Supplementation of lactobacillus-fermented rapeseed meal in broiler diet reduces Campylobacter jejuni cecal colonization and limits the l-tryptophan and l-histidine biosynthesis pathways

Khattak, F; Galgano, SG; Pedersen,, N. R; Hui, Yan; Matthiesen,, R; Houdijk, JGM

Published in:
Journal of the Science of Food and Agriculture

DOI:
10.1002/jsfa.13378

First published: 23/02/2024

Document Version
Peer reviewed version

Khattak Farina (Orcid ID: 0000-0001-8206-5788)
Galgano Salvatore (Orcid ID: 0000-0002-5600-3206)

Supplementation of lactobacillus fermented rapeseed meal in broiler diet reduces *Campylobacter Jejuni* caecal colonisation and limits the L-tryptophan and L-histidine biosynthesis pathways

Farina Khattak,[a*] Salvatore Galgano,[a] Ninfa Rangel Pedersen,[b] Yan Hui,[c] Rikke Matthiesen,[b] and Jos Houdijk [a]

[a] Monogastric Science Research Centre, SRUC, Edinburgh, UK, [b] Fermentationexperts AS, Denmark, [c] University of Copenhagen, Department of Food Science, Frederiksberg, Denmark

*(Email: Farina.Khattak@sruc.ac.uk)

Running title: Lactobacillus fermented rapeseed meal impact on Campylobacter jejuni

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jsfa.13378

Abstract

BACKGROUND: *Campylobacter jejuni (C. jejuni),* a widely distributed global foodborne pathogen primarily linked with contaminated chicken meat, poses a significant health risk. Lowering this pathogen abundance in poultry meat is challenging but essential. This study assessed the impact of *lactobacillus*-fermented rapeseed meal (LFRM) on broilers exposed to *C. jejuni* contaminated litter, evaluating growth performance, *Campylobacter* levels, and metagenomic profile.

RESULTS: By day 35, the litter contamination successfully colonised broilers with Campylobacter spp., particularly *C. jejuni.* In the grower phase, LFRM improved ($P < 0.05$) body weight and daily weight gain, resulting in a 9.2% better feed conversion ratio during the pre-challenged period (days 13 to 20). LFRM also reduced *C. jejuni* concentration in the caeca ($P < 0.05$), without altering alfa and beta diversity. However, metagenomic data analysis revealed LFRM targeted reduction in the abundance of *C. jejuni* biosynthetic pathways of L-tryptophan and L-histidine and gene families associated with transcription and virulence factors whilst also possibly leading to a selected stress-induced resistance mechanisms.

CONCLUSION: The study demonstrated that LFRM inclusion improved growth and decreased caecal *Campylobacter* spp. concentration and relative abundance of pivotal *C. jejuni* genes. Performance benefits likely resulted from LFRM metabolites. At the molecular level, LFRM may have reduced *C. jejuni* colonisation, likely by decreasing abundance of energy transduction and L-histidine and L-tryptophan biosynthesis genes, otherwise required for bacterial survival and increased virulence.

Keywords: Lacto-fermented rapeseed meal, campylobacter, broilers, metagenomics

INTRODUCTION

The 2019 EU One Health Zoonoses report highlights campylobacteriosis as the first most reported bacterial zoonosis in humans.[1] Broiler chickens are potential reservoirs for *Campylobacter* species, such as *C. jejuni* or *C. coli,* which are pathogenic to humans. [2] While chickens are commonly asymptomatic after colonisation, the manifestation of symptoms and the presence of the entity also vary depending on the season and country, potentially resulting in 20 to 100% of the flocks being positive at slaughter.[3] This poses a subsequent risk for consumers. [4,5]

Classic-antimicrobial interventions on-farm, such as in-feed antibiotics to decrease *Campylobacter* load, are not easily implemented due to country-depended legislation, but most importantly, due to the possible development of antibiotic resistance. [6,7] Consequently, a significant effort has been put in place to develop alternatives to classic antibiotics, such as prebiotics, bacteriophages, plant extracts, organic acids, and medium-chain fatty acids, with demonstrated bactericidal activity against *Campylobacter* spp. [8–12]

The One Health approach emphasizes the interconnectedness between human and animal health and the environment, stressing the impact of each on the others. This concept is crucial for *Campylobacter* control in poultry, addressing zoonotic transmission risks, environmental impacts of farming, and the necessity for collaborative efforts to reduce antibiotics. Strategies like fermented rapeseed meal align with One Health principles, concurrently addressing animal health and food safety for comprehensive control measures. Amongst the tested antimicrobial alternatives, [9,13–15] studies have shown that feeding fermented rapeseed meals (FRMs) could promote gut health by increasing *Lactobacillus* spp., organic acids [16] and decreasing *E. coli*, and *Salmonella* spp. in broiler chickens, [17] pigs, [18] rabbits [19] and minks. [20] The positive effects of FRMs could be linked to the fermentation process typical of their manufacture, which could increase the bioavailability of protein, promote the

synthesis of vitamins and antioxidants, reduce the content of antinutritive compounds such as glucosinolates, phytates, and non-starch polysaccharides[21–24] and help improving nutrient digestibility, growth and immune response in turkey, [23] rabbits, [19] and fish. [25,26]

Rapeseed meal, especially when it has undergone a fermentation process, has generated significant attention as a substitute protein source for monogastric animals. The nutritional benefits of feeding lactobacillus fermented rapeseed meal (LFRM) have already been demonstrated. [27] Most of these growth-promoting benefits were associated with increased feed digestibility and utilization by reducing the negative impact of anti-nutritional components and promoting gut morphology and antioxidant capacity in the body. [28] However, the role of LFRM in reducing pathogenic bacteria is not well understood, and its impact on biosynthetic pathways remains unexplored. Implementing the applied approach of using nutritional interventions to reduce pathogens in the animal gut can reduce reliance on antibiotics for disease prevention and treatment but can also significantly improve overall animal health and productivity, ultimately leading to safer animal products and better economic returns for commercial farmers. The current study is thus designed to explore the effect of supplementing LFRM in broilers that are artificially exposed to *C. jejuni* infection and, investigate its ability to reduce *C. jejuni* load, and assess its impact via both colony forming unit (CFU) enumeration and short-read metagenomic analysis whilst also evaluating the performance of birds through the production cycle.

MATERIALS AND METHODS

Lacto-fermented rapeseed meal (LFRM)

The LFRM, a complementary feed ingredient produced by Fermentationexperts A/S, is rapeseed meal fermented with lactic acid bacilli, *Lactiplantibacillus plantarum* and two *Pediococcus* species (*P. pentosaceus* and *P. acidilactici)* and multi-enzyme product

containing xylanase, β-glucanase, and phytase. The final product contains an added probiotic bacterium *Enterococcus faecium*, a lactic acid content of 5.5% and a protein content of about 34%. The product is commercially known as EP100i, and the chemical composition of LFRM is presented in Table 2.

Animals, diets, housing, and *C. jejuni* challenge

The study was carried out in compliance with the ARRIVE guidelines. The *in-vivo* experiment was carried out under the Animal Scientific Procedures Act (1986) and approved by the ethical review committee of Scotland's Rural College (SRUC).

A total of 144 Ross 308 male day-old broilers were allocated randomly to two dietary treatments, with twelve birds per pen and six replicates per treatment. Moreover, cloacal swabs from one bird per pen were carried out on arrival (d0) to confirm the absence of *C. jejuni* after inoculation of the swab on charcoal cefoperozone deoxycholate agar (CCDA, PO0119 Oxoid, Ltd. Ireland) and inoculation at 42°C ±1°C under microaerophilic conditions (CN0025A, Oxoid Ltd., Ireland) for 48 hours. The study followed a randomised complete block design. Basal iso-nitrogenous and iso-energetic wheat-soyabean meal control diets (Treatment 1, T1 Table 1) were manufactured as one batch for each feeding phase, i.e., starter (day 0 to 13), grower (day 13 to 20) and finisher (day 20 to d35). Treatment 2 (T2) was generated by incorporating 3% and 4% LFRM on the top of the base diet of T1 during the grower and finisher phases, respectively. Diets were manufactured with coccidiostat (Maxiban) but contained no veterinary antibiotics. All diets were in the form of mash and were formulated to meet the breed's nutrient requirements. Birds had *ad libitum* access to feed and water. Birds were reared on fresh wood shavings in clean floor pens, having a stocking density of 33kg/m^2 at d42. On d20, all broilers were artificially infected with the *C. jejuni* ATCC33291 strain (7 x 10^6 CFU g^{-1}) through a seeded litter tray procedure.[11] Farm

and technical staff responsible for animal care, sample collection, and analytical analysis were blinded to treatment allocations. Blinding of treatments was done by randomly assigning a unique number to each treatment, i.e., on feed bags, floor pens, and sampling pots.

