunity activation may be the main mode of action of this *aroA*-deleted *E.coli* vaccine [15]. Therefore, it was hypothesized that the vaccine may act, at least partially, via the intestinal microbiota, since it is known to affect mucosal immunity in broilers [16]. Thus, the cecum microbiota was assessed by Next-Generation Sequencing. The analysis identified 472 bacterial genera in the cecum. Of these, the relative number of bacteria differed between treatment groups for 37 genera on day 3, 29 genera on day 14 and 23 genera differed between groups on day 25. Of these genera, *E. coli* was increased in the vaccinated group in relation to the control on day 3 (Fig. 1).

Main coordinate analysis indicated a clear differentiation of bacterial communities from 'Control' and 'Vaccine' groups. The overall differences between groups were larger on day 3 and were reduced with time (Fig. 2A). Regarding bacterial richness in the cecum, microbial diversity was reduced by vaccination in all time points (Fig. 2B).

3.2. Vaccine-induced TCR Vβ1$^+$ lymphocyte stimulation does not occur in caecal tonsils

TCR Vβ1$^+$ lymphocytes occur in greater number in response to the *E. coli* vaccine (15). Indeed, vaccination induced rapid immune responses, following the trend of the changes in the microbiota. Interestingly, TCR αβ Vβ1$^+$ cells – which are known to be relevant for mucosal IgA production [17] – seemed to commit to the vaccine response at D3. At this date, more vaccinated animals showed a non-normal distribution of TCR Vβ1$^+$ lengths in spleen than animals in the control group (χ^2, $P = 0.06$) (Fig. 3A). No differences in TCR lengths were found in caecal tonsils on D7 or on dates 7 or 14 in the spleen (not shown). This measurement is an indicator of lymphocyte clonality. Higher clonality induced by vaccination on D3 translated into higher numbers of TCR Vβ1$^+$ in the blood of broilers on D7. More animals in the vaccinated group crossed the top threshold for this immune cell in broiler blood (Fig. 3B) [18]. Regarding other immune cells, there was a transient reduction in peripheral blood leukocyte counts, which was mainly contributed by CD8$^+$CD28$^+$ lymphocytes and by monocytes (Fig. S1).

3.3. Anti-E. coli IgA is mainly directed to the respiratory mucosae

Vaccination was followed by a rapid immune engagement, with the production of TCR Vβ1$^+$ cells. Since these cells are expected to have effects on mucosal immunity, a challenge trial was performed to assess if this translated into protection, and whether there was IgA involvement [18]. Mucosal IgA were titred from two *in vivo* trials. The average results between these trials show that vaccination induced higher levels of specific *E. coli* IgA in the tracheal wash following immunization (Fig. 3C). Also, hours after immunization there was a peak in IL-6 production in the lung (Fig. 3D) – a cytokine that is involved in plasma cell survival [19]. In a secondary trial, birds were challenged by spray with the field isolate of the bacteria with or without previous vaccination. IgA was measured against the LPS of the challenge strain. Higher IgA in tears occurred simultaneously with lower liver lesion scores in the vaccinated group (Fig. 3E), but IgA was reduced in the tracheal wash of these animals (Fig. 3F). No significant *E. coli* lesions were found in the myocardium. Lung lesions indicated damages caused both by the bacteria and by IBV, which was used to assist in bacterial colonization. Therefore, its assessment was not useful. Macroscopically, pericarditis was obvious in the presence or absence of vaccination following challenge (Fig. 4).

4. Discussion

Immunization against avian colibacillosis using an *aroA*-deleted vaccine has been shown to induce protection of birds. Mechanisms of action of this vaccine have only recently started to be understood, as its protection bears no correlation to serum antibody titres, for instance [20,21]. The present work aimed to further elucidate mechanisms through which the vaccine can induce its effects. Anecdotally, intestinal parameters are improved following vaccination, prompting us to assess its direct and indirect impacts in the intestine of broilers.

