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ORIGINAL ARTICLE

A carvacrol-based product reduces *Campylobacter jejuni* load and alters microbiota composition in the caeca of chickens

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Abstract

Aim: This study was conducted to test the ability of a carvacrol-based formulation (Phodé, France) to decrease the *C. jejuni* caecal load in inoculated broiler chickens and to study the impact of the *C. jejuni* inoculation alone or combined with the product, on the caecal microbiota.

Methods and Results: On day 1, chickens were either fed a control feed or the same diet supplemented with a carvacrol-based product. On day 21, the carvacrol-supplemented chickens and half of the non-supplemented chickens were inoculated with *C. jejuni* (10^8 CFU). Quantitative PCR was used to quantify *C. jejuni* in chicken caecal samples and 16S rRNA gene sequencing was carried out at 25, 31 and 35 days of age. A significant decrease of 1.4 log of the *C. jejuni* caecal load was observed in 35-day-old chickens supplemented with the product, compared to the inoculated and unsupplemented group ($p < 0.05$). The inoculation with *C. jejuni* significantly increased the population richness, Shannon and Simpson diversity and altered beta-diversity. Compared to the control group, the *C. jejuni* inoculation causes significant changes in the microbiota. The carvacrol-based product associated with *C. jejuni* inoculation increased the diversity and strongly modified the structure of the microbial community. Functional analysis by 16S rRNA gene-based predictions further revealed that the product up-regulated the pathways involved in the antimicrobial synthesis, which could explain its shaping effect on the caecal microbiota.

Conclusions: Our study confirmed the impairment of the caecal bacterial community after inoculation and demonstrated the ability of the product to reduce the *C. jejuni* load in chickens. Further investigations are needed to better understand the mode of action of this product to promote the installation of a beneficial microbiota to its host.

Significance and Impact of the Study: Results suggested that this product could be promising to control *C. jejuni* contamination of broilers.

KEYWORDS

16Sr RNA gene sequencing, broiler chicken, *Campylobacter jejuni*, essential oil, feed additive, foodborne disease, microbiome, PICRUSt2

INTRODUCTION

Zoonoses are diseases transmitted from animals to humans by direct or indirect contact or through food consumption. In the European Union, *Campylobacter* spp. is the most important zoonotic bacteria involved in foodborne gastrointestinal diseases with more than 246,000 cases of campylobacteriosis in 2018 (European Food Safety Authority and European Centre for Disease Prevention and Control, 2019). Chicken meat contamination occurs mainly during evisceration at the slaughterhouse (Herman et al., 2003). The European Food Safety Authority (EFSA) estimated that 37.5% of fresh broiler meat were contaminated in the EU (European Food Safety Authority and European Centre for Disease Prevention and Control, 2019).

Campylobacter spp. are Gram-negative, microaerophilic, spirally curved bacteria that mainly live as commensal organisms in the gastrointestinal tract of birds (Silva et al., 2011; Vandamme et al., 2015). Chickens are natural hosts for *Campylobacter* species and especially *C. jejuni* (Williams et al., 2016). *C. jejuni* colonizes primarily the caeca, but also the large intestine and the cloaca reaching 10^6–10^9 CFU/g of contents (Dhillon et al., 2006; Meunier et al., 2016). Although *C. jejuni* is considered as a commensal, studies have shown that this bacterium can cross the epithelial intestinal barrier and disseminate to internal organs (e.g. liver and spleen) (Cox et al., 2009; Meade et al., 2009). Several researchers suggested an alteration in the intestinal physiology of chickens (modification of intestinal villi and cryptae), as well as an activation of the immune system (Awad et al., 2015; Humphrey et al., 2014; Lamb-Rosteski et al., 2008). However, the impact of *C. jejuni* colonization after inoculation or exposure with infected birds on the structure of the microbiota remains unclear and might be modulated by the genetic origin of the chickens (Connerton et al., 2018; Thibodeau et al., 2015). Unfortunately, previously cited studies limited their scope on *C jejuni* impairment of microbiota taxonomy and structure diversity. The study of the functional pathways of caecal microbiota together with its composition and diversity could provide a better understanding of the interactions of *C. jejuni* within the caecal microbial community and therefore health consequences.

In 2013, Romero-Barrios et al. proposed a mathematical model suggesting that a reduction of the *C. jejuni* load by two to three log at the farm level could reduce human infections by, respectively, 76%–90% (Romero-Barrios et al., 2013). The use of essential oil as bactericidal ingredients in chicken feed might be a promising strategy to reach that goal. Indeed, the antibacterial properties of essential oil compounds have been widely described against a large range of pathogenic bacteria (Du et al., 2015). The positive effect of carvacrol (2-methyl-5-[1-methylethyl]-phenol), a monoterpenic phenol component of the essential oils isolated from *Origanum vulgare* (oregano) and *Thymus vulgare* (thyme), against *C. jejuni* has been demonstrated in vitro (Anderson et al., 2009; Kollanoor Johny et al., 2010) and in vivo (Allaoua et al., 2018; Arsi et al., 2014; Szott et al., 2020). Due to their lipophilic properties, essential oil compounds such as carvacrol, easily cross the biological membranes (Zotti et al., 2013), resulting in an early absorption in the stomach and proximal small intestine (Michiels et al., 2008). Fixing carvacrol and thymol on silica was not sufficient to bring them to the distal part of the digestive tract of chickens, where *C. jejuni* grows (Du et al., 2015). An appropriate formulation is thus necessary to release the carvacrol in the caeca to inhibit *C. jejuni* development. We previously demonstrate in vitro the antimicrobial activity of a carvacrol-based formulation against *C. jejuni* and evidenced a delay in the release of this formulated carvacrol into the caeca (Allaoua et al., 2018). However, the effectiveness of this formulation to reduce the *C. jejuni* load in vivo remains to be established. Furthermore, previous studies reported the effect of oregano essential oil or thymol mixed with carvacrol (Bortoluzzi et al., 2017; Ruan et al., 2021; Zhu et al., 2019) on the chicken caecal microbiota, but to our knowledge, none mentioned the effect of carvacrol either alone, pure or formulated.

Thus, the aim of this study was to (i) test the ability of the carvacrol-based formulation to decrease the *C. jejuni* caecal load on broiler chickens after inoculation and (ii) study the impact of the inoculation with *C. jejuni* alone or combined with the carvacrol-based product, on the caecal microbiota structure and predicted metabolic functions.

MATERIALS AND METHODS

Birds and experimental design

The in vivo assay was conducted in accordance with the current guidelines of the directive 2010/63/EU of the European Parliament and of the Council, in the facilities of the UE-1277 Plateforme d'Infectiologie Experimentale (PFIE, INRAE, 2021. Infectiology of the farm, model and wild animal facility, Centre Val de Loire, Nouzilly, France). All the experimental procedures were approved by the Loire Valley ethical review board (CEEA VdL, committee number 19, n°02110.02). The experimental procedure is detailed in Figure 1.

One hundred male ROSS 308 broiler chickens of 1 day old were used and housed in the breeding rooms underregulated environment: temperature from 32°C for 1-day-old chicks to 25°C for 35-day-old chickens and

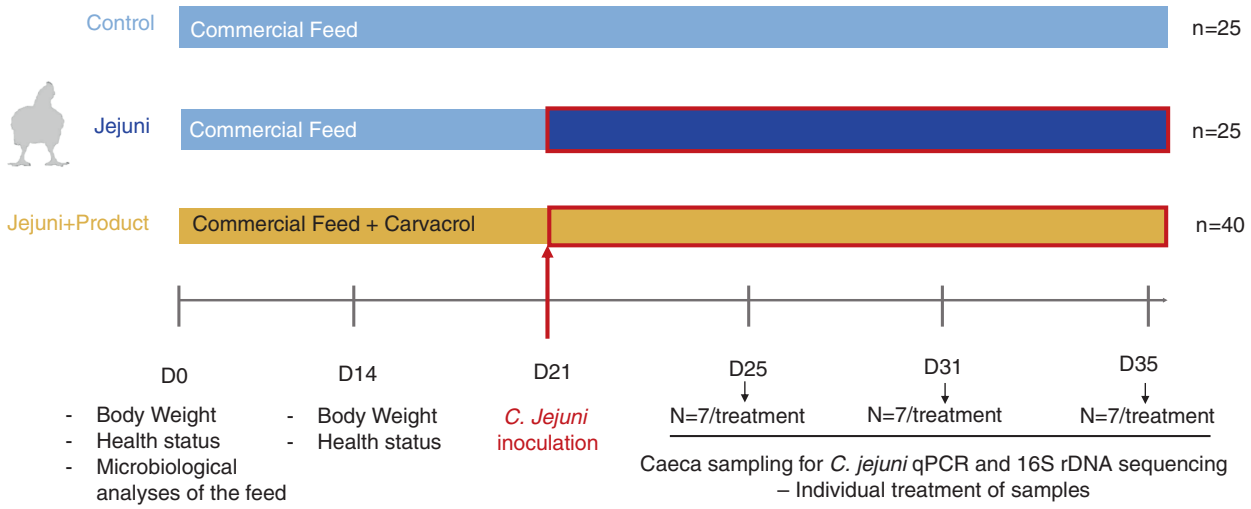

FIGURE 1 Experimental procedure of the in vivo challenge on chickens. Control: non-inoculated and non-supplemented group, Jejuni: *C. jejuni* inoculated and non-supplemented group, Jejuni + product: *C. jejuni* inoculated and supplemented group with the carvacrol-based product at 2.5 kg/ton of feed

relative humidity at 60%. The environment of the animals was enriched by placing resting mats and suspended metal plates.