Growth performance

Weights of birds and feed were recorded on d0, d13, d20, and d35 and used to calculate the average body weight (ABW), average daily weight gain (ADWG), average daily feed intake (ADFI), and feed conversion ratio (FCR) per pen. Days 0 to 20 and 20 to 35 are reported as pre- and post-challenge periods, respectively.

Sample collection

At d35, nine birds/pen were humanly euthanised via cervical dislocation, and both caeca were collected. The caecal content of one bird per pen was artificially expelled under sterile conditions and immediately stored at 80°C for downstream gDNA isolation and library preparation for shotgun metagenomic sequencing. The caecal content of the remaining 8 birds per pen was pooled together through four sets of two birds per pen (n = 48 samples per treatment) and was immediately used for *C. jejuni* CFU enumeration.

Enumeration of *Campylobacter* spp.

The concentration of *C. jejuni* (CFU g^{-1}) was calculated through CFU enumeration carried out on caecal content isolated as previously described [29], approximately 1 g of caecal content was 1:10 diluted (10^{-1}) in sterile phosphate-saline buffer (PBS), thus four further 10-fold serial dilutions were carried (i.e., from 10^{-1} to 10^{-5}). Therefore, 100 µL of each dilution was plated on CCDA plates and incubated at 42°C ± 1°C for 48 hours under microaerophilic conditions as described above. After incubation, the colonies per plate were counted, and the concentration of *C. jejuni* (CFU g^{-1} of content) was calculated by multiplying the

number of colonies on each plate by the dilution factor of that plate and by dividing this by the volume used for each plate.

Genomic DNA isolation, library preparation, shotgun metagenomic sequencing and bioinformatic analysis.

Genomic DNA was isolated from caecal content using the Bead-Beat Micro AX Gravity Kit (A&A, Gdynia, Poland) according to the manufacturer's instructions. The concentration and purity of the purified DNA were evaluated using Qubit fluorometer (ThermoFisher Scientific). The gDNA was then subjected to 150 bp pair-end shotgun metagenome sequencing on the Novaseq platform (Illumina), which was carried out by a commercial sequencing service provider (Novogene, Cambridge, UK). Raw shotgun metagenomic reads were filtered, taking into account both broiler and eventual human host contaminations via using KneadData, which integrated FastQC, [30] Trimmomatic [31] and Bowtie2. [32] Thus, quality-filtered reads were used to assign the taxonomy via the MetaPhlAn V3 pipeline, [33] which allowed the creation of total sum scaling (TSS) normalised taxonomy tables at phylum, genus, and species levels. The latter was used in Vegan V2.6-4 [34] in R to generate both α and β diversity metrics. The abundance of microbial metabolic pathways and gene families were profiled via functional profiling of the metagenome using HUMAnN 3.0, [35] outputting copies per million (CPM), therefore taking into account the sequencing depth for each sample.

Statistical analysis

The study design was a randomised complete block design with 6 blocks and 6 treatments. The pen was the experimental unit for all the parameters measured. The possible significant differences in growth performance were evaluated by the analysis of variance in a GenStat 19 statistical software package (IACR, Rothamsted, Hertfordshire, UK) with a $P<0.05$ level

of significance. Log_{10} transformed CFU concentration, the output of the CFU enumeration and α-diversity metrics were analysed via fitting a linear mixed model (LMM) in R V4.3.1[36] using the function "*lmer()*" from the package *lme4*[37], and calculating the *P* values through type III ANOVA via Satterthwaite's degrees of freedom method, using the R package *lmerTest.* [38] The LMM was fitted with "treatment" as a categorical fixed effect and the hierarchy of block/pen as a random effect, or block only when the number of observations was equally abundant as the number of pens (n=12). The differential abundance at the taxonomical level was analysed via a linear mixed model fitted through the MaAsLin2 package[39] following total sum scaling (TSS) normalisation via MetaPhlAn V3, and Log_{10} transformation, with treatment as a fixed effect and block as random effect. Single species significance was assessed via LMM using *lme4* as previously described. Bray-Curtis dissimilarity matrices (β-diversity) were analysed via PERMANOVA and depicted via principal coordinate analysis (PCoA) plots Vegan V2.6-4. MaAsLin2 was also used to analyse metagenomic data via fitting a zero-inflated negative binomial regression (ZINB), with treatment as a fixed effect and block as random effect and applying a cumulative sum scaling (CSS) normalisation to the input files, to take into account of the bias typical of both sampling depth and dominating high abundant features (i.e., unintegrated). In this case, the pathways abundance table, a subset of the pathway's abundance table, and a subset of the gene families abundance table, both containing only features related to *C. jejuni* were analysed via MaAsLin2 as per above. MaAsLin2 outputs were accompanied by both a *P* value and a *Q* value, the latter accounting for the false discovery rate, therefore, significantly differential abundant results were considered when both *P* and *Q* values were < 0.05, whilst tendency was defined as $0.05 < P, Q < 0.1$.

RESULTS

Growth performance

The growth performance data during the starter (0 to 13 d), grower (13 to 20 d), and finisher (20 to 35 d) phases are presented in Table 3. The growth performance for each treatment group was similar ($P > 0.05$) during the starter phase, i.e., prior to LFRM supplementation. During the grower phase, birds fed diets supplemented with 3% LFRM were, on average, 8.5% heavier at d20, resulting in a 13.2% greater ADWG than the control ($P < 0.05$). The increase in ABW in LFRM birds was also reflected in a 9.2% better FCR during 13 to 20d (pre-challenge period). However, the differences were not significant when compared to the control group ($P < 0.1$). At the end of the *Campylobacter*-challenged finisher phase, during which ADWG and FCR were similar between treatments, ABW of LFRM birds tended to be heavier than control birds (+5%; $P < 0.1$). No differences were observed when ADFI data were compared between the treatment groups during different phases of growth ($P > 0.05$). The birds remained healthy throughout the trial period, and the overall mortality was low (0.69%) and not treatment associated.

Caecal *C. jejuni* concentration

None of the cloacal swabs carried out at day 0 were positive for *Campylobacter* spp. after inoculation of CCDA plates. The seeded litter challenge model was successful in the horizontal transfer of *Campylobacter* spp. in birds within pens, as indicated by the presence of the pathogen in all pens at d35. As depicted in Figure 1, the LMM was carried out on CFU enumeration of *Campylobacter* spp. showed a significant reduction in the caecal content bacterial concentration ($F(1,41)=15.13$, $P < 0.05$) in the LFRM group (7.19 ± 0.58 Log_{10} CFU g^{-1}) compared to the control group (7.67 ± 0.31 Log_{10} CFU g^{-1}).