The peripheral blood immunophenotype induced by poultry vaccination with *aroA*-deleted *E. coli* has been investigated in another opportunity [15]. In that occasion and in the present work, there was a marked effect of the vaccine on CD4$^+$TCR Vβ1$^+$ cells. These cells are

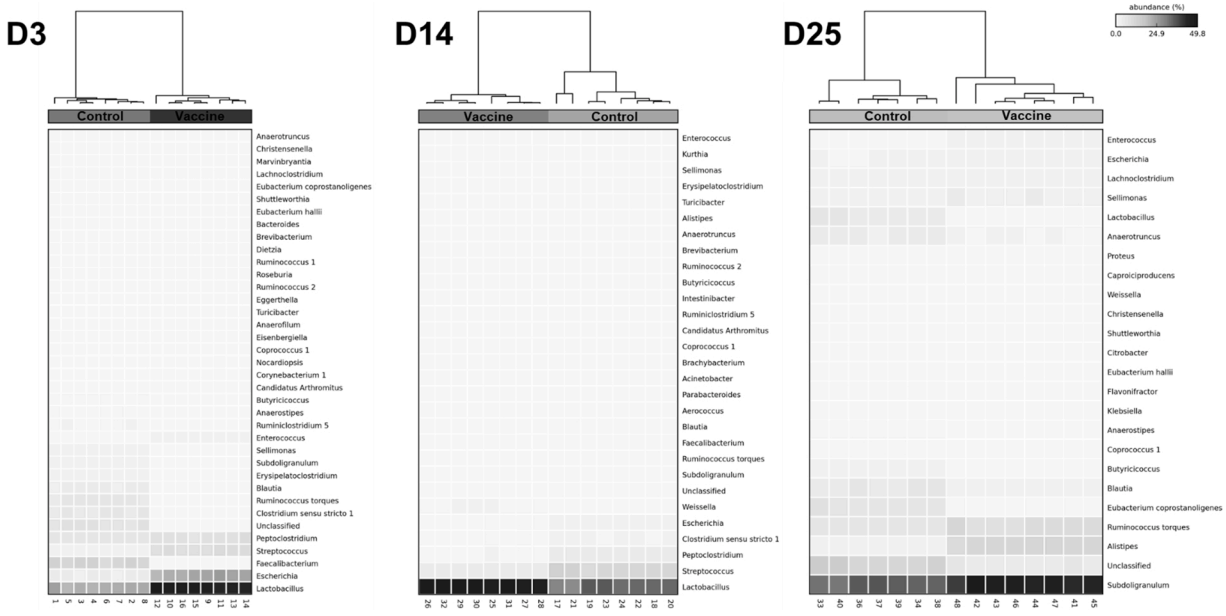

Fig. 1. Vaccination increased *Escherichia* in the cecum on D3 post-immunization. Caecum bacterial genera that differed between groups on day 3 (D3), on day 14 (D14) and on day 25 (D25). The phylogenetic tree above the heatmap shows the proximity between each sample. It is noteworthy that samples within each treatment group clustered together. Darker colours indicate higher abundance in the caecal contents.