On the first day of the experiment (D1), the absence of enteropathogenic *Escherichia*, *Salmonella* spp., *Campylobacter* spp. and *Clostridium perfringens* was checked by enumeration in the caecal content of 10 chickens with, respectively, BromoCresol Purple medium, Xylose-Lysine-Deoxycholate agar medium, Charcoal Cefoperazone Deoxycholate Agar and Glucose Yeast medium. Chickens were allocated to two groups: 50 chickens received a non-supplemented soybean meal-wheat-corn-based diet (Table 1) and the 40 others received the same commercial feed supplemented with 2.5 kg/ton of feed (0.25%) of a product formulated and provided by Phodé (France). The product (Phodé, France) is composed of 1%–2% of carvacrol (active ingredient), 3%–5% of surfactants (solubilizing agents), 40%–45% of monoglycerides (stabilizing agents), 4%–7% of water and 15%–25% cellulose (adsorbent carrier) and 20%–30% of silica (caking inhibitor). A previous pharmacokinetic study demonstrated that the carvacrol-based product was not absorbed in the upper part of the digestive tract but was released in the caeca and large intestine (Allaoua et al., 2018). On D14, the health status of three animals/group were checked for the absence of enteropathogenic *Escherichia*, *Salmonella* spp., *Campylobacter* spp. and *Clostridium perfringens* by enumeration in the caecal content.

The absence of enteropathogenic *Escherichia*, *Salmonella* spp., *Campylobacter* spp. and *Clostridium perfringens* was also checked by enumeration in 400 g of feed on the first day of the test. The feed intake and the body-weight measurements were carried out at the beginning of the test (mean body weight on D1: 0.051 ± 0.004 kg) and on 21, 25, 31 and 35-day-old chickens. On D21, the chickens with the non-supplemented feed were separated into two groups, a group of non-inoculated and non-treated chickens (Control group $n = 24$) and one group with inoculated and non-supplemented chickens (Jejuni group $n = 23$). The chickens of the Jejuni and Jejuni + Product

TABLE 1 Chemical composition of basal diet

Ingredient (%)	
Corn	**28.01**
Wheat	30
Soybean meal	34.2
Soybean oil	4
Calcium carbonate	0.94
Dicalcium phosphate	1.88
Salt	0.4
Methionin	0.17
Vitamin + mineral + anticoccidial premix	0.4
Total	
Calculated nutritional composition (%)	
Crude protein	21.34
Lysin	1.02
Methionin	0.43
Fat	5.96
Calcium	0.97
Phosphore	0.71
AME (kcal/kg)	2936

Abbreviation: AME, apparent metabolizable energy.

groups were orally inoculated with 1.10^8 CFU (equivalent dose to that found in the caeca of the broiler chickens) of *C. jejuni* per chicken (CJ-MDR1, a multidrug-resistant wild-type strain of *C. jejuni*, ANSES, France) on D21. Chickens were inoculated at 21 days because it is known that maternal antibodies stay in the chick's blood circulation for 14–21 days post-hatch (King et al., 2010). It is only at this stage that the chicks are considered immunologically mature. Euthanasia were carried out using CO_2 or by cervical dislocation depending on the age of the animals. Necropsies were performed on seven chickens of each group ($n = 7$) on D25, 31 and 35 (4, 10 and 14 days post-inoculation). During those autopsies, both caeca of each chicken were recovered, frozen in liquid nitrogen and then stored at −80°C. The remaining chickens were euthanized at the end of the procedure.

qPCR evaluation of the *C. jejuni* caecal load

Caeca were emptied and rinsed with 1X Phosphate Buffered Saline (PBS) solution prepared from 10X PBS (Invitrogen, Fisher Scientific). Then, DNA was extracted from 200 mg of caecal content, using the QiAmp Fast DNA Stool Mini Kit (Qiagen) according to the protocol of Josefsen et al. and the supplier's instructions (Josefsen et al., 2015). The expression of the N-benzoylglycine amidohydrolase (hippuricase, hipO) gene has been used to differentiate strains of *C. jejuni* and *C. coli*. In order to specifically quantify *C. jejuni*, the primers and probe designed by LaGier et al. (2004) were chosen to amplify the hipO gene. The two primers were Cj-F1 TGCTAGTGAGGTTGCAAAAGAATT and Cj-R1 TCATTTCGCAAAAAATCCAAA, and the probe was Cj-probe [6FAM]ACGATGATTAAATTCACAATTTTTTT CGCCAAA[TAM]. QPCR reactions were performed on Get-TQ platform (Toulouse, France) on a 96-well plate on a StepOne Plus thermocycler (Thermo Fisher Scientific). The qPCR assays were carried out in a 12 µl volume using the TaqMan Fast Advanced Mastermix (Thermo Fisher Scientific, Life technologies). Each sample was treated individually, they were never pooled ($n = 7$/group/time). The qPCR reaction contained 1X TaqMan Fast Advanced Mastermix, the two primers (10 µmol L^{-1}), the probe (250 µmol L^{-1}) and 2 µl of template DNA. Thermal cycling conditions were as follows: one cycle at 95°C for 20 min, followed by 40 cycles at 95°C for 1 min and 60°C for 20 min.

A standard curve was first made using 1/10 serial dilutions of a 10^9 CFU/ml *C. jejuni* stock solution. After centrifugation, the pellets were collected to obtain a similar matrix as the samples. DNA was extracted as described above and quantified using qPCR.

16 rDNA amplicon sequencing

On each individual sample, the V3 and V4 hypervariable regions of the 16S rRNA gene were amplified with indexed and adaptor-linked universal primers (343F: 5'CTTTCCCTACACGACG CTCTTCC GATCTACGGRAGGCAGCAG and 784R: 5'GGAGTTC AGACGTGT GCTCTTCCGATCTTACCAGGGTATCT AATCCT). The amplicons were sequenced on Illumina MiSeq at the GeT PLAGE platform. Paired-end reads of 250 bp were obtained. The raw sequences were analysed using the Galaxy-supported FROGS pipeline (Escudié et al., 2018) to process the 3,041,045 16S ribosomal RNA gene amplicon sequences obtained. Amplicons without any ambiguous base, with a length between 400 and 500 nucleotides and matching with V3 and V4 proximal PCR primer sequences, were kept for clustering. Reads were clustered into OTUs (Operational Taxonomic Units) using the iterative growth process SWARM (Mahé et al., 2014). Chimaera was detected using VSEARCH (Rognes et al., 2016) and then discarded. The remaining OTUs were filtered to keep OTUs present at least in 2 samples. The taxonomic affiliation of the OTUs (Operational Taxonomic Units) was performed with the BLAST algorithm against the Silva 138 database (Quast et al., 2013). The mean number of reads per sample was 34,907 (min: 20,675 to max: 61,415). A phyloseq object (McMurdie & Holmes, 2013) containing the OTUs table (OTU abundance for each sample with taxonomic classification) as well as sample information was built for subsequent analysis. Diversity indexes (α-diversity), including Shannon and Inverse Simpson, were calculated with the phyloseq R package after the rarefaction of the OTU table at 20,675 sequences. PICRUSt2 analysis was used to predict the caecal microbial community functions with the unrarefied OTU abundance table as input (Douglas et al., 2020). Following PICRUSt2 authors' recommendations, OTUs with a weighted Nearest Sequenced Taxon Index (NSTI) score higher than 0.15 were discarded due to low prediction quality. Relative predicted abundance of MetaCyc pathways and prediction based on Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto, 2000) were normalized by dividing the abundance of each pathway by the sum of all pathway abundances per sample.

Statistical analysis

Data analyses were conducted using the R software (release 4.0.3, The R foundation for statistical computing). Root square transformed taxonomic relative abundances at phylum, family and genus levels, diversity indexes

(number of observed OTU, Shannon and InvSimpson indexes), log-transformed qPCR data and body weight and feed intake for each time point were analysed with ANOVA. Age, treatment and their interaction were considered as fixed effects. P values were adjusted using the Hochberg methods. When the main effect was significant, post hoc comparisons with a Tukey adjustment for multiple comparisons were made (R package emmeans). To compare the bacterial community structures and MetaCyc pathways resulting matric across all samples, a non-Metric Distance Scaling (nMDS) analysis was conducted with a Bray–Curtis dissimilarity index on the total sum scaling normalized matrix. A PERMANOVA (vegan package) was then used to test whether composition among groups was similar or not.

Data access

All raw sequences were deposited in the NCBI Sequence Read Archive (accession no. PRJNA736580).

RESULTS

Body weight of animals

All animals were in good health and no group showed body weight or feed intake reduction. Body weight of chickens is presented in Figure 2.

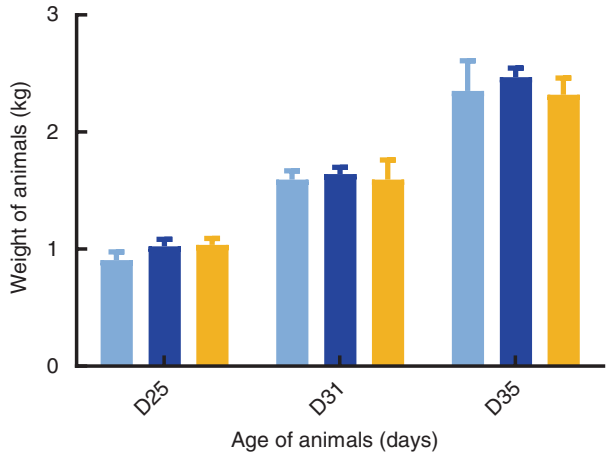

FIGURE 2 Effect of *C. jejuni* inoculation and supplementation of the feed with the carvacrol-based product on body weight of chickens ($n = 7$ per group). The light blue bars represent the control group, non-inoculated and non-supplemented. The dark blue bars represent the Jejuni group, that is chickens inoculated with *C. jejuni* and non-supplemented with the product. The yellow bars represent the Jejuni + product group, that is chickens inoculated with *C. jejuni* and supplemented with the carvacrol-based product at 2.5 kg/ton of feed

Efficacy of *C. jejuni* inoculation

Chickens were inoculated with 10^8 CFU of *C. jejuni*. In order to assess *C. jejuni* load in the control and inoculated chickens, a qPCR experiment using primer designed for specific amplification of hipO gene was performed. As expected, chickens from the Control group were exempt from *C. jejuni* throughout the test (Figure 3). There was no significant effect of the sampling age, but a significant impact on the group and a significant interaction between day and group ($p < 0.05$ after Bonferroni correction). Indeed, the carvacrol-based product significantly decreased by 1.4 log ($p < 0.05$) the *C. jejuni* caecal load after 35 days while no significant difference was observed at 25 and 31 days. On 31-day-old chickens, the carvacrol-based formulation induced a non-significant decrease of 0.77 log of the targeted bacteria load. The analysis of the relative abundance of the OTU affiliated to *Campylobacter* by sequencing the V3–V4 region of the 16S rRNA gene leads to the same conclusions (Figure 9). Of note, this latter genus and the *Campylobacteraceae* family were the unique taxa of the *Campylobacterota* phylum.