Metagenomic analysis

Microbiota

In terms of α-diversity, neither Shannon nor Pielou's index revealed any significant differences between the control and the LFRM group, similarly, the Bray-Curtix dissimilarity index did not show any significant difference among the microbial communities, according to the β-diversity. At phylum level (Figure 2), there were no significant differences between control and LFRM groups, whilst Firmicutes were the most abundant phylum in both control (49.85 % ± 21.75 %) and LFRM (54.9 % ± 17.38 %), followed by Bacteroidetes with a relative abundance of 29.2 % ± 20.18 % in control and 35.42 % ± 12.66 % in the LFRM group. Proteobacteria was the third most abundant genus (13.12 % ± 6.97 % in control and 7.42 % ± 6.64 % in LFRM), followed by Actinobacteria (7.8 % ± 16.32 % in control and 2.23 % ± 2.59 % in LFRM) and Chlamydiae (0.03 % ± 0.03 % in both control and LFRM). Similarly, no significant differences were found in the relative abundance at genus level (Figure 3), whose five most predominant components in control and LFRM were *Alistipes*, (29.2 % ± 20.18 %, 35.05 % ± 11.94 %), an unclassified Firmicutes genus (17.7 % ± 8.28 %, 23.06 % ± 14.34 %), *Escherichia* (8.66 % ± 4.32 %, 5.54 % ± 5.87 %), *Lachnoclostridium* (5.86 % ± 4.4 %, 7.45 % ± 4.29 %) and *Flavonifractor* (7.29 % ± 10.11 %, 5.97 % ± 1.64 %). At species level, *C. jejuni* was 4.46 % ± 5 % abundant in the control group and 1.88 % ± 2.61 % in LFRM (Figure 4). However, the $\log_{10}$ transformed abundance was not found to be significantly different between groups ($F(1,10)=1.45$, $P = 0.256$).

Microbiome

We did not observe any statistical differences in α- and β- diversity calculated on the abundance of pathways or gene families through the experimental groups (Figures 5, 6 and 7). Hereafter, each pathway or gene family is accompanied by the average CPM ± standard

deviation in the control and LFRM groups, respectively, in brackets. At a community level, a total of ten pathways were differentially abundant in the control and LFRM groups ($P < 0.05$, Q < 0.05, Supplementary Information 1), amongst which 4 were not annotated to the used database for the alignment at the time of writing, and therefore classed as unintegrated by HUMAnN 3.0. The remaining 6 pathways were significantly more abundant in the control group compared to LFRM ($P < 0.05$, $Q < 0.05$). All values depicted in brackets are for control, followed by LFRM. The super-pathway of geranylgeranyl diphosphate biosynthesis II (via MEP) of *Bifidobacterium animalis* (0.37 CPM ± 0.61 CPM, 1.77 CPM ± 2.52 CPM), the thiamine diphosphate salvage II (98.33 CPM ± 25.32 CPM, 115.77 CPM ± 24.32 CPM), gluconeogenesis III (135.51 CPM ± 16.01 CPM, 149.16 CPM ± 20.32 CPM), the pyrimidine deoxyribonucleotides de novo biosynthesis IV (16.77 CPM ± 11.99 CPM, 14.95 CPM ± 15.59 CPM), the 5-aminoimidazole ribonucleotide biosynthesis II (626.83 CPM ± 89.94 CPM, 699.04 CPM ± 56.89 CPM) and the super-pathway of 5-aminoimidazole ribonucleotide biosynthesis (626.83 CPM ± 89.94 CPM, 699.04 CPM ± 56.89 CPM). In parallel, a total of 12 pathways were significantly differentially abundant ($P < 0.05$, Q < 0.05) between control and LFRM group (Supplementary Information 2), of which 1 was classed as unintegrated after HUMAnN 3.0 alignment. The remaining 11 pathways were significantly more abundant in the control compared to the LFRM group, and were the L. histidine biosynthesis pathway (Figure 8, 6.61 CPM ± 10.09 CPM, 3.13 CPM ± 6.92 CPM), the 5 aminoimidazole ribonucleotide biosynthesis I pathway (5.97 CPM ± 8.82 CPM, 2.98 CPM ± 5.84 CPM), the L-tryptophan biosynthesis pathway (Figure 9, 4.84 CPM ± 6.72 CPM, 2.31 CPM ± 5.36 CPM), the super-pathway of pyrimidine ribonucleotides *de novo* biosynthesis (5.18 CPM ± 7.6 CPM, 2.39 CPM ± 5.05 CPM), the thiamine diphosphate salvage II pathway (3.92 CPM ± 5.22 CPM, 1.78 CPM ± 4.36 CPM), the octanoyl acyl carrier protein biosynthesis pathway (4.56 CPM ± 6.1 CPM, 1.85 CPM ± 4.28 CPM), the

heme b biosynthesis II oxygen independent pathway (4.25 CPM ± 6.15 CPM, 2.19 CPM ± 4.95 CPM), the pyruvate fermentation to isobutanol engineered pathway (4.83 CPM ± 7.34 CPM, 2.42 CPM ± 5.1 CPM), the super-pathway of sulphur oxidation (8.65 CPM ± 9.71 CPM, 5.48 CPM ± 10.55 CPM), the gondoate biosynthesis anaerobic (8.14 CPM ± 11.1 CPM, 4.13 CPM ± 8.37 CPM), and the pre Q0 biosynthesis (4.73 CPM ± 7.53 CPM , 2.11 CPM ± 4.5 CPM).

At *C. jejuni* level, a total of 28 gene families were found to be significantly differentially abundant in the two experimental conditions ($P < 0.05$, $Q < 0.05$, Supplementary Information 3), of which 19 were more abundant in the control compared to LFRM and 9 were more abundant in the LFRM group compared to the control. Amongst the gene families found to be more abundant in the control group, 5 were not aligned to any known gene family at the time of writing, 1 was aligned to a putative lipoprotein (D2MY87), and 1 was aligned to DUF2165 family protein. The remaining 12 gene families more abundant in the control were the A0A0B6UXT5 membrane protein 9 3.52 CPM ± 4.11 CPM, 2.14 CPM ± 3.7 CPM), the filamentous hemagglutinin domain protein (Figure 10 A,1.57 CPM ± 3.23 CPM, 0.24 CPM ± 0.6 CPM), the 50S ribosomal protein L14 (2.57 CPM ± 3.31 CPM, 1.51 CPM ± 3.25 CPM), the ribosome silencing factor UPI000257FDBE (1.46 CPM ± 1.81 CPM, 1.43 CPM ± 1.98 CPM), the TipAS antibiotic recognition domain (9.34 CPM ± 7.04 CPM, 9.34 CPM ± 3.49 CPM), the MetQ NlpA family ABC transporter substrate binding protein (1.41 CPM ± 2.02 CPM, 0.49 CPM ± 1.12 CPM), the Group 3 truncated haemoglobin ctb (1.63 CPM ± 2.24 CPM, 1.14 CPM ± 2.09 CPM), the arsenical resistance operon repressor (Figure 10 B, 6.48 CPM ± 9.89 CPM, 5.74 CPM ± 12.19 CPM), the enoyl acyl carrier protein reductase FMN (1.41 CPM ± 1.68 CPM, 0.9 CPM ± 1.94 CPM), the methyltransferase A0A1J6QWD4 (1.67 CPM ± 2.18 CPM, 0.86 CPM ± 1.93 CPM), and the phosphoribosylformylglycinamidine synthase subunit PurS (2.14 CPM ± 3.9 CPM, 1.43

CPM ± 2.11 CPM). Within the 9 gene families more abundant in the LFRM group, 3 were not assigned to any known gene family at the time of writing, whereas the remaining 6 gene families were the membrane protein A0A2X2R173 (19.28 CPM ± 24.25 CPM, 29.94 CPM ± 65.31 CPM), the protein translocase subunit SecE (2.28 CPM ± 3.32 CPM, 2.56 CPM ± 3.45 CPM), the RNA polymerase sigma factor A0A1L1WRX1 (2.84 CPM ± 3.07 CPM, 24.28 CPM ± 26.29 CPM), the exodeoxyribonuclease V subunit alpha (0.24 CPM ± 0.39 CPM, 1.23 CPM ± 0.62 CPM), the adenyltransferase A9J069 (Figure 10 C, 2.71 CPM ± 3.09 CPM, 5.55 CPM ± 2.86 CPM) and the AadE gene (Figure 10 D, 2.62 CPM ± 2.91 CPM, 7.66 CPM ± 4.58 CPM).