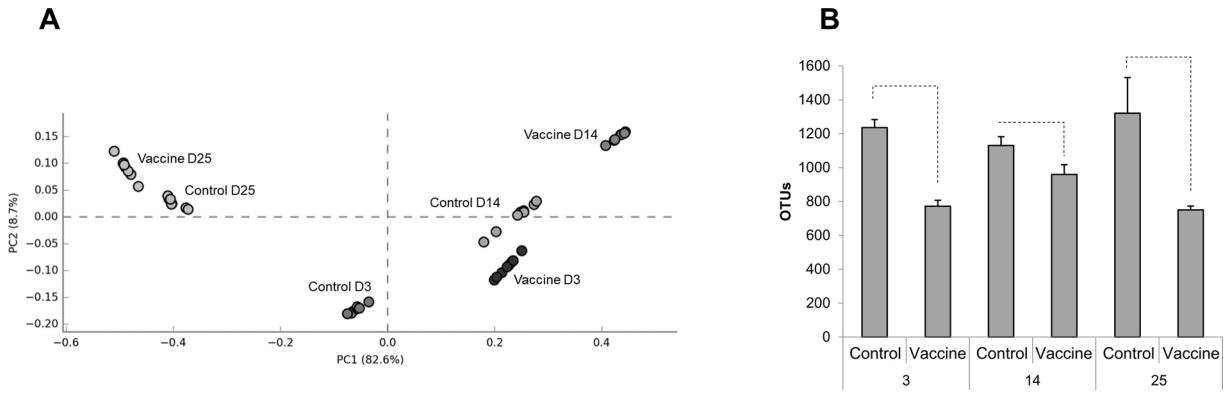

Fig. 2. Vaccination altered the overall composition of cecum microbiota. (A) Main coordinate analysis of caecal bacterial phyla. The graph indicates the distance of the overall composition of the cecum microbiota between animals, each represented by a dot. Larger distances between dots indicate higher variations between their microbiota. Bray-Curtis's method was used for designing the figure. Notice that variations within the horizontal axis are the most important effects, as they represent over 82 % of the changes in the microbiota. (B) Bacterial community richness was reduced by vaccination. Figure based on number of OTUs. Statistically significant differences between groups on a given date are indicated by the dotted lines. Analysis by two-way ANOVA with Tukey's post hoc test.