Diversity and structure dynamics of the caecal bacterial community

The α- and β-diversity of caecal microbiota

To evaluate the effect of *C. jejuni* inoculation and carvacrol-based product on caecal bacterial communities,

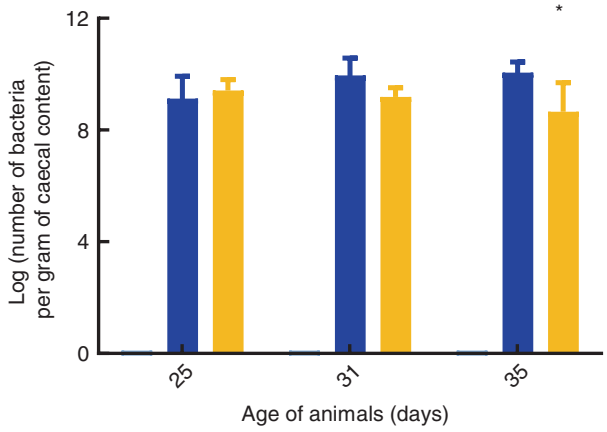

FIGURE 3 Kinetic of *C. jejuni* carriage in chickens after supplementation of the feed with the carvacrol-based product ($n = 7$ per group). Star represents the level of significance ($p < 0.05$). The light blue bars represent the control group, non-inoculated and non-supplemented. The dark blue bars represent the Jejuni group, that is chickens inoculated with *C. jejuni* and non-supplemented with the product. The yellow bars represent the Jejuni + product group, that is chickens inoculated with *C. jejuni* and supplemented with the carvacrol-based product at 2.5 kg/ton of feed

we first assessed α-diversity using the richness index, evaluated as the number of observed OTUs, and Shannon and InvSimpson indices (Figure 4). As expected, in the Control group, the diversity indices were stable across the time of sampling except for a slight decrease of the Shannon index between D31 and D35. A significant interaction between age and group was observed. Compared with the Control group, the inoculation with *C. jejuni* significantly increased the population richness at 31 and 35 days and its diversity at 35 days according to the two indices.

Supplementation with carvacrol-based product in the feed of the inoculated animals increased the Shannon diversity of the caecal bacterial community from 25 days on compared to the inoculated group (Jejuni group). The same results were observed for InvSimpson, except at 35 days of age, where the differences between the inoculated animals receiving or not carvacrol-based product were not significant anymore. Finally, adding the carvacrol-based product to the feed appeared to stabilize the diversity with age in inoculated animals since no effect of age was observed for this group.

As a next step, we analysed the consequence of *C. jejuni* inoculation and the effect of the addition of the carvacrol-based product in the feed in inoculated animals on beta-diversity of the caecal bacterial community. Besides the age-related effect, the non-metric multidimensional scaling (nMDS) plot indicated that the community structure strongly differed between groups as highlighted by the clear separation of the three groups (Figure 5) and the results of the multilevel pairwise adonis (Figure S2). This separation was effective from 25 days of age. An age-related change was observed in the three groups (Figure 5) and the results of the multilevel pairwise adonis (Figure S2). This separation was effective from 25 days of age. An age-related change was observed in the three groups. The treatment with the carvacrol-based product in *C. jejuni* inoculated chickens led to the greatest change in community structure compared to the two other groups.

Core bacterial community

To assess the effect of our treatments on the core bacterial community, an Euler diagram (Larsson et al., 2020) was displayed at each age, to represent shared OTUs in each experimental group based on the OTUs present in at least 50% of the 63 individuals, that is representative of the core bacterial community (Figure 6). The addition of the carvacrol-based product in the feed of the inoculated animals lead to a specific microbial signature, as indicated by the majority of unique OTUs (81, 107 and 109 OTUs out of the 416 OTUs at, respectively, 25, 31 and 35 days of age).

Relative abundances of the dominant phyla

To better characterize the microbial signature of treatments on the chicken caecal community, we analysed the taxa relative abundance at the phylum, family and genus levels.

A total of 10 different phyla could be identified in the chicken caeca but only 3 had a relative abundance above 0.5%. As expected, *Campylobacterota* phylum was observed only in the two groups inoculated with *C. jejuni* (Jejuni and Jejuni + Product groups). The major phyla in the caecal content of the chickens were the *Firmicutes* and the *Proteobacteria*, whatever the age or the treatment (respectively between 70% and 95% and between 3% and 29%, Figure 7, Table S1). The age-related increase in *Firmicutes* at the expense of *Proteobacteria* in the Control group was not observed in the chickens inoculated with *C. jejuni*. The addition of the carvacrol-based product seemed to

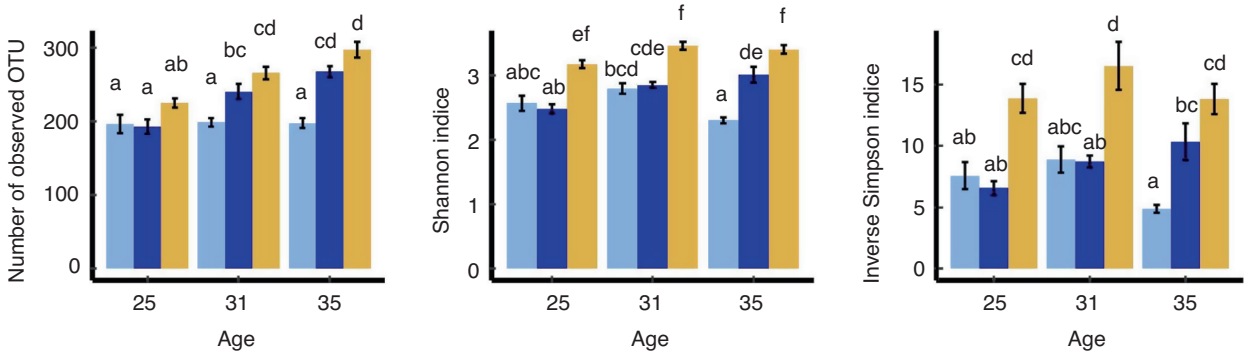

FIGURE 4 Effect of *C. jejuni* inoculation and supplementation of the feed with the carvacrol-based product on chicken caecal bacterial community α-diversity at 25, 31 and 35 days of age ($n = 7$ per group). Letters represent pairwise contrasts ($p < 0.05$). The light blue bars represent the control group, non-inoculated and non-supplemented. The dark blue bars represent the Jejuni group, that is chickens inoculated with *C. jejuni* and non-supplemented with the product. The yellow bars represent the Jejuni + product group, that is chickens inoculated with *C. jejuni* and supplemented with the carvacrol-based product at 2.5 kg/ton of feed

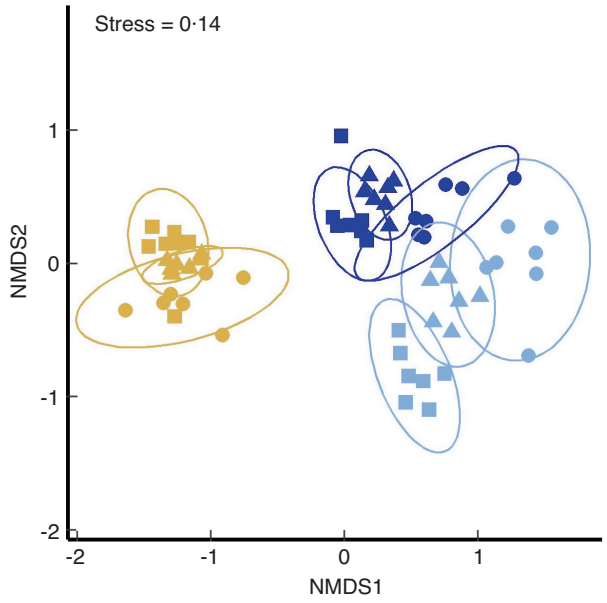

FIGURE 5 Effect of *C. jejuni* inoculation and supplementation of the feed with the carvacrol-based product on chicken caecal bacterial community β-diversity at 25, 31 and 35 days of age: nMDS two-dimensional representation of caecal bacterial community using Bray–Curtis distance matrix ($n = 7$ per group). The light blue signs represent the control group, non-inoculated and non-supplemented. The dark blue signs represent the Jejuni group, that is chickens inoculated with *C. jejuni* and non-supplemented with the product. The yellow signs represent the Jejuni + product group, that is chickens inoculated with *C. jejuni* and supplemented with the carvacrol-based product at 2.5 kg/ton of feed. ●, chickens of 25 days old; ▲, chickens of 31 days old; ■, chickens of 35 days old

restore this age-related dynamic at least for the *Firmicutes* phylum.

Differentially abundant caecal bacterial families

There were 79 families present in the chicken caecal ecosystem of which 5 were classified as 'unknown family'. However, only 13 families had a relative abundance of over 0.5% in at least one experimental group and were further considered for statistical analysis (Paës et al., 2020) (Figure 8a, Table S1). Compared to the Control group, the inoculation significantly halved the relative abundance of *Enterobacteriaceae* on 25 days chickens and doubled that of *Lachnospiraceae* and *Ruminococcaceae* at 35 days of age while the establishment of the *Monoglobaceae* family was observed from D31 (Figure 8b). The addition of the carvacrol-based product in the feed of the inoculated animals further decreased the *Enterobacteriaceae* abundance on D35. Treatment with the carvacrol-based product modified the *Oscillospiraceae* to *Ruminococcaceae* balance. In the Control and Jejuni groups, the *Oscillospiraceae* were present in greater quantity, whereas in the group that received the product the *Ruminococcaceae* predominated (Figure 8b). Interestingly, upon treatment with the carvacrol-based product, we observed also the establishment of the *Butyricicoccaceae* family in the inoculated animals with an abundance of up to 2%.