DISCUSSION

The One Health significance of the multi-drug resistant organism (MDRO) *Campylobacter* spp., both as a zoonotic agent and related to the spread of antimicrobial resistance, requires the development of new nutritional strategies to lower its carriage in live animals. Amongst nutritional strategies, fermented rapeseed meal has been used to improve growth, nutrient digestibility, [40] gut health [20,41–43] and to control *Salmonella* spp.[44] To our knowledge, this is the first study to demonstrate that LFRM supplementation in a broiler diet has the ability to reduce caecal concentration of *C. jejuni*. This observed positive effect can be attributed to the metabolites present in LFRM, which may inhibit the growth dynamics of various bacteria.[45–47] A possible mechanism of action could be related to faster nutrient absorption in the small intestine, which in turn could lead to reduced substrate availability for some specific microbes in the lower gastrointestinal tract. [47] It can thus be speculated that rapid absorption in the small intestine results in fewer undigested residues reaching the large intestine, where microbes depend on these fermentation residues for growth and survival. Moreover, during the LFRM fermentation process, some of the dietary fibre is solubilised which would otherwise resist digestion by the host endogenous enzymes, thus increasing

their solubility and decreasing viscosity. Consequently, it alters the pH and nutrient composition of the gut, making the gut environment less favourable for the growth of certain specific microbial species. [48] Moreover, the probiotic species typical of fermented feedstuff like LFRM may hinder the proliferation of microorganisms such as *Campylobacter* spp. This is likely achieved by modulating the rest of the microbiota, for example, by providing intermediate metabolites for cross-feeding. Additionally, they generate molecules such as bacteriocins and hydrogen peroxide, further inhibiting microbial growth. [49,50] These positive effects have been demonstrated in other monogastric animals. A significant decrease in fungi, *E. coli*, *Salmonella* spp., and *Clostridium perfringens* in faeces of sows fed fermented rapeseed-meal diets has been observed. This is also accompanied by enhancements in the host-immune system and health, including a reduction in diarrhoetic symptoms and mortality rates. [40] In accord with these findings, we also observed an improved ABW and ADWG in birds fed LFRM during the pre-challenged grower phase. From a nutritional point of view, the fermentation process may enhance the quality of the feed due to a) the reduction of anti-nutritional factors such as glucosinolates, tannins, and phytate compounds,[17,51,52] b) the reduction of fibre content [53–55], c) the increase in protein digestibility [56] and d) the increase in the level of lactic acid bacteria (LAB).[57,58]

One of the reasons for the positive impact of LAB in fermented feed is due to their role in preventing the establishment of harmful bacteria via pH reduction through the production of organic molecules such as lactic acid, [59,60] direct competition for the same resources, and production of active molecules such as bacteriocins[61,62]. Past studies have shown that fermented liquid feed, characterised by a high *Lactobacillus* count and a pH of 4, was associated with a reduced occurrence of *Salmonella* spp. in pigs [63,64] and a decreased vulnerability to *Campylobacter* spp. and *Salmonella* spp. in broilers [65] However, other researchers demonstrated no significant interactions between fermented liquid feed and the

levels of *Salmonella enteritidis* and *Campylobacter* spp. after simultaneous challenge. [65] Furthermore, research on mink [20], rabbits[19] and poultry[41] has also shown a reduction in pathogenic bacteria in animals receiving fermented rapeseed meal.

All the birds in the current study were artificially challenged with *C. jejuni* at day 20 to mimic commercial pathogen dynamics. Indeed, regardless of the species, [66] *Campylobacter* is rarely detected in commercial broiler flocks of less than 3 weeks of age, likely due to the persistence of maternal antibodies. [67] However, commercially, the whole flock becomes colonised within 4 to 7 days after the first bird is infected, without any apparent clinical sign in the host until slaughter age. [68–70] *Campylobacter* colonisation is reported to negatively influence the expression of nutrient transporter genes in the chicken gut, [71] thus could potentially negatively affect bird's final body weight.[72] Nevertheless, our study indicated no negative effect on growth performance. Instead, it suggested that birds fed LFRM tended to be heavier at the end of the challenged phase (days 20 to 35), which was likely a carry-over effect of the positive impact on weight gain observed by the end of the grower phase.

The current metagenomic analysis revealed that, at *C. jejuni* level, the L-histidine and the L-tryptophan biosynthesis pathways were less abundant in LFRM compared to the control group. Tryptophan is an essential amino acid involved in protein synthesis and other biological processes that humans and animals need to obtain from food,[73] and it has been established that the tryptophan biosynthetic pathway is required for pathogenic bacteria to cause disease. [74,75] In many bacteria, tryptophan is synthesised *de novo,* [76] whilst commensal pathogenic are auxotrophic for tryptophan production, and a biosynthetic deficit causes bacteria to lose virulence within the host organism.[77] On the other hand, intracellular pathogens, such as *C. jejuni*, could also uptake amino acids from the host to fulfil their metabolic needs. [78] The significance of these molecules is demonstrated by the well-documented defence mechanisms against *Chlamydia*, *Leishmania* and *Mycobacterium*,

during which the host limits their access to tryptophan, leading to a decreased bacterial ability to establish and maintain infections, where intracellular pathogens are starved of tryptophan by the action of the degradative enzyme indoleamine 2,3-dioxygenase. [79]

Like tryptophan, histidine biosynthesis is a crucial pathway for various cellular processes. It is, therefore, reasonable to assume that the availability of histidine could influence the growth of *Campylobacter* species. However, the specific relationship between the histidine biosynthesis pathway in *Campylobacter* and its role in bacterial growth and virulence has not been yet elucidated. Histidine, also an essential amino acid, plays a crucial role in protein formation and catalytic processes and is significant in regulating various cellular functions. [80–82] Histidine is synthesised *de novo* in plants and bacteria and performs multifaceted functions, and research has shown that the enzymes responsible for its production are potential targets for tuberculosis treatment.[26,83–85]Therefore, our findings could inform on the possible decreased histidine availability linked to the decreased abundance of *C. jejuni* in the LFRM group.

A total of 29 *C. jejuni* gene families were differentially abundant between control and LFRM groups, most of them encoding for membrane, ribosomal, and carrier proteins. [86] The filamentous hemagglutinin domain protein was more abundant in the control group. This gene is known in *Bordetella pertussis* for encoding for a surface-exposed protein acting as a main virulence attachment factor, [86] while in *C. jejuni* it has been shown to affect host cell binding, [87] thus a possible decrease in the LFRM group could signify a decreased ability to invade host cells, which could be at the basis of the lower pathogen concentration observed in chickens administered LFRM. Interestingly, the arsenical resistance operon repressor was also more abundant in the control group, in comparison to LFRM. *Campylobacter* spp. are highly resistant to arsenic compounds, commonly found in both environment and livestock-feed additives, and in *Campylobacter* spp., the transcriptional repressor *ArsR* has been

demonstrated to inhibit the expression of the *ars* operon. [88] Our findings could indicate that a lower abundance of *ArsR*-like genes was found to be consequential to LFRM administration, which could potentially translate into a higher expression of arsenic resistance genes within this group. This could potentially inform the response mechanism of *C. jejuni* to extreme stress, during which the bacterium deploys several resistance mechanisms in order to increase its chances of survival. In parallel, we also found that the *aadE* gene, encoding for the aminoglycoside 6-adenylyltransferase (AadE) protein, which confers resistance to aminoglycoside in *Campylobacter* spp.,[89,90] was more abundant in the LFRM group than control, which could corroborate the above theoretical stress-response mechanism from this genus. Finally, the gene encoding for adenyltransferase A9J069 was also more abundant in the LFRM group, which possibly belonging to the same gene clade as *aadE*, as it also confers resistance to aminoglycosides in *Campylobacter* spp.[90]

Our findings suggest that strategies hindering the bacterial production of tryptophan and histidine in the gut could decrease the microbial pathogenicity, likely aiding in the reduction of *Campylobacter* growth. These insights stem from metagenomic data and have the potential to lay the foundation for understanding the mechanism of action of LFRM when incorporated into a broiler diet.