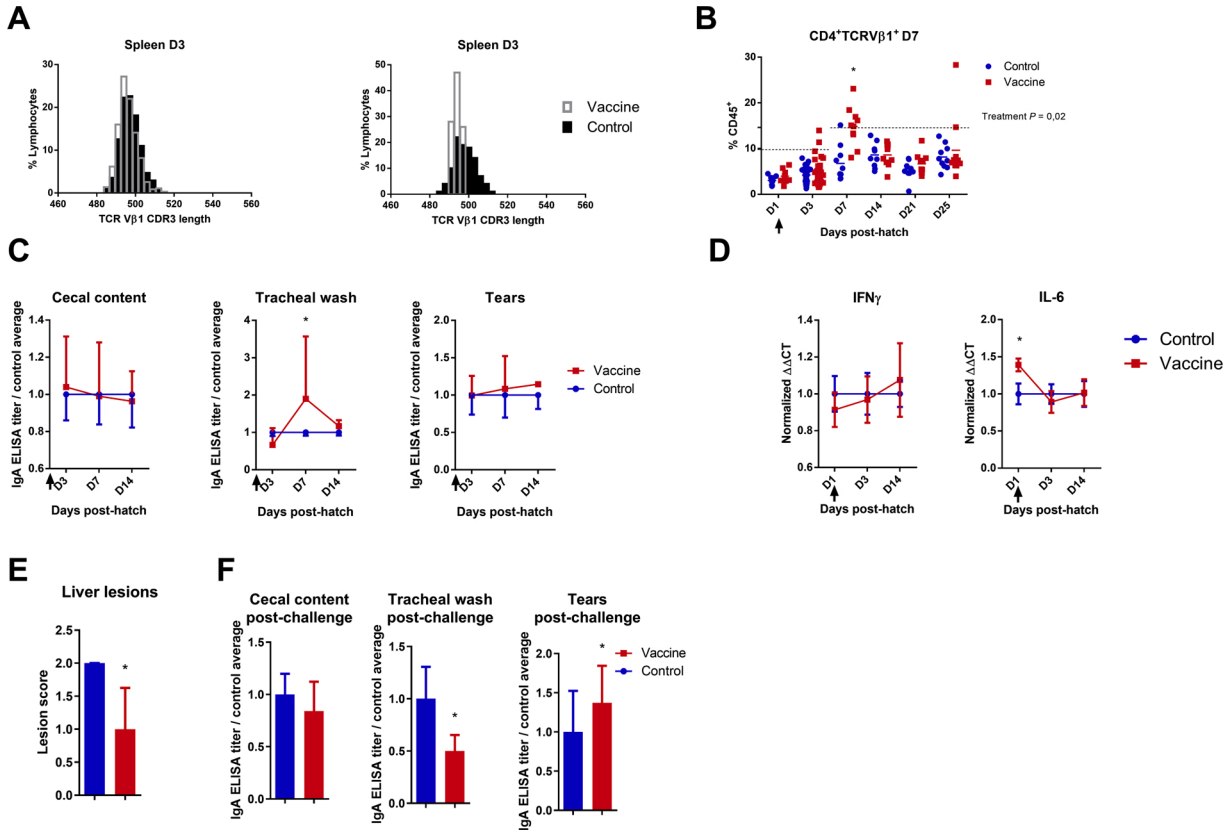

Fig. 3. Vaccine-induced TCR Vβ1+ responses seemed directed to the respiratory mucosae. (A) TCR Vβ1 length distribution was altered by vaccination in the spleen. Left: Distribution of TCR lengths was different between groups on day 3. Analysis by comparison of Gaussian curve fits for each group. The graph indicates the percentage of lymphocytes with a given TCR length (in base pairs) in all animals of that group. SD not shown for clarity. Right: Extreme illustrative results of animals with a Gaussian distribution of TCR lengths in the spleen ('Control') or with a non-normal distribution ('Vaccine'). D, day. (B) The peripheral immunophenotype was altered by vaccination. The graph presents the percentage of $CD4^{+}$TCR $V\beta1^{+}$ cells in relation to total leukocytes ($CD45^{+}$). The asterisk indicates statistical difference between groups on the given date by two-way ANOVA with Fisher's post hoc test. Main treatment effects are indicated by the *P* next to the graph. Each dot represents an animal and the horizontal bar shows the average for each group. The dotted line indicates the top threshold for the percentage of $CD4^{+}$TCR $V\beta1^{+}$ cells in broilers in the different age ranges [18]. (C) Vaccination induced higher respiratory IgA. Vaccine given on D1. Statistical analysis by two-way ANOVA with Fisher's post-hoc test. The asterisk indicates statistically significant differences on the give date. (D) Vaccination induced IL-6 mRNA production in the lung. Statistical analysis by two-way ANOVA with Fisher's post-hoc test. The asterisk indicates statistically significant differences on the give date. Results were calculated by the ΔΔCt method. (E) Vaccination prevented liver lesions following challenge. Graph shows lesion scores. Statistical analysis by two-tailed Student's *t* test. (F) Following *E. coli* challenge, specific IgA was altered in the respiratory mucosae, but not in the intestine.

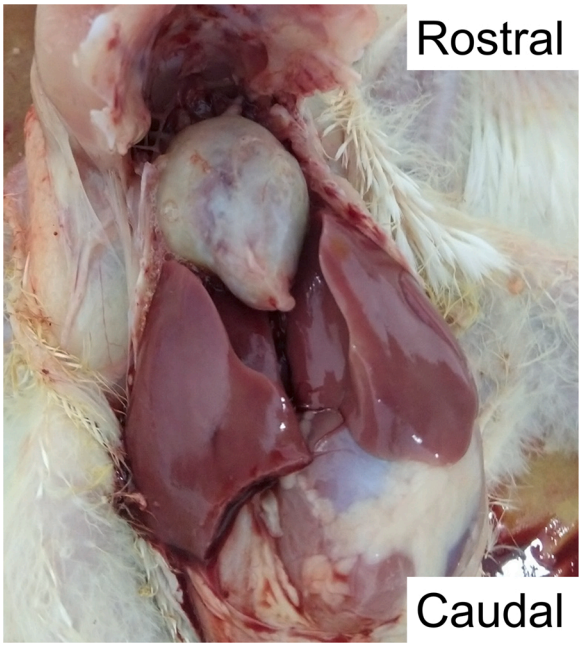

Fig. 4. Representative image of pericarditis following *E. coli* experimental infection. Lesions occurred in both vaccinated and unvaccinated animals.