Differentially abundant caecal bacterial genera

16S RNA gene sequences analyses revealed a total of 206 genera in the chicken caecal ecosystem of which 8 had multi-affiliation and 38 were classified as unknown genus is given a BLAST identity threshold of 97%. Thirty-five identified genera had a relative abundance of over 0.5% in at least one experimental group and were further considered for statistical analysis (Paës et al., 2020) (Figure 9, Table S1). Compared to the control group, the inoculation decreased the *Escherichia-Shigella* proportion at 25 days of age, those of *Rombustia* at 25 and 31 days of age, and the *Clostridioides* proportion at 31 days. *Paludicola* genus was increased with inoculation from D25, *Agatobacter* genus was increased on D31 while *Lactobacillus*, *Selimonas* and *Eisenbergiella* were increased from D31. The supplementation of feed with the carvacrol-based product hindered the increase of *Agatobacter* and *Eisenbergiella* observed in the non-treated inoculated animals and further decreased the *Escherichia-Shigella* proportion. Moreover, compared to the two other groups, *C. jejuni* inoculation associated with formulated carvacrol supplementation led to a decrease in the relative abundance of *Klebsiella* and *Salmonella* and *Oscillobacter*. Conversely, the development of *[Ruminococcus] torques group*, *Faecalibacterium*, *Subdoligranulum*, *Butyricicoccus*, *Blautia*, *Shuttleworthia*, *Tyzzerella* and *Lachnospiraceae GCA-900066575 group* was favoured in the caecal microbiota by the supplementation of inoculated chickens with the carvacrol-based product.

Predicted functions of the caecal bacterial community

To evaluate whether the shift in the microbiota composition following the *C. jejuni* inoculation alone or coupled with the addition of the carvacrol-based product would affect the microbiota functions, we identified the predicted MetaCyc and KEGG pathways using PICRUSt2 (Douglas et al., 2020). The nMDS plot of the 376 identified pathways (Figure 10) highlighted a separation between groups. According to PERMANOVA pairwise comparisons (Figure S2), the separation between groups was effective from 25 days of age. A total of 171 out of the 376 MetaCyc pathways, revealed a significant effect on the

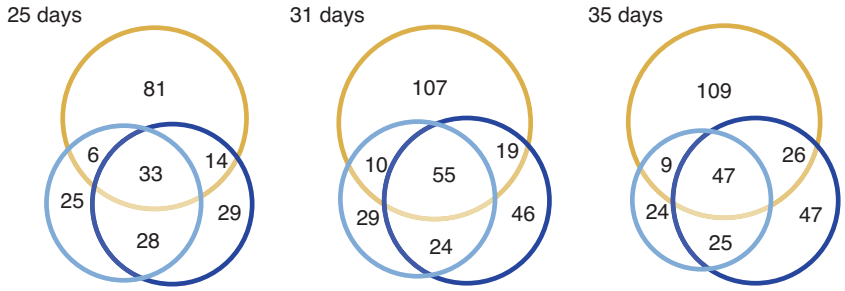

FIGURE 6 Effect of *C. jejuni* inoculation and supplementation of the feed with the carvacrol-based product on the core caecal bacterial community of chickens at 25, 31 and 35 days of age ($n = 7$ per group). Euler diagram with shared caecal bacterial OTUs between experimental groups based on the OTUs present in at least 50% of the 63 individuals. The light blue circles represent the control group, non-inoculated and non-supplemented. The dark blue circles represent the Jejuni group, that is chickens inoculated with *C. jejuni* and non-supplemented with the product. The yellow circles represent the Jejuni + Product group, that is chickens inoculated with *C. jejuni* and supplemented with the carvacrol-based product at 2.5 kg/ton of feed.

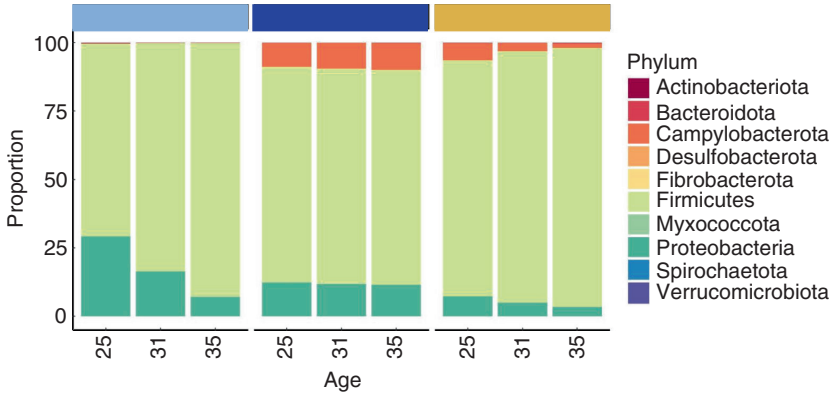

FIGURE 7 Effect of *C. jejuni* inoculation and feed supplementation with the carvacrol-based product on relative abundance of the phyla in the caecal bacterial community of chickens at 25, 31 and 35 days of age ($n = 7$ per group). The light blue rectangle represent the control group, non-inoculated and non-supplemented. The dark blue rectangle represents the Jejuni group, that is chickens inoculated with *C. jejuni* and non-supplemented with the product. The yellow rectangle represents the Jejuni + product group, that is chickens inoculated with *C. jejuni* and supplemented with the carvacrol-based product at 2.5 kg/ton of feed. Only 3 out of the 10 phyla had a relative abundance above 0.5%

group (p-adjusted <0.05) while 53 exhibited a significant age effect (Table S1). To get a general vision of metabolic potential shift according to the group, we aggregated MetaCyc pathways to predict level3 KEGG pathways. A total of 165 out of the 257 level3 KEGG pathways revealed significant differences (p-adjusted <0.05) with the group while only 17 exhibited a significant age effect (Table S1). Focusing on significant KEGG level3 pathways involved in degradation and biosynthesis pathways, we could observe two kinds of the response of microbiota metabolic potential to *C. jejuni* inoculation (Figure 11 and Table S1). The first metabolic response occurred only rapidly after inoculation (from the fourth day onwards, i.e. D25). The predicted relative abundance of genes involved in lysine and zeatin biosynthesis were increased, whereas those in penicillin and cephalosporin, carotenoid, flavone and flavonol biosynthesis, atrazine and fluorobenzoate degradation were decreased. A second profile corresponding to a later response was observed from 10 to 15 days after inoculation (D31–D35). It included the increase of predicted relative abundance of genes involved in the biosynthesis of folate, streptomycin, amino acids (phenylalanine, tyrosine and tryptophan) and the decrease of those involved in the degradation of other amino acids (lysine, valine, leucine and isoleucine), of monoterpen degradation (geraniol, limonene and pinene), of aromatic compound utilization (benzoate, aminobenzoate, ethylbenzene, nitrotoluene, chlorocyclohexane and chlorobenzene degradation) and carbon fixation pathways.

Interestingly, supplementation with the carvacrol-based product in animals inoculated with the *C. jejuni* led to the greatest changes in predicted pathway structure compared to the two other groups. The increased pathways involved antimicrobial synthesis (tetracycline, butirosin, neomycin, streptomycin, penicillin and cephalosporin, bacterial toxins), bacterial structural compound syntheses

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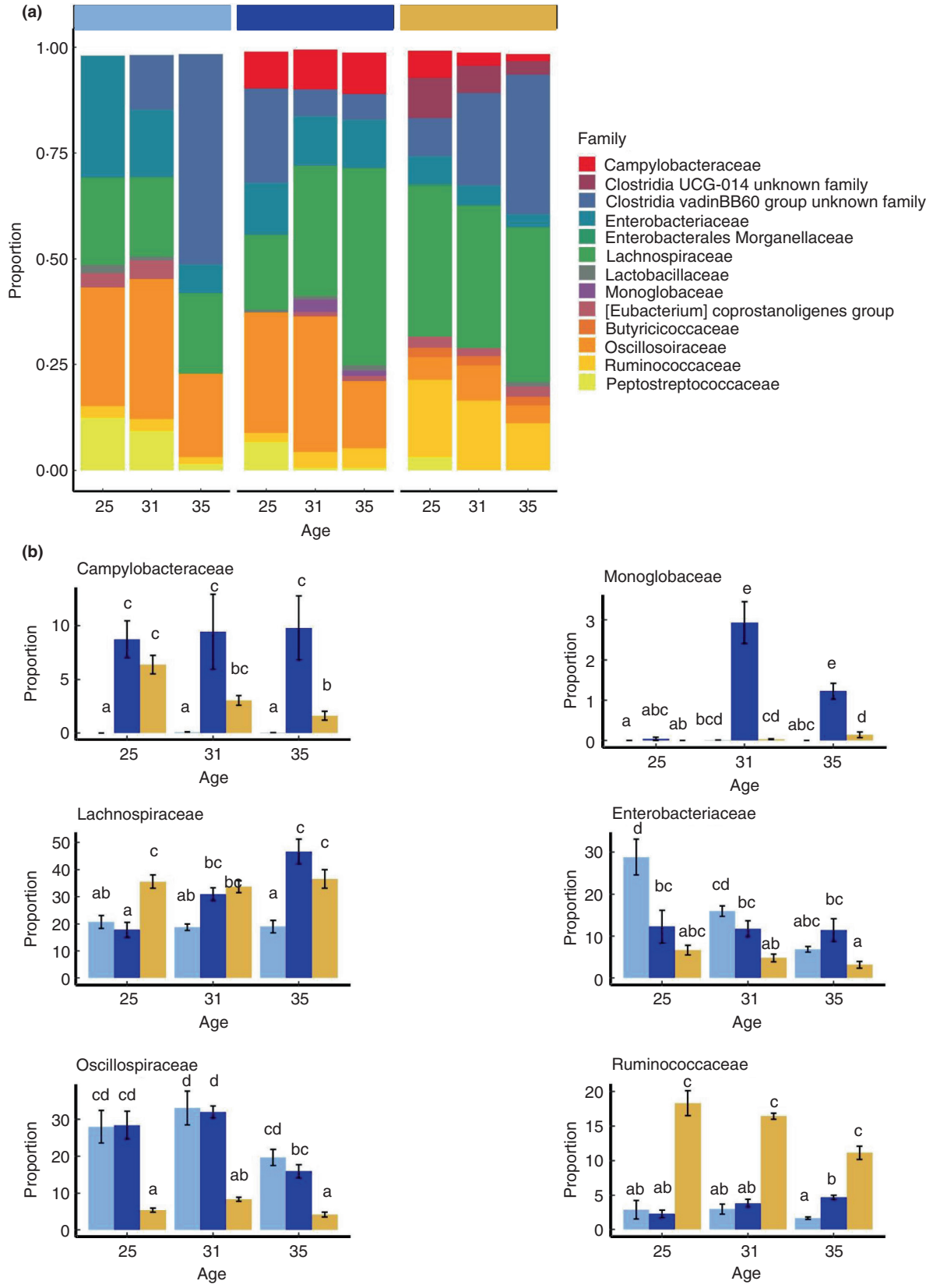

FIGURE 8 Effect of *C. jejuni* inoculation and feed supplementation with the carvacrol-based product on the relative abundance of the families in the caecal bacterial community of chickens at 25, 31 and 35 days of age ($n = 7$ per group). (a) Stacked plot with families above 0.5% of relative abundances. (b) Detailed evolution of the selected family. The light blue bars represent the control group, non-inoculated and non-supplemented. The dark blue bars represent the Jejuni group, that is chickens inoculated with *C. jejuni* and non-supplemented with the product. The yellow bars represent the Jejuni + Product group, that is chickens inoculated with *C. jejuni* and supplemented with the carvacrol-based product at 2.5 kg/ton of feed.