CONCLUSIONS

Our study demonstrates that supplementing broiler diets with LFRM resulted in increased body weight during the grower phase and a reduction in the concentration of *C. jejuni* near-harvest. Likely, these positive effects primarily originated from the fermentation-metabolites within the LFRM, which are known to improve nutrient digestibility, elevate the level of lactic acid, and modulate immunity levels. Our study supports the view that incorporating LFRM in broiler diets could potentially decrease the risk of

campylobacteriosis from poultry. Our findings could also indicate that, in response to the LFRM treatment, *C. jejuni* virulence could decrease, which might have been related to the observed lower pathogen concentration in the same group. However, an increased stress-induced resistance could have been induced in response to the treatment. We also speculate that strategies that have the ability to limit L-tryptophan and L-histidine biosynthetic pathways can restrict *Campylobacter* growth and potentially combat its colonisation and could likely be utilised as a target for non-antibiotic solutions to help reduce human cases of campylobacteriosis.

ACKNOWLEDGEMENTS

The work was partially funded by Fermentationexperts A/S, who have received money through the PPILOW project, which has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 816172 and SRUC. SRUC receives support from the Scottish Government. The authors would also like to acknowledge Prof. Dennis Sandris /Josue Leonardo Castro Mejia and his team from the University of Copenhagen for running the shotgun metagenomic analysis in their lab.

CONFLICT OF INTEREST

The authors declare that no known conflicts of interest are associated with this publication.

REFERENCES

1 EFSA. The European Union One Health 2019 Zoonoses Report. *EFSA Journal* **19** (2021).

2 Friis C, Wassenaar TM, Javed MA, Snipen L, Lagesen K, Hallin PF, Newell DG, Toszeghy M, Ridley A, Manning G, and Ussery DW. Genomic characterization of campylobacter jejuni strain M1. *PLoS One* **5** (2010).

3 Jacobs-Reitsma W, Lyhs U, and Wagenaar J. Campylobacter in the Food Supply. 3rd Edition. Nachamkin ISCMBMJ, ed. American Society for Microbiology (ASM).; 2008.

4 Little CL, Richardson JF, Owen RJ, Pinna E De, and Threlfall EJ. Prevalence, characterisation and antimicrobial resistance of Campylobacter and Salmonella in raw poultrymeat in the UK, 2003-2005. *Int J Environ Health Res* **18**:403–414 (2008).

5 Jorgensen F, Bailey R, Williams S, Henderson P, Wareing DRA, Bolton FJ, Frost JA, Ward L, and Humphrey TJ. Prevalence and numbers of Salmonella and Campylobacter spp. on raw, whole chickens in relation to sampling methods. *Int J Food Microbiol* Elsevier; **76**:151–164 (2002).

6 Munita JM and Arias CA. Mechanisms of Antibiotic Resistance. *Microbiol Spectr* **4**:1–37 (2016).

7 Galgano S, Conway L, Dalby N, Fellows A, and Houdijk JGM. Encapsulated peracetic acid as a valid broad-spectrum antimicrobial alternative, leading to beneficial microbiota compositional changes and enhanced performance in broiler chickens. *J Anim Sci Biotechnol* **14** (2023).

8 Hermans D, Martel A, Deun K Van, Immerseel F Van, Heyndrickx M, Haesebrouck F, and Pasmans F. The Cinnamon-Oil Ingredient trans-Cinnamaldehyde Fails To Target Campylobacter jejuni Strain KC 40 in the Broiler Chicken Cecum Despite Marked In Vitro Activity. *J Food Prot* Elsevier; **74**:1729–1734 (2011).

9 Hermans D, Martel A, Deun K van, Verlinden M, Immerseel F van, Garmyn A, Messens W, Heyndrickx M, Haesebrouck F, and Pasmans F. Intestinal mucus protects Campylobacter jejuni in the ceca of colonized broiler chickens against the bactericidal effects of medium-chain fatty acids. *Poult Sci* Elsevier; **89**:1144–1155 (2010).

10 Khattak F and Galgano S. Role of pre-, pro- and syn-biotics in reducing zoonotic pathogen abundance, lowering antimicrobial resistance, and improving food safety: Old concepts with a new perspective. In: ONeill HM, Burton Emily, Scholey D, eds. Vol:33. CABI international; 2023.

11 Khattak F, Paschalis V, Green M, Houdijk GM, Soultanas P, and Mahdavi J. TYPLEX® Chelate, a novel feed additive, inhibits Campylobacter jejuni biofilm formation and cecal colonization in broiler chickens. *Poult Sci* Elsevier; **97**:1391–1399 (2018).

12 Zhang Y, Lin Y, Galgano S, Houdijk J, Xie W, Jin Y, Lin J, Song W, Fu Y, Li X, Chui W, Kan W, Jia C, Hu G, and Li T. Recent Progress in Phage Therapy to Modulate Multidrug-Resistant Acinetobacter baumannii, Including in Human and Poultry. *Antibiotics* Multidisciplinary Digital Publishing Institute; **11**:1406 (2022).

13 Zhang Y, Lin Y, Galgano S, Houdijk J, Xie W, Jin Y, Lin J, Song W, Fu Y, Li X, Chui W, Kan W, Jia C, Hu G, and Li T. Recent Progress in Phage Therapy to Modulate Multidrug-Resistant Acinetobacter baumannii, including in Human and Poultry. Antibiotics. MDPI; 2022.

14 Galgano S, Conway L, Fellows A, and Houdijk J. Impact of precursor-derived peracetic acid on post-weaning diarrhea, intestinal microbiota, and predicted microbial functional genes in weaned pigs. *Front Microbiol* **15** (2024).

15 Galgano S, Conway L, Maggio F Di, Farthing K, Dalby N, Fellows A, and Houdijk JGM. Precursor-derived in-water peracetic acid impacts on broiler performance, gut microbiota and antimicrobial resistance genes. *Poult Sci* Elsevier; **102**:102368 (2023).

16 Sugiharto S and Ranjitkar S. Recent advances in fermented feeds towards improved broiler chicken performance, gastrointestinal tract microecology and immune responses: A review. Animal Nutrition. KeAi Communications Co.; p. 1–10 2019.

17 Chiang G, Lu WQ, Piao XS, Hu JK, Gong LM, and Thacker PA. Effects of Feeding Solid-state Fermented Rapeseed Meal on Performance, Nutrient Digestibility, Intestinal Ecology and Intestinal Morphology of Broiler Chickens. *Asian-Australasian Journal of Animal Sciences* **23**:263–271 (2010).

18 Missotten JAM, Michiels J, Degroote J, and Smet S De. Fermented liquid feed for pigs: An ancient technique for the future. J Anim Sci Biotechnol. BioMed Central Ltd.; 2015.

19 Wlazło Ł, Kowalska D, Bielański P, Chmielowiec-Korzeniowska A, Ossowski M, Łukaszewicz M, Czech A, and Nowakowicz-Dębek B. Effect of fermented rapeseed meal on the gastrointestinal microbiota and immune status of rabbit (Oryctolagus cuniculus). *Animals* MDPI AG; **11**:1–14 (2021).

20 Wlazło Ł, Nowakowicz-Dębek B, Czech A, Chmielowiec-Korzeniowska A, Ossowski M, Kułażyński M, Łukaszewicz M, and Krasowska A. Fermented rapeseed meal as a component of the mink diet (Neovison vison) modulating the gastrointestinal tract microbiota. *Animals* MDPI AG; **11** (2021).

21 Shi C, He J, Wang J, Yu J, Yu B, Mao X, Zheng P, Huang Z, and Chen D. Effects of Aspergillus niger fermented rapeseed meal on nutrient digestibility, growth performance and serum parameters in growing pigs. *Animal Science Journal* Blackwell Publishing; **87**:557–563 (2016).

22 Kasprowicz-Potocka M, Zaworska A, Frankiewicz A, Nowak W, Gulewicz P, Zduńczyk Z, and Juśkiewicz J. The Nutritional Value and Physiological Properties of Diets with Raw and Candida utilis-Fermented Lupin Seeds in Rats. *Food Technol Biotechnol* **53**:286–297 (2015).

23 Drażbo A, Kozłowski K, Ognik K, Zaworska A, and Jankowski J. The effect of raw and fermented rapeseed cake on growth performance, carcass traits, and breast meat quality in turkey. *Poult Sci* Elsevier; **98**:6161–6169 (2019).