crucial in helping B lymphocytes to produce IgA in the mucosae, and their number in blood is directly correlated to intestinal counts [17,18]. Therefore, induction of $CD4^+$TCR $V\beta1^+$ is a likely mechanism for this vaccine, which is administered via spray [22,23]. Activation of TCR $V\beta1^+$ cells was explored by TCR length analysis. This technique allows the identification of clonal expansion of T cells [10]. The caecal tonsils and the spleen were probed for TCR $V\beta1^+$ clonal expansion. In the present study, some degree of TCR clonality was induced in the spleen at 3d post-vaccination, as expected; clonal expansion of T lymphocytes start as early as 24 h after antigen exposure [24]. However, oligoclonal expansion of TCR $V\beta1^+$ lymphocytes did not appear in the caecal tonsils. Therefore, the main site of clonal expansion following vaccination seems not to be the caecal tonsils. In corroboration, anti-*E. coli* IgA production was more pronounced in the respiratory secretions than in intestinal contents. Also, IL-6 mRNA production was induced in the lungs hours post-vaccination. Clonal expansion in the spleen correlated with rapid changes in IL-6 production. IL-6 is important in the defence against air sac disease in birds [25]. IL-6 is increased in poultry within 1 h in response to *E. coli,* having been shown that it induces the formation of antibodies against *E. coli* antigens [26,27].

Another possible role of the vaccine in the intestine could be to mediate changes in the microbiota [28]. Vaccinated animals had the largest differences in microbiota constitution in relation to the control group soon after vaccination (D3). This difference of microbiota between groups waned with time, possibly partly reflecting the time span from vaccination. With regards to specific bacterial genera, abundance of *E. coli* was increased five-fold in vaccinated animals on day 3. By day 14, both groups already showed similar numbers of cecum *E. coli*. Although it is not possible to affirm with certainty, this is an indication that caecal *E. coli* found on day 3 were originated from the vaccine – its sudden decrease is in accordance with the incapacity of *aroA*-deleted *E. coli* in surviving in the host [29].

Several mechanisms mediate how the commensal microbiota affects the host. Direct immune sensing of the microbiota through TLR5 has a pivotal role in the immunity against influenza, for instance [30]; mucosal integrity may be altered by short-chain fatty acids (SCFA) produced by the microbiota [31]; commensal bacterial products can foster the development of IgA^+ plasma cells through effects on dendritic cells [32]. Therefore, it is relevant that *E. coli* spray vaccination concentrated the microbiota into fewer genera and increased cecal *Lactobacillus*, since some of its species are capable of bacteriocin production, and this genus can directly activate immune cells, inducing Th1 cytokines, for instance [33,34]. Lactobacilli are able to induce antibody formation following immunization of poultry with other antigens [34]. Similarly, *Streptococcus* was also present at higher levels in the vaccinated group on day 3. Both *Streptococcus* and *Lactobacillus* are Firmicutes of the Bacillus family. These bacteria have been associated with improved mucosal immune responses to rotavirus and to influenza vaccines in human beings [35,36]. These changes in the microbiota constituted the most important consequences of vaccination to the intestinal health in the present trial.

5. Conclusion

Overall, the results presented here indicate the relative importance of different mucosae on the mechanisms of action of spray vaccination of broilers with an *aroA*-deleted *E. coli*. Changes in TCR $V\beta1^+$ lymphocyte activation – seen by spectratyping – and specific IgA production indicate that the gut-associated lymphoid tissue is not a predominant immune site following vaccination; immunity to the vaccine seems to arise at the respiratory-associated lymphoid sites or at other secondary lymphoid tissues, such as the spleen. Vaccination did induce consistent changes to the intestinal microbiota, however, which may account for the improved intestinal health reported in the field following immunization; the altered intestinal microbiota may then indirectly affect the outcomes of *E. coli* vaccination in poultry.

Declaration of Competing Interest

GS and ECM are affiliated to Zoetis, which sponsored the study. There was no interference from Zoetis or from its representatives in the selection of published results.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cimid.2021.101612.

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