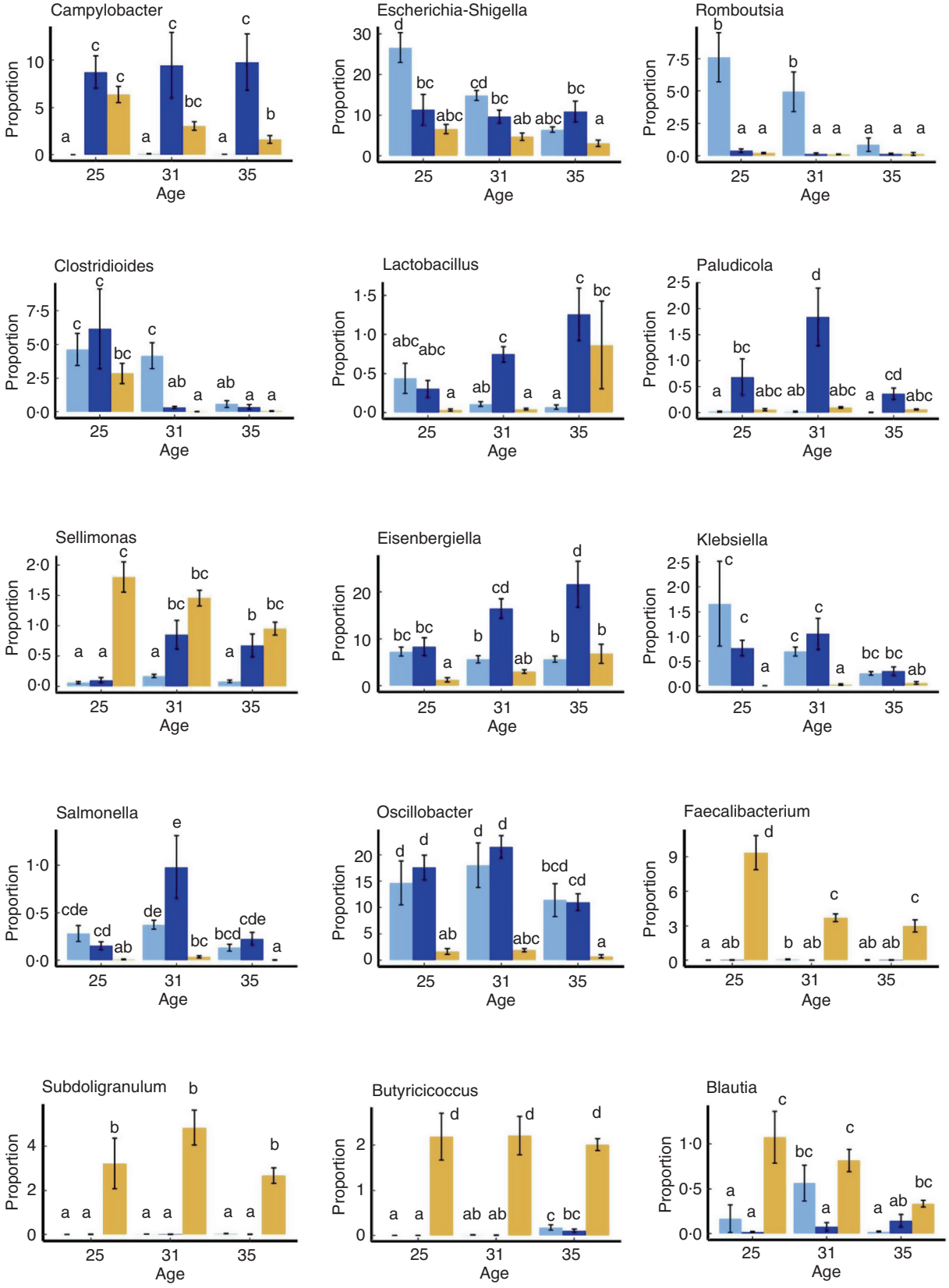

FIGURE 9 Effect of *C. jejuni* inoculation and feed supplementation with the carvacrol-based product on the relative abundance of the 27 most abundant (>0.1%) genera in the caecal bacterial community of chickens at 25, 31 and 35 days of age ($n = 7$ per group). The light blue bars represent the Control group, non-inoculated and non-supplemented. The dark blue bars represent the Jejuni group, that is chickens inoculated with *C. jejuni* and non-supplemented with the product. The yellow bars represent the Jejuni + Product group, that is chickens inoculated with *C. jejuni* and supplemented with the carvacrol-based product at 2.5 kg/ton of feed.

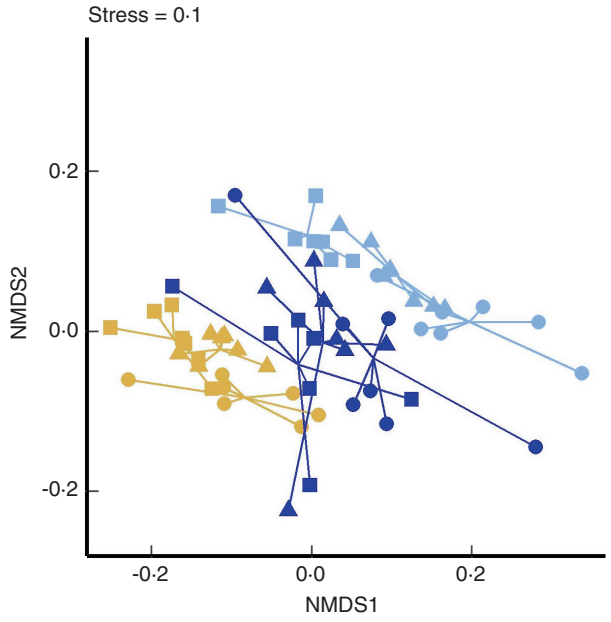

FIGURE 10 Effect of *C. jejuni* inoculation and carvacrol-based product on chicken caecal bacterial community predicted MetaCyc pathways using PIRCRUSt2 at 25, 31 and 35 days of age ($n = 7$ per group): nMDS two-dimensional representation based on Bray–Curtis metrics. The light blue signs represent the control group, non-inoculated and non-supplemented. The dark blue signs represent the Jejuni group, that is chickens inoculated with *C. jejuni* and non-supplemented with the product. The yellow signs represent the Jejuni + Product group, that is chickens inoculated with *C. jejuni* and supplemented with the carvacrol-based product at 2.5 kg/ton of feed. ●, chickens of 25 days old; ▲, chickens of 31 days old; ■, chickens of 35 days old.

such as peptidoglycan, fatty acids and lysine biosynthesis. The down-regulated pathways involved amino acid degradation (lysine, valine, leucine and isoleucine), aromatic compound utilization (benzoate, aminobenzoate, ethylbenzene, nitrotoluene, chlorocyclohexane and chlorobenzene degradation), monoterpen degradation (geraniol, limonene and pinene) and carbon fixation pathways. Altogether, the difference in microbiota composition according to *C. jejuni* inoculation alone or in broiler chickens treated with the carvacrol-based product was associated with major modifications of the predicted metabolic functions.

DISCUSSION

C. jejuni is the leading cause of bacterial zoonosis responsible for gastroenteritis in humans. Contamination occurs mainly through the consumption of contaminated chicken meat by contact with digestive content at the time of evisceration. The aim of this study was first to evaluate the ability of a carvacrol-based product to decrease the *C. jejuni* caecal load in inoculated broiler chickens and second to study the impact of the *C. jejuni* inoculation alone or combined with the carvacrol-based product on the caecal microbiota. Although we did not test the carvacrol-based product alone, we demonstrated that the product was effective in reducing the *C. jejuni* load in the caeca of inoculated animals by 1.4 log after 35 days of treatment. These beneficial effects of the carvacrol-based product were associated with a shift of the gut microbiota composition and predicted metabolic function potentially leading to beneficial effects on the chicken host.

The chicken caecal microbiota

In chickens, the microbiota colonizes the gut intestinal tract from the hatch and it stabilizes around 3 weeks (Diaz Carrasco et al., 2019). In accordance with previous studies on chicken caecal microbiota, we confirmed the predominance of *Firmicutes*, while the *Bacteroidota* and *Proteobacteria* were present in lower abundances (Awad et al., 2016; Han et al., 2016; Hankel et al., 2019; Kumar et al., 2018; Wei et al., 2013). The most represented families in the control group were *Lachnospiraceae*, *Enterobacteriaceae*, *Peptostreptococcaceae* and an unknown family of the *Clostridiales* vadin BB60 group. According to the literature, the proportions of these families are subject to variations due to differences in diet, breeding environment and genetics.