24 He R, Ju X, Yuan J, Wang L, Girgih A, and Aluko R. Antioxidant activities of rapeseed peptides produced by solid state fermentation. *Food Res Int* **491**:432–438 (2012).

25 Dossou S, Koshio S, Ishikawa M, Yokoyama S, Basuini MF El, Zaineldin AI, Mzengereza K, Moss A, and Dawood MAO. Effects of replacing fishmeal with fermented and non-fermented rapeseed meal on the growth, immune and antioxidant responses of red sea bream (Pagrus major). *Aquac Nutr* Blackwell Publishing Ltd; **25**:508–517 (2019).

26 Dossou S, Koshio S, Ishikawa M, Yokoyama S, Dawood MAO, Basuini MF El, Olivier A, and Zaineldin AI. Growth performance, blood health, antioxidant status and immune response in red sea bream (Pagrus major) fed Aspergillus oryzae fermented rapeseed meal (RM-Koji). *Fish Shellfish Immunol* Academic Press; **75**:253–262 (2018).

27 Satessa GD, Tamez-Hidalgo P, Kjærulff S, Vargas-Bello-pérez E, Dhakal R, and Nielsen MO. Effects of increasing doses of lactobacillus pre-fermented rapeseed product with or without inclusion of macroalgae product on weaner piglet performance and intestinal development. *Animals* MDPI AG; **10** (2020).

28 Hu Y, Wang Y, Li A, Wang Z, Zhang X, Yun T, Qiu L, and Yin Y. Effects of fermented rapeseed meal on antioxidant functions, serum biochemical parameters and intestinal morphology in broilers. *Food Agric Immunol* Taylor and Francis Ltd.; **27**:182–193 (2016).

29 Khattak F, Galgano S, and Houdijk J. Bacterial concentration and Campylobacter spp. quantification differ when fresh or ultrafrozen samples are analysed over time using molecular biology and culture-based methods. *PLoS One* Public Library of Science; **17** (2022).

30 Andrew S. FastQC A Quality Control Tool for High Throughput Sequence Data. 2016.

31 Bolger AM, Lohse M, and Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* Oxford Academic; **30**:2114–2120 (2014).

32 Langmead B and Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nature Methods 2012 9:4* Nature Publishing Group; **9**:357–359 (2012).

33 Blanco-Míguez A, Beghini F, Cumbo F, McIver LJ, Thompson KN, Zolfo M, Manghi P, Dubois L, Huang KD, Thomas AM, Nickols WA, Piccinno G, Piperni E, Punčochář M, Valles-Colomer M, Tett A, Giordano F, Davies R, Wolf J, Berry SE, Spector TD, Franzosa EA, Pasolli E, Asnicar F, Huttenhower C, and Segata N. Extending and improving metagenomic

taxonomic profiling with uncharacterized species using MetaPhlAn 4. *Nat Biotechnol* (2023).

34 Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Szoecs E, and Wagner H. vegan: Community Ecology Package. 2020.

35 Beghini F, McIver LJ, Blanco-Míguez A, Dubois L, Asnicar F, Maharjan S, Mailyan A, Manghi P, Scholz M, Thomas AM, Valles-Colomer M, Weingart G, Zhang Y, Zolfo M, Huttenhower C, Franzosa EA, and Segata N. Integrating taxonomic, functional, and strain-level profiling of diverse microbial communities with biobakery 3. *Elife* eLife Sciences Publications Ltd; **10** (2021).

36 R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria; 2022.

37 Bates D, Mächler M, Bolker BM, and Walker SC. Fitting linear mixed-effects models using lme4. *J Stat Softw* **67** (2015).

38 Kuznetsova A, Brockhoff PB, and Christensen RHB. lmerTest Package: Tests in Linear Mixed Effects Models . *J Stat Softw* **82** (2017).

39 Mallick H, Rahnavard A, McIver LJ, Ma S, Zhang Y, Nguyen LH, Tickle TL, Weingart G, Ren B, Schwager EH, Chatterjee S, Thompson KN, Wilkinson JE, Subramanian A, Lu Y, Waldron L, Paulson JN, Franzosa EA, Bravo HC, and Huttenhower C. Multivariable association discovery in population-scale meta-omics studies. *PLoS Comput Biol* Public Library of Science; **17**:e1009442 (2021).

40 Grela ER, Czech A, Kiesz M, Wlazło Ł, and Nowakowicz-Dębek B. A fermented rapeseed meal additive: Effects on production performance, nutrient digestibility, colostrum immunoglobulin content and microbial flora in sows. *Animal Nutrition* KeAi Communications Co.; **5**:373–379 (2019).

41 Szmigiel I, Konkol D, Korczyński M, Łukaszewicz M, and Krasowska A. Changes in the microbial composition of the cecum and histomorphometric analysis of its epithelium in broilers fed with feed mixture containing fermented rapeseed meal. *Microorganisms* MDPI AG; **9**:1–18 (2021).

42 Czech A, Grela ER, and Kiesz M. Dietary fermented rapeseed or/and soybean meal additives on performance and intestinal health of piglets. *Sci Rep* Nature Research; **11** (2021).

43 Czech A, Nowakowicz-Debek B, Łukaszewicz M, Florek M, Ossowski M, and Wlazło Ł. Effect of fermented rapeseed meal in the mixture for growing pigs on the gastrointestinal tract, antioxidant status, and immune response. *Sci Rep* Nature Research; **12** (2022).

44 Ashayerizadeh A, Dastar B, Shams Shargh M, Sadeghi Mahoonak A, and Zerehdaran S. Fermented rapeseed meal is effective in controlling Salmonella

enterica serovar Typhimurium infection and improving growth performance in broiler chicks. *Vet Microbiol* Elsevier; **201**:93–102 (2017).

45 Stecher B and Hardt WD. Mechanisms controlling pathogen colonization of the gut. *Curr Opin Microbiol* Elsevier Current Trends; **14**:82–91 (2011).

46 Ravindran V and Kornegay ET. Acidification of weaner pig diets: A review. *J Sci Food Agric* **62**:313–322 (1993).

47 Winsen RL Van, Urlings BAP, Lipman LJA, Snijders JMA, Keuzenkamp D, Verheijden JHM, and Knapen F Van. Effect of Fermented Feed on the Microbial Population of the Gastrointestinal Tracts of Pigs. *Appl Environ Microbiol* **67**:3071–3076 (2001).

48 Zhao J, Bai Y, Zhang G, Liu L, and Lai C. Relationship between dietary fiber fermentation and volatile fatty acids' concentration in growing pigs. *Animals* MDPI AG; **10** (2020).

49 Moens F, Weckx S, and Vuyst L De. Bifidobacterial inulin-type fructan degradation capacity determines cross-feeding interactions between bifidobacteria and Faecalibacterium prausnitzii. *Int J Food Microbiol* **231**:76–85 (2016).

50 Moens F, Verce M, and Vuyst L De. Lactate- and acetate-based cross-feeding interactions between selected strains of lactobacilli, bifidobacteria and colon bacteria in the presence of inulin-type fructans. *Int J Food Microbiol* **241**:225–236 (2017).

51 Xu FZ, Zeng XG, and Ding XL. Effects of replacing soybean meal with fermented rapeseed meal on performance, serum biochemical variables and intestinal morphology of broilers. *Asian-Australas J Anim Sci* **25**:1734–1741 (2012).

52 Tripathi MK and Mishra AS. Glucosinolates in animal nutrition: A review. *Anim Feed Sci Technol* Elsevier; **132**:1–27 (2007).

53 Khempaka S, Thongkratok R, Okrathok S, and Molee W. An evaluation of cassava pulp feedstuff fermented with a. oryzae, on growth performance, nutrient digestibility and carcass quality of broilers. *Journal of Poultry Science* Japan Poultry Science Association; **51**:71–79 (2014).