Effect of the *C. jejuni* inoculation on the caecal microbiota

In accordance with previous studies (Sofka et al., 2015; Thibodeau et al., 2015), the *C. jejuni* inoculation resulted in an increase in the richness and α-diversity while β-diversity was altered in the caecal microbiota 4 days after inoculation. The increase of diversity was related to a disruption of taxonomic balance. Indeed, although *C. jejuni* are naturally hosted in the caeca of broiler chickens (Newell, 2002), their massive introduction through inoculation led to a decrease in the abundance of *Firmicutes*, as previously observed by Sofka et al. (2015). In the present study, we further observed important modifications within the *Fimicutes* phylum with an establishment of *Monoglobaceae* family member and dominance of the *Lachnospiraceae* at the expense of members of the *Clostridia* vadimBB60 group unknown family under inoculation. According to Awad et al. (2016), the positive relationship between the presence of *Campylobacter* and the *Lachnospiraceae* proportion may be explained by cross-feeding properties since *Campylobacter* are known

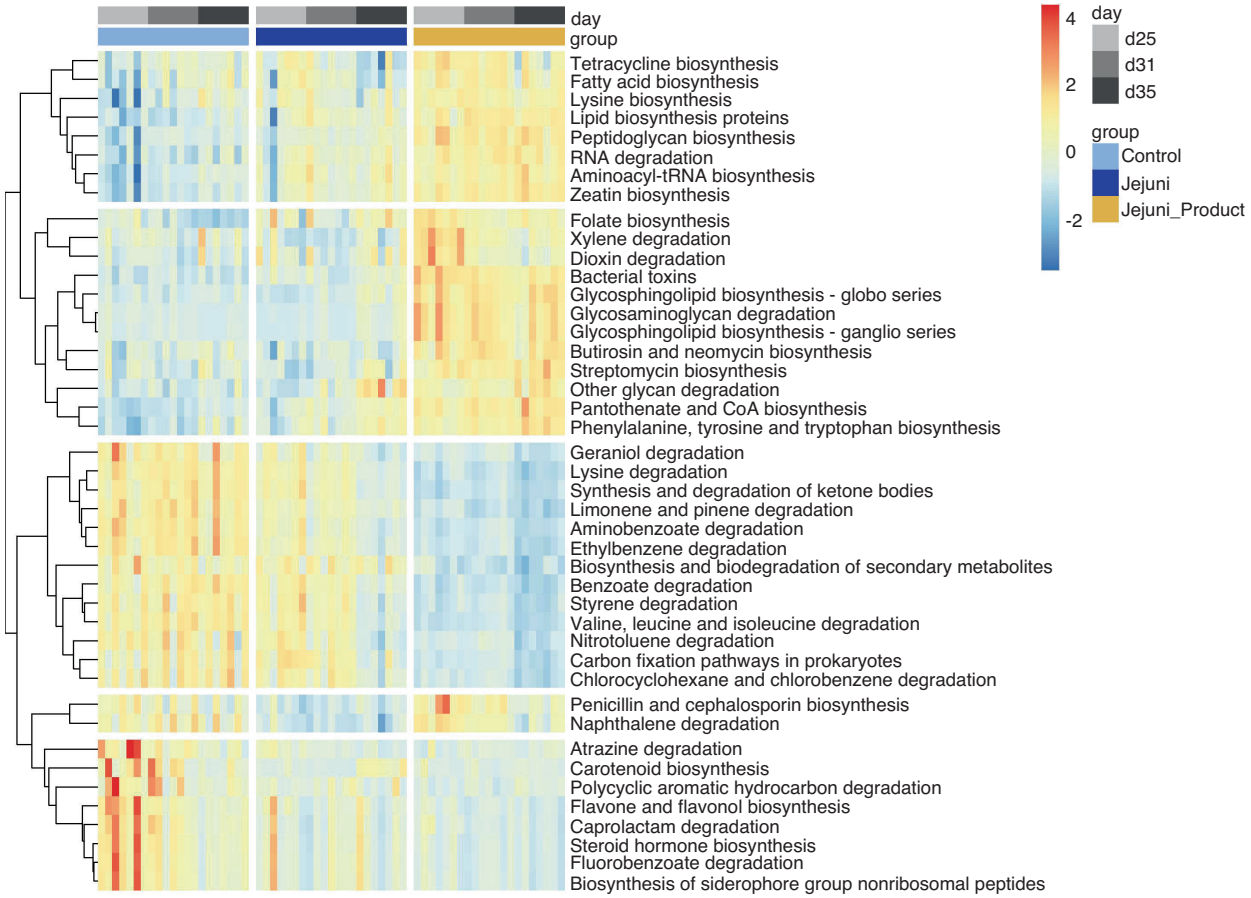

FIGURE 11 Effect of *C. jejuni* inoculation and carvacrol-based product on chicken caecal bacterial community predicted level 3 KEGG degradation and biosynthesis pathways at 25, 31 and 35 days of age. Heatmap represents the relative abundance of level 3 KEGG pathways (rows) in individual samples (columns). The colours represent the row-scaled relative abundance from low (blue) to high values (red). Level 3 pathways (rows) were clustered by the Ward algorithm. Control: non-inoculated and non-supplemented group, Jejuni: *C. jejuni* inoculated and non-supplemented group, Jejuni + product: *C. jejuni* inoculated and carvacrol-based product supplemented group ($n = 7$ per group)

to use the organic acid produced by *Lachnospiraceae* as an energy source. Sakaridis et al. (2018) found that caecal samples with higher *Campylobacter* loads had higher *Enterobacteriaceae* proportions, and on the contrary Sofka et al. (2015) and Connerton et al. (2018) showed a decrease in the *Enterobacteriaceae* count in *Campylobacter*-positive chickens. It seems that the effect of the presence of *Campylobacter* on *Enterobacteriaceae* proportion depends on the genera that are most commonly found in the study. Indeed, when *Escherichia-Shigella* is the main genus, the proportion of *Enterobacteriaceae* decreases at the same time as the number of *Campylobacter* increases in the chicken microbiota samples (as we observed at 25 and 31 days) (Connerton et al., 2018; Sofka et al., 2015). We hypothesize that the genera *Campylobacter* and *Escherichia-Shigella* might belong to the same niche and therefore must be in competition. Fifteen days after inoculation, an increase of *Lactobacillus*, *Paludicola*, *Sellimonas* and *Eisenbergiella* proportion was still observed. According to the literature, although *Campylobacter* inoculation resulted in a taxonomic rearrangement of the composition of the microbiota, no clear pattern is yet identifiable (Connerton et al., 2018; Kaakoush et al., 2014; Thibodeau et al., 2015). Besides altering the composition of caecal microbiota, we observed that *C. jejuni* inoculation led to either an early or late effect on the metabolic potential of the caecal bacterial community. To our knowledge, no data are available on *C. jejuni* impact on microbiota functionality. These predicted gene relative abundance evolution with time may reflect an adaptation of microbiota to the installation of the exogenous *C. jejuni*.

Effect of the carvacrol-based product on the caecal microbiota of inoculated animals

Our carvacrol-based product added to the feed was effective in reducing the load of *C. jejuni* in chicken ceca by 1.4

log. This is consistent with our previous pharmacokinetic study, demonstrating that the carvacrol-based product was not absorbed in the upper part of the digestive tract while it was released in the caeca and large intestine, the main sites for *C. jejuni* development (Allaoua et al., 2018). In addition to the beneficial effect on reducing *C. jejuni* abundance, the product decreased the abundance of potentially pathogenic bacteria such as *Escherichia-Shigella*, *Salmonella* and *Klebsiella*. An effective action of *Origanum vulgare* essential oil and carvacrol against enteropathogens like *Salmonella* and *Klebsiella* were demonstrated in vitro (Fournomiti et al., 2015; Mellencamp et al., 2011) and in vivo with a supplemented feed (Bauer et al., 2019). Carvacrol has been shown to increase the sensitivity of *Salmonella enterica* serovar Typhimurium to antibiotics (Kollanoor Johny et al., 2010). We demonstrated here the same effects when the carvacrol-based product was administered through the feed. Additionally, in chickens inoculated with *C. jejuni*, the carvacrol-based treatment restored the proportion of *Firmicutes* relative abundance and peculiarly the relative abundance of its genera *Eisenbergiella* and *Paludicola* to levels similar to those found in the control group.

Interestingly, the addition of the carvacrol-based product in the feed of the animals inoculated with *C jejuni* increased the diversity and strongly altered the structure of the bacterial community, leading to a specific microbial signature with a majority of OTUs found exclusively in this group. The differences in microbiota structure can be explained by (i) the exclusive presence in this group of *Faecalibacterium*, *Shuttleworthia*, *Subdoligranulum* and *Tyzzerella* and (ii) the increase of *Blautia* and *Butyricicoccus* and the reduced proportion of *Oscillobacter* compared to the inoculated chicken that did not receive the carvacrol-based product. *Faecalibacterium* and *Subdoligranulum* genera are well known to be butyrate producers which appear to be deleterious for *Campylobacter* growth (Ahsan et al., 2016; Cresci et al., 2017). Indeed, *C. jejuni* is able to sense the microbiota-derived butyrate via the BumSR to-component signal transduction system (Goodman et al., 2020). A bactericidal effect of butyrate on *C. jejuni* has been demonstrated in vitro (Van Deun et al., 2008) while a negative correlation between caecal butyrate content and *C. jejuni* load has been reported in broiler in vivo (Hankel et al., 2019). Altogether this could explain the negative correlation observed in our study between *Campylobacter* and *Faecalibacterium/Subdoligranulum* proportion. Additionally, Subdoligranulum also produces lactic, succinic and acetic acids that appear to have a bacteriostatic effect on enteropathogens (Bjerrum et al., 2006). *Faecalibacterium* and *Shuttleworthia* are also considered beneficial bacteria because they were associated with a body weight increase of chickens (Lee et al., 2017).

In our study, the observed shift in the composition of the caecal microbiota induced by the carvacrol-based product in chickens inoculated with *C. jejuni* was associated with important alteration of gut microbial predicted metabolism and functions. It could partly be explained by the microbiota functional enrichment in pathways involved in the biosynthesis of antimicrobial synthesis and bacteriocin (i.e. bacterial toxins) as previously observed in suckling piglets supplemented with benzoic acid and essential oils (Zhai et al., 2020). Besides support of gut barrier integrity, immune activation and nutrient competition, secretion of antimicrobial products allow the gut microbiota with colonization resistance (Ducarmon et al., 2019). Altogether, these mechanisms might explain both the resistance to *C. jejuni* development, the associated decrease of enteropathogens (Sassone-Corsi et al., 2016) and the microbiota structure specificity induced by the carvacrol-based product in chickens inoculated with *C. jejuni*. In inoculated chickens, the carvacrol-based product down-regulated the predicted degradation pathways of lysine, valine, leucine and isoleucine while up-regulated biosynthesis of lysine, phenylalanine, tyrosine and tryptophan. In accordance, Li et al. observed in piglets group supplemented with a mix of carvacrol and thymol, an increased concentration of several amino acids such as valine, isoleucine and alanine (Li et al., 2018). Knowing that the amino acid and protein composition and content was similar between the three groups (same diet), these results suggest that the carvacrol-based treatment in inoculated chickens could contribute to microbiota amino acid sparing in favour of protein biosynthesis. The monoterpen (geraniol, limonene and pinene) degradation pathways were down-regulated in inoculated and carvacrol-based product treated animals. Taking into account the bacteriostatic and bactericidal activities of carvacrol resulting from its monoterpenic structure (Suntres et al., 2015), our results might suggest tolerance of the selected bacterial community to essential oil compounds.

We can conclude that the carvacrol-based product provided to chickens from hatching is efficient to limit the development of *C. jejuni* after inoculation. Furthermore, our specific formulation allows the carvacrol to reach the caeca where it might select beneficial bacteria and promote the establishment of a beneficial bacterial community. Besides the direct bactericidal action of carvacrol, predicted metabolism and function analysis based on 16S rRNA gene abundance has allowed us to propose an indirect pathway of action of the carvacrol-based product via the selection of an adapted bacterial population producing antimicrobial substances that confer resistance to colonization. Further studies are needed to confirm the first results observed with PICRUSt2 on predicted pathways through whole

microbiota genome shotgun sequencing and thorough analysis with and without *Campylobacter* colonization. In particular, this would provide a better understanding of the ability of certain microbiota to cope with the presence of carvacrol and identify new antimicrobial molecules active against *C. jejuni* and derived from chicken microbiota.

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CONFLICT OF INTEREST

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REFERENCES

Ahsan, U., Cengiz, Ö., Raza, I., Kuter, E., Chacher, M. F. A., Iqbal, Z., et al. (2016) Sodium butyrate in chicken nutrition: the dynamics of performance, gut microbiota, gut morphology, and immunity. *World's Poultry Science Journal* 72, 265–275. doi:http://dx.doi.org/10.1017/s0043933916000210

Allaoua, M., Etienne, P., Noirot, V., Carayon, J.-L., Téné, N., Bonnafé, E. et al. (2018) Pharmacokinetic and antimicrobial activity of a new carvacrol-based product against a human pathogen, campylobacter jejuni. *Journal of Applied Microbiology*, 125, 1162–1174. https://doi.org/10.1111/jam.13915

Anderson, R.C., Krueger, N.A., Byrd, J.A., Harvey, R.B., Callaway, T.R., Edrington, T.S. et al. (2009) Effects of thymol and diphenyliodonium chloride against campylobacter spp. during pure and mixed culture in vitro. *Journal of Applied Microbiology*, 107, 1258–1268.

Arsi, K., Donoghue, A.M., Venkitanarayanan, K., Kollanoor-Johny, A., Fanatico, A.C., Blore, P.J. et al. (2014) The efficacy of the natural plant extracts, thymol and carvacrol against campylobacter colonization in broiler chickens. *Journal of Food Safety*, 34, 321–325.

Awad, W.A., Mann, E., Dzieciol, M., Hess, C., Schmitz-Esser, S., Wagner, M. et al. (2016) Age-related differences in the luminal and mucosa-associated gut microbiome of broiler chickens and shifts associated with campylobacter jejuni infection. *Frontiers in Cellular and Infection Microbiology*, 6. http://dx.doi.org/10.3389/fcimb.2016.00154

Awad, W.A., Molnár, A., Aschenbach, J.R., Ghareeb, K., Khayal, B., Hess, C. et al. (2015) Campylobacter infection in chickens modulates the intestinal epithelial barrier function. *Innate Immunity*, 21, 151–160.

Bauer, B.W., Radovanovic, A., Willson, N.-L., Bajagai, Y.S., Hao Van, T.T., Moore, R.J. et al. (2019) Oregano: a potential prophylactic treatment for the intestinal microbiota. *Heliyon*, 5, e02625. http://dx.doi.org/10.1016/j.heliyon.2019.e02625

Bjerrum, L., Engberg, R.M., Leser, T.D., Jensen, B.B., Finster, K. & Pedersen, K. (2006) Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and culture-based techniques. *Poultry Science*, 85, 1151–1164. http://dx.doi.org/10.1093/ps/85.7.1151

Bortoluzzi, C., Pedroso, A.A., Mallo, J.J., Puyalto, M., Kim, W.K. & Applegate, T.J. (2017) Sodium butyrate improved performance while modulating the cecal microbiota and regulating the expression of intestinal immune-related genes of broiler chickens. *Poultry Science*, 96, 3981–3993. https://doi.org/10.3382/ps/pex218

Connerton, P.L., Richards, P.J., Lafontaine, G.M., O'Kane, P.M., Ghaffar, N., Cummings, N.J. et al. (2018) The effect of the timing of exposure to campylobacter jejuni on the gut microbiome and inflammatory responses of broiler chickens. *Microbiome*, 6, 88. https://doi.org/10.1186/s40168-018-0477-5

Cox, N.A., Richardson, L.J., Buhr, R.J. & Fedorka-Cray, P.J. (2009) Campylobacter species occurrence within internal organs and tissues of commercial caged Leghorn laying hens. *Poultry Science*, 88, 2449–2456. https://doi.org/10.3382/ps.2009-00195

Cresci, G.A.M., Mayor, P.C. & Thompson, S.A. (2017) Effect of butyrate and lactobacillus GG on a butyrate receptor and transporter during campylobacter jejuni exposure. *FEMS Microbiology Letters*, 364, fnx046. https://doi.org/10.1093/femsle/fnx046

Dhillon, A.S., Shivaprasad, H.L., Schaberg, D., Wier, F., Weber, S. & Bandli, D. (2006) Campylobacter jejuni infection in broiler chickens. *Avian Diseases*, 50, 55–58. https://doi.org/10.1637/7411-071405R.1

Diaz Carrasco, J.M., Casanova, N.A. & Fernández Miyakawa, M.E. (2019) Microbiota, gut health and chicken productivity: what is the connection? *Microorganisms*, 7, 374. https://doi.org/10.3390/microorganisms7100374

Douglas, G.M., Maffei, V.J., Zaneveld, J.R., Yurgel, S.N., Brown, J.R., Taylor, C.M. et al. (2020) PICRUSt2 for prediction of metagenome functions. *Nature Biotechnology*, 38, 685–688. https://doi.org/10.1038/s41587-020-0548-6

Du, E., Gan, L., Li, Z., Wang, W., Liu, D. & Guo, Y. (2015) In vitro antibacterial activity of thymol and carvacrol and their effects on broiler chickens challenged with Clostridium perfringens. *Journal of Animal Science and Biotechnology*, 6, 58.

Ducarmon, Q.R., Zwittink, R.D., Hornung, B.V.H., van Schaik, W., Young, V.B. & Kuijper, E.J. (2019) Gut microbiota and colonization resistance against bacterial enteric infection. *Microbiology and Molecular Biology Reviews*, 83, e00007–e00019. https://doi.org/10.1128/MMBR.00007-19

Escudié, F., Auer, L., Bernard, M., Mariadassou, M., Cauquil, L., Vidal, K. et al. (2018) FROGS: find, rapidly, OTUs with galaxy solution. *Bioinformatics*, 34, 1287–1294. https://doi.org/10.1093/bioinformatics/btx791

European Food Safety Authority and European Centre for Disease Prevention and Control. (2021) The European Union one health 2019 zoonoses report. *EFSA Journal* 19, e06406. http://dx.doi.org/10.2903/j.efsa.2021.6406

Fournomiti, M., Kimbaris, A., Mantzourani, I., Plessas, S., Theodoridou, I., Papaemmanouil, V. et al. (2015) Antimicrobial activity of essential oils of cultivated oregano (Origanum vulgare), sage (Salvia officinalis), and thyme (Thymus vulgaris) against clinical isolates of Escherichia coli, Klebsiella oxytoca, and Klebsiella pneumoniae. *Microbial Ecology in Health and Disease*, 26, 23289. https://doi.org/10.3402/mehd.v26.23289

Goodman, K.N., Powers, M.J., Crofts, A.A., Trent, M.S. & Hendrixson, D.R. (2020) Campylobacter jejuni BumSR directs a response to butyrate via sensor phosphatase activity to impact transcription and colonization. *The Proceedings of the National Academy of Sciences*, 117, 11715–11726. https://doi.org/10.1073/pnas.1922719117

Han, Z., Willer, T., Pielsticker, C., Gerzova, L., Rychlik, I. & Rautenschlein, S. (2016) Differences in host breed and diet influence colonization by campylobacter jejuni and induction of local immune responses in chicken. *Gut Pathogens*, 8, 56. http://dx.doi.org/10.1186/s13099-016-0133-1

Hankel, J., Jung, K., Kuder, H., Keller, B., Keller, C., Galvez, E. et al. (2019) Caecal microbiota of experimentally campylobacter jejuni-infected chickens at different ages. *Frontiers in Microbiology*, 10. https://doi.org/10.3389/fmicb.2019.02303

Herman, L., Heyndrickx, M., Grijspeerdt, K., Vandekerchove, D., Rollier, I. & De Zutter, L. (2003) Routes for campylobacter contamination of poultry meat: epidemiological study from hatchery to slaughterhouse. *Epidemiology and Infection*, 131, 1169–1180. http://dx.doi.org/10.1017/s0950268803001183

Humphrey, S., Chaloner, G., Kemmett, K., Davidson, N., Williams, N., Kipar, A. et al. (2014) Campylobacter jejuni is not merely a commensal in commercial broiler chickens and affects bird welfare. *MBio*, 5, e01364–e01314. http://dx.doi.org/10.1128/mbio.01364-14

INRAE (2021) *PFIE—Accueil*. Available at: https://www6.val-de-loire.inrae.fr/pfie/ [Accessed 10th March 2022].