54 Skrede G, Herstad O, Sahlstrøm S, Holck A, Slinde E, and Skrede A. Effects of lactic acid fermentation on wheat and barley carbohydrate composition and production performance in the chicken. *Anim Feed Sci Technol* Elsevier; **105**:135–148 (2003).

55 Sugiharto S, Yudiarti T, and Isroli I. Functional properties of filamentous fungi isolated from the indonesian fermented dried cassava, with particular application on poultry. *Mycobiology* Korean Society of Mycology; **43**:415–422 (2015).

56 Borresen EC, Henderson AJ, Kumar A, Weir TL, and Ryan EP. Fermented Foods: Patented Approaches and Formulations for Nutritional Supplementation and Health Promotion. .

57 Engberg RM, Hammershøj M, Johansen NF, Abousekken MS, Steenfeldt S, and Jensen BB. Fermented feed for laying hens: Effects on egg production, egg quality, plumage condition and composition and activity of the intestinal microflora. *Br Poult Sci* **50**:228–239 (2009).

58 Canibe N and Jensen BB. Fermented liquid feed—Microbial and nutritional aspects and impact on enteric diseases in pigs. *Anim Feed Sci Technol* Elsevier; **173**:17–40 (2012).

59 Heres L, Engel B, Urlings HAP, Wagenaar JA, and Knapen F Van. Effect of acidified feed on susceptibility of broiler chickens to intestinal infection by Campylobacter and Salmonella. *Vet Microbiol* Elsevier; **99**:259–267 (2004).

60 Loh TC, Law FL, Foo HL, Goh YM, Zulkifli I, and Malaysia P. Effects of feeding a fermented product on egg production, faecal microflora and faecal pH in laying hens. J Anim Feed Sci. 2007.

61 Savvidou SE, Beal JD, Brooks PH, and Ragione RM La. Liquid feed fermented with Lactobacillus salivarius reduces susceptibility of broiler chickens to Salmonella enterica typhimurium Sal 1344 nalr. *In Proceedings of the British Society of Animal Science* Cambridge University Press.; p. 201–201 2009.

62 Sun H, Tang JW, Fang CL, Yao XH, Wu YF, Wang X, and Feng J. Molecular analysis of intestinal bacterial microbiota of broiler chickens fed diets containing fermented cottonseed meal. *Poult Sci* **92**:392–401 (2013).

63 Wolf PJ van der, Wolbers WB, Elbers ARW, Heijden HMJF van der, Koppen JMCC, Hunneman WA, Schie FW van, and Tielen MJM. Herd level husbandry factors associated with the serological Salmonella prevalence in finishing pig herds in The Netherlands. *Vet Microbiol* Elsevier; **78**:205–219 (2001).

64 Wolf PJ Van Der, Bongers JH, Elbers ARW, Franssen FMMC, Hunneman WA, Exsel ACA Van, and Tielen MJM. Salmonella infections in finishing pigs in The Netherlands: bacteriological herd prevalence, serogroup and antibiotic resistance of isolates and risk factors for infection. *Vet Microbiol* Elsevier; **67**:263–275 (1999).

65 Heres L, Engel B, Knapen F Van, Jong MCM De, Wagenaar JA, and Urlings HAP. Fermented liquid feed reduces susceptibility of broilers for Salmonella enteritidis. *Poult Sci* Elsevier; **82**:603–611 (2003).

66 Umar S, Maiyah AT, and Mushtaq A. Campylobacter infections in poultry: Update on challenges and potential immune interventions. Worlds Poult Sci J. Cambridge University Press; p. 381–390 2016.

67 Cawthraw SA and Newell DG. Investigation of the Presence and Protective Effects of Maternal Antibodies Against Campylobacter jejuni in Chickens. *Avian Dis* **54**:86–93 (2010).

68 Kaino K, Hayashidani H, Kaneko K, and Ogawa M. Intestinal colonization of Campylobacter jejuni in chickens. *The Japanese Journal of Veterinary Science* **50**:489–494 (1988).

69 Sahin O, Kassem II, Shen Z, Lin J, Rajashekara G, and Zhang Q. Campylobacter in Poultry: Ecology and Potential Interventions. *Avian Dis* **59**:185–200 (2015).

70 Barrios PR, Reiersen J, Lowman R, Bisaillon JR, Michel P, Fridriksdóttir V, Gunnarsson E, Stern N, Berke O, McEwen S, and Martin W. Risk factors for Campylobacter spp. colonization in broiler flocks in Iceland. *Prev Vet Med* Elsevier; **74**:264–278 (2006).

71 Awad WA, Aschenbach JR, Ghareeb K, Khayal B, Hess C, and Hess M. Campylobacter jejuni influences the expression of nutrient transporter genes in the intestine of chickens. *Vet Microbiol* Elsevier; **172**:195–201 (2014).

72 Awad WA, Molnár A, Aschenbach JR, Ghareeb K, Khayal B, Hess C, Liebhart D, Dublecz K, and Hess M. Campylobacter infection in chickens modulates the intestinal epithelial barrier function. *Innate Immun* SAGE Publications Ltd; **21**:151–160 (2015).

73 Khattak F and Helmbrecht A. Effect of different levels of tryptophan on productive performance, egg quality, blood biochemistry, and caecal microbiota of hens housed in enriched colony cages under commercial stocking density. *Poult Sci* Oxford University Press; **98**:2094–2104 (2019).

74 Shaun Lott J. The tryptophan biosynthetic pathway is essential for Mycobacterium tuberculosis to cause disease. Biochem Soc Trans. Portland Press Ltd; p. 2029–2037 2020.

75 Wellington S, Nag PP, Michalska K, Johnston SE, Jedrzejczak RP, Kaushik VK, Clatworthy AE, Siddiqi N, McCarren P, Bajrami B, Maltseva NI, Combs S, Fisher SL, Joachimiak A, Schreiber SL, and Hung DT. Asmall-molecule allosteric inhibitor of Mycobacterium tuberculosis tryptophan synthase. *Nat Chem Biol* Nature Publishing Group; **13**:943–950 (2017).

76 Shaun Lott J. The tryptophan biosynthetic pathway is essential for Mycobacterium tuberculosis to cause disease. Biochem Soc Trans. Portland Press Ltd; p. 2029–2037 2020.

77 Smith DA, Parish T, Stoker NG, and Bancroft GJ. Characterization of auxotrophic mutants of Mycobacterium tuberculosis and their potential as vaccine candidates. *Infect Immun* **69**:1142–1150 (2001).

78 Dwivedy A, Ashraf A, Jha B, Kumar D, Agarwal N, and Biswal BK. De novo histidine biosynthesis protects Mycobacterium tuberculosis from host

IFN-γ mediated histidine starvation. *Commun Biol* Nature Research; **4** (2021).

79 Al-Younes HM, Gussmann J, Braun PR, Brinkmann V, and Meyer TF. Naturally occurring amino acids differentially influence the development of Chlamydia trachomatis and Chlamydia (Chlamydophila) pneumoniae. *J Med Microbiol* **55**:879–886 (2006).

80 Kohen R, Yamamoto Y, Cundy KC, and Ames BN. Antioxidant activity of carnosine, homocarnosine, and anserine present in muscle and brain (histidine/lipid peroxidation/8-hydroxydeoxyvuanoslne). Proc. NatI. Acad. Sci. USA. 1988.

81 Jackson IMD, Ampola MG, and Reichlin S. Hypothalamic and Brain Thyrotropin-Releasing Hormone Content and Pituitary-Thyroid Function in Histidine-Deficient Rats. Endocrinology. 1977.

82 Liao SM, Du QS, Meng JZ, Pang ZW, and Huang RB. The multiple roles of histidine in protein interactions. *Chem Cent J* BioMed Central Ltd.; **7** (2013).

83 Lunardi J, Nunes J, Bizarro C, Basso L, Santos D, and Machado P. Targeting the Histidine Pathway in Mycobacterium tuberculosis. *Curr Top Med Chem* **13**:2866–2884 (2013).

84 Monti S, D'Ambrosio K, Simon G De, and D'Ambrosio K. L-Histidinol Dehydrogenase as a New Target for Old Diseases. *Curr Top Med Chem* **16**:2369-2378. (2016).