Josefsen, M.H., Andersen, S.C., Christensen, J. & Hoorfar, J. (2015) Microbial food safety: potential of DNA extraction methods for use in diagnostic metagenomics. *Journal of Microbiological Methods*, 114, 30–34.

Kaakoush, N.O., Sodhi, N., Chenu, J.W., Cox, J.M., Riordan, S.M. & Mitchell, H.M. (2014) The interplay between campylobacter and helicobacter species and other gastrointestinal microbiota of commercial broiler chickens. *Gut Pathogens*, 6, 18. https://doi.org/10.1186/1757-4749-6-18

Kanehisa, M. & Goto, S. (2000) KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Research*, 28, 27–30. https://doi.org/10.1093/nar/28.1.27

King, M.O., Owen, J.P. & Schwabl, H.G. (2010) Are maternal antibodies really that important? Patterns in the immunologic development of altricial passerine house sparrows (passer domesticus). *PLoS One*, 5, e9639. https://doi.org/10.1371/journal.pone.0009639

Kollanoor Johny, A., Darre, M.J., Donoghue, A.M., Donoghue, D.J. & Venkitanarayanan, K. (2010) Antibacterial effect of trans-cinnamaldehyde, eugenol, carvacrol, and thymol on salmonella enteritidis and campylobacter jejuni in chicken cecal contents in vitro. *Journal of Applied Poultry Research*, 19, 237–244.

Kumar, S., Chen, C., Indugu, N., Werlang, G.O., Singh, M., Kim, W.K. et al. (2018) Effect of antibiotic withdrawal in feed on chicken gut microbial dynamics, immunity, growth performance and prevalence of foodborne pathogens. *PLoS One*, 13, e0192450. https://doi.org/10.1371/journal.pone.0192450

LaGier, M.J., Joseph, L.A., Passaretti, T.V., Musser, K.A. & Cirino, N.M. (2004) A real-time multiplexed PCR assay for rapid detection and differentiation of campylobacter jejuni and campylobacter coli. *Molecular and Cellular Probes*, 18, 275–282.

Lamb-Rosteski, J.M., Kalischuk, L.D., Inglis, G.D. & Buret, A.G. (2008) Epidermal growth factor inhibits campylobacter jejuni-induced claudin-4 disruption, loss of epithelial barrier function, and Escherichia coli translocation. *Infection and Immunity*, 76, 3390–3398.

Larsson, J., Godfrey, A. J. R., Gustafsson, P., algorithms), Eberly, D. H. (geometric, code), Huber, E. (root solver, ggrepel), Slowikowski, K. et al. (2020) *eulerr: Area-Proportional Euler and Venn Diagrams with Ellipses*. https://CRAN.R-project.org/package=eulerr

Lee, K.-C., Kil, D.Y. & Sul, W.J. (2017) Cecal microbiome divergence of broiler chickens by sex and body weight. *Journal of Microbiology*, 55, 939–945. https://doi.org/10.1007/s12275-017-7202-0

Li, Y., Fu, X., Ma, X., Geng, S., Jiang, X., Huang, Q. et al. (2018) Intestinal microbiome-metabolome responses to essential oils in piglets. *Frontiers in Microbiology*, 9, 1988. https://doi.org/10.3389/fmicb.2018.01988

Mahé, F., Rognes, T., Quince, C., Vargas, C. de, and Dunthorn, M. (2014) Swarm: robust and fast clustering method for amplicon-based studies. *PeerJ*, 2, e593. https://doi.org/10.7717/peerj.593

McMurdie, P.J. & Holmes, S. (2013) Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*, 8, e61217. https://doi.org/10.1371/journal.pone.0061217

Meade, K.G., Narciandi, F., Cahalane, S., Reiman, C., Allan, B. & O'Farrelly, C. (2009) Comparative in vivo infection models yield insights on early host immune response to campylobacter in chickens. *Immunogenetics*, 61, 101–110.

Mellencamp, M., Koppien-Fox, J., Lamb, R., and Dvorak, R. (2011) Antibacterial and antioxidant activity of oregano essential oil. *International Conference on the Epidemiology and Control of Biological, Chemical and Physical Hazards in Pigs and Pork*. Maastricht, Netherlands. https://doi.org/10.31274/safepork-180809-673

Meunier, M., Guyard-Nicodème, M., Dory, D. & Chemaly, M. (2016) Control strategies against campylobacter at the poultry production level: biosecurity measures, feed additives and vaccination. *Journal of Applied Microbiology*, 120, 1139–1173.

Michiels, J., Missotten, J., Dierick, N., Fremaut, D., Maene, P. & De Smet, S. (2008) In vitro degradation and in vivo passage kinetics of carvacrol, thymol, eugenol and trans-cinnamaldehyde along the gastrointestinal tract of piglets. *Journal of the Science of Food and Agriculture*, 88, 2371–2381. https://doi.org/10.1002/jsfa.3358

Newell, D.G. (2002) The ecology of campylobacter jejuni in avian and human hosts and in the environment. *International Journal of Infectious Diseases*, 6, S16–S21. https://doi.org/10.1016/S1201-9712(02)90179-7

Paës, C., Gidenne, T., Bébin, K., Duperray, J., Gohier, C., Guené-Grand, E. et al. (2020) Early introduction of solid feeds: ingestion level matters more than prebiotic supplementation for shaping gut microbiota. *Frontiers in Veterinary Science*, 7, 261. https://doi.org/10.3389/fvets.2020.00261

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P. et al. (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research*, 41, D590–D596. https://doi.org/10.1093/nar/gks1219

Rognes, T., Flouri, T., Nichols, B., Quince, C. & Mahé, F. (2016) VSEARCH: a versatile open source tool for metagenomics. *PeerJ*, 4, e2584. https://doi.org/10.7717/peerj.2584

Romero-Barrios, P., Hempen, M., Messens, W., Stella, P., and Hugas, M. (2013) Quantitative microbiological risk assessment (QMRA) of food-borne zoonoses at the European level. *Food Control*, 29, 343, 349. http://dx.doi.org/10.1016/j.foodcont.2012.05.043

Ruan, D., Fan, Q., Fouad, A.M., Sun, Y., Huang, S., Wu, A. et al. (2021) Effects of dietary oregano essential oil supplementation on growth performance, intestinal antioxidative capacity, immunity, and intestinal microbiota in yellow-feathered chickens. *Journal of Animal Science*, 99. https://doi.org/10.1093/jas/skab033

Sakaridis, I., Ellis, R.J., Cawthraw, S.A., van Vliet, A.H.M., Stekel, D.J., Penell, J. et al. (2018) Investigating the association between the caecal microbiomes of broilers and campylobacter burden. *Frontiers in Microbiology*, 9. https://doi.org/10.3389/fmicb.2018.00927

Sassone-Corsi, M., Nuccio, S.-P., Liu, H., Hernandez, D., Vu, C.T., Takahashi, A.A. et al. (2016) Microcins mediate competition among Enterobacteriaceae in the inflamed gut. *Nature*, 540, 280–283. https://doi.org/10.1038/nature20557

Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P.A. & Teixeira, P. (2011) Campylobacter spp. as a foodborne pathogen: a review. *Frontiers in Microbiology*, 2, 200. https://doi.org/10.3389/fmicb.2011.00200

Sofka, D., Pfeifer, A., Gleiss, B., Paulsen, P. & Hilbert, F. (2015) Changes within the intestinal flora of broilers by colonisation with Campylobacter jejuni. *Berliner und Münchener Tierärztliche Wochenschrift*, 128, 104–110.

Suntres, Z.E., Coccimiglio, J. & Alipour, M. (2015) The bioactivity and toxicological actions of carvacrol. *Critical Reviews in Food Science and Nutrition*, 55, 304–318. https://doi.org/10.1080/10408398.2011.653458

Szott, V., Reichelt, B., Alter, T., Friese, A. & Roesler, U. (2020) In vivo efficacy of carvacrol on campylobacter jejuni prevalence in broiler chickens during an entire fattening period. *European Journal of Microbiology and Immunology*, 10, 131–138. https://doi.org/10.1556/1886.2020.00011

Thibodeau, A., Fravalo, P., Yergeau, É., Arsenault, J., Lahaye, L. & Letellier, A. (2015) Chicken caecal microbiome modifications induced by campylobacter jejuni colonization and by a non-antibiotic feed additive. *PLoS One*, 10, e0131978. https://doi.org/10.1371/journal.pone.0131978

Van Deun, K., Pasmans, F., Ducatelle, R., Flahou, B., Vissenberg, K., Martel, A. et al. (2008) Colonization strategy of campylobacter jejuni results in persistent infection of the chicken gut. *Veterinary Microbiology*, 130, 285–297. https://doi.org/10.1016/j.vetmic.2007.11.027

Vandamme, P., Dewhirst, F. E., Paster, B. J., and On, S. (2015) "Campylobacter," In Bergey's manual of systematics of archaea and bacteria. http://onlinelibrary.wiley.com/book/10.1002/9781118960608

Wei, S., Morrison, M. & Yu, Z. (2013) Bacterial census of poultry intestinal microbiome. *Poultry Science*, 92, 671–683. https://doi.org/10.3382/ps.2012-02822

Williams, L.K., Fonseca, B.B. & Humphrey, T.J. (2016) Campylobacter jejuni in poultry: a commensal or a pathogen? In: *Campylobacter spp. and related organisms in poultry*. Cham: Springer, pp. 75–87.

Zhai, H., Luo, Y., Ren, W., Schyns, G. & Guggenbuhl, P. (2020) The effects of benzoic acid and essential oils on growth performance, nutrient digestibility, and colonic microbiota in nursery pigs. *Animal Feed Science and Technology*, 262, 114426. https://doi.org/10.1016/j.anifeedsci.2020.114426

Zhu, N., Wang, J., Yu, L., Zhang, Q., Chen, K. & Liu, B. (2019) Modulation of growth performance and intestinal microbiota in chickens fed plant extracts or virginiamycin. *Frontiers in Microbiology*, 10, 1333. https://doi.org/10.3389/fmicb.2019.01333

Zotti, M., Colaianna, M., Morgese, M.G., Tucci, P., Schiavone, S., Avato, P. et al. (2013) Carvacrol: from ancient flavoring to neuromodulatory agent. *Molecules*, 18, 6161–6172. https://doi.org/10.3390/molecules18066161

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