85 Alifano P, Fani R, Lio`, P, Lio`, L, Lazcano A, Bazzicalupo M, Carlomagno MS, and Bruni CB. Histidine Biosynthetic Pathway and Genes: Structure, Regulation, and Evolution. Microbiol Rev. 1996.

86 Kimura,' A, Mountzouros KT, Relman DA, Falkow S, and Cowell' JL. Bordetella pertussis Filamentous Hemagglutinin: Evaluation as a Protective Antigen and Colonization Factor in a Mouse Respiratory Infection Model. Infect Immun. 1990.

87 Kivistö RI, Kovanen S, Skarp-De Haan A, Schott T, Rahkio M, Rossi M, and Hänninen ML. Evolutionand comparative genomics of Campylobacter jejuni ST-677 clonal complex. *Genome Biol Evol* Oxford University Press; **6**:2424–2438 (2014).

88 Wang L, Jeon B, Sahin O, and Zhang Q. Identification of an arsenic resistance and arsenic-sensing system in Campylobacter jejuni. *Appl Environ Microbiol* **75** (2009).

89 Pinto-Alphandary H, Mabilat C, and Courvalin P. Emergence of aminoglycoside resistance genes aadA and aadE in the genus Campylobacter. *Antimicrob Agents Chemother* **34** (1990).

90 Zhang X, Zhou Q, Tang M, Pu J, Zhang J, Lu J, Zhang Y, and Gao Y. Aminoglycoside Resistance and Possible Mechanisms in Campylobacter Spp.

Isolated From Chicken and Swine in Jiangsu, China. *Front Microbiol* Frontiers Media S.A.; **12**:2975 (2021).

Table Legends

Figure Legends

Table 1. The ingredients and analysed nutrient composition (g kg^{-1}) of control diets fed to broilers from day 0 to 35 post-hatch

Items (g kg^{-1})	STARTER	GROWER	FINISHER
Barley	104.0	83.0	71.0
Wheat	500.0	550.0	600.0
Soya bean meal	260.0	230.0	190.0
Full fat soya	50.0	50.0	50.0
L Lysine HCl	4.0	3.0	3.0
DL-methionine	4.0	3.5	3.0
L-threonine	1.5	1.5	1.5
Soya Oil	40.0	45.0	47.5
Limestone	12.5	12.5	12.5
Monocalcium phosphate	15.0	12.5	12.5
Salt	2.5	2.5	2.5
Sodium bicarbonate	1.5	1.5	1.5
Vitamin-Trace minerals premix[ɬ]	4.0	4.0	4.0
Maxiban G160 Premix	0.6	0.6	0.6
Ronozyme WX	0.2	0.2	0.2
Ronozyme P 5000 (CT)	0.2	0.2	0.2
Analysed Nutrient Composition (g kg−1)			
Dry matter	899.0	898.2	899.5
Fat (ether extract)	63.9	68.5	71.1
Crude Protein*	218.4	206.4	191.4
Fibre	3.08	3.02	2.97
Ash	6.02	5.68	5.50

[ɬ]The vitamin and trace minerals premix provided (units kg^{-1} diets): retinol 16 000 iu; cholecalciferol 33 000 iu; tocolpherol 75 iu; thiamine 3 mg; riboflavin 10 mg; pyridoxine 3 mg; cyanocobalamin 15 μg; phylloquinone 5 mg; nicotinic acid 60 mg; pantothenic acid 14.5 mg; folic acid 1.5 mg; biotin 275 μg; choline chloride 250 mg; iron 20 mg; copper 10 mg; m100 mg; cobalt 1 mg; zinc 82 mg; iodine 1 mg; selenium 0.2 mg; molybdenum 0.5 mg.

*Crude protein = Nitrogen x 6.25

Table 2. The chemical analysis of LFRM (EP100i)

Nutrient	g kg^{-1}
Dry Matter	890
Crude Protein	340
Crude Fat	38
Crude Fiber	118
Crude Ash	70
Sugars	32

Table 3. Effect of dietary treatments on growth performance of broilers

Parameters	T1	T2	SED	P value
Starter phase				
ABW d0 (bird kg^{-1})	0.042	0.042	0.000	0.940
ABW d13 (bird kg^{-1})	0.308	0.319	0.016	0.519
ADWG d0-13 (bird kg^{-1})	0.020	0.021	0.001	0.521
ADFI d0-13 (bird kg^{-1})	0.032	0.033	0.001	0.155
FCR d0-13	1.544	1.569	0.053	0.641
Grower Phase (pre-challenged phase)				
ABW d20 (bird kg^{-1})	0.680	0.738	0.020	0.014
ADWG d13-20 (bird kg^{-1})	0.053	0.06	0.003	0.031
ADFI d13-20 (bird kg^{-1})	0.076	0.077	0.004	0.828
FCR d13-20	1.432	1.300	0.116	0.280
Finisher phase (post-challenged phase)				
ABW d35 (bird kg^{-1})	2.113	2.220	0.052	0.066
ADWG d20-35 (bird kg^{-1})	0.096	0.097	0.003	0.680
ADFI d20-35 (bird kg^{-1})	0.15	0.151	0.006	0.858
FCR d20-35	1.565	1.563	0.062	0.973
Overall performance				
ADWG d0-35 (bird kg^{-1})	0.059	0.061	0.001	0.154
ADFI d0-35 (bird kg^{-1})	0.091	0.092	0.004	0.741
FCR d0-35	1,537	1.494	0.046	0.537

T1, Control; T2, LFRM at 3% and 4% in diets during the grower and finisher phase, respectively. ABW, Average body weight; ADWG, Average daily weight gain; ADFI, Average daily feed intake; FCR, feed to gain ratio (ADFI/ADWG); P values, Significantly different ($P \leq 0.05$) or trending ($0.05 < P \leq 0.10$).

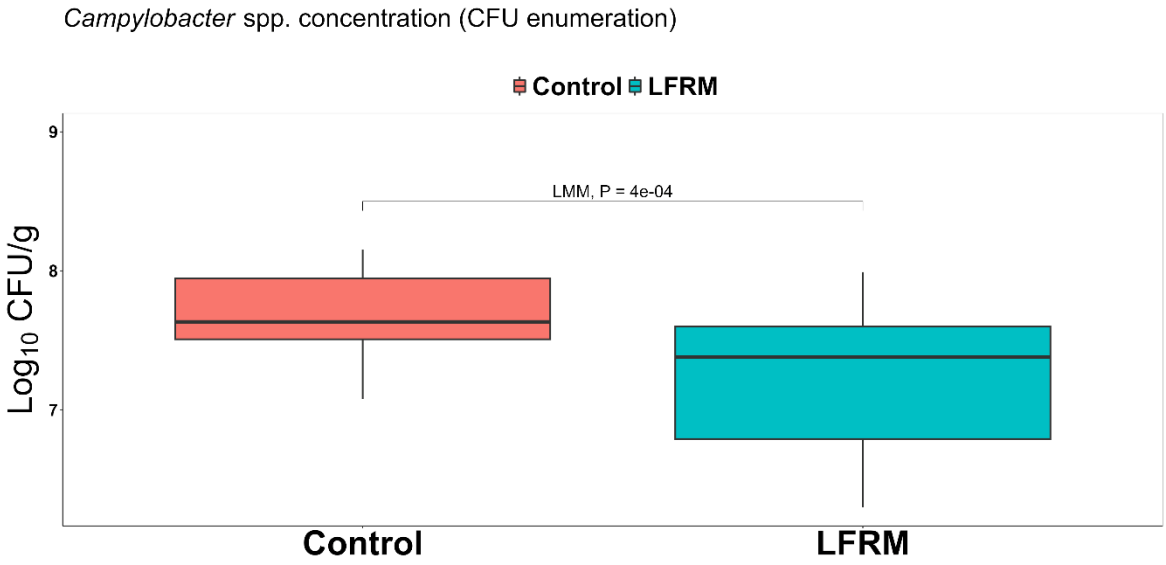

Figure 1. Caecal CFU enumeration of *Campylobacter spp.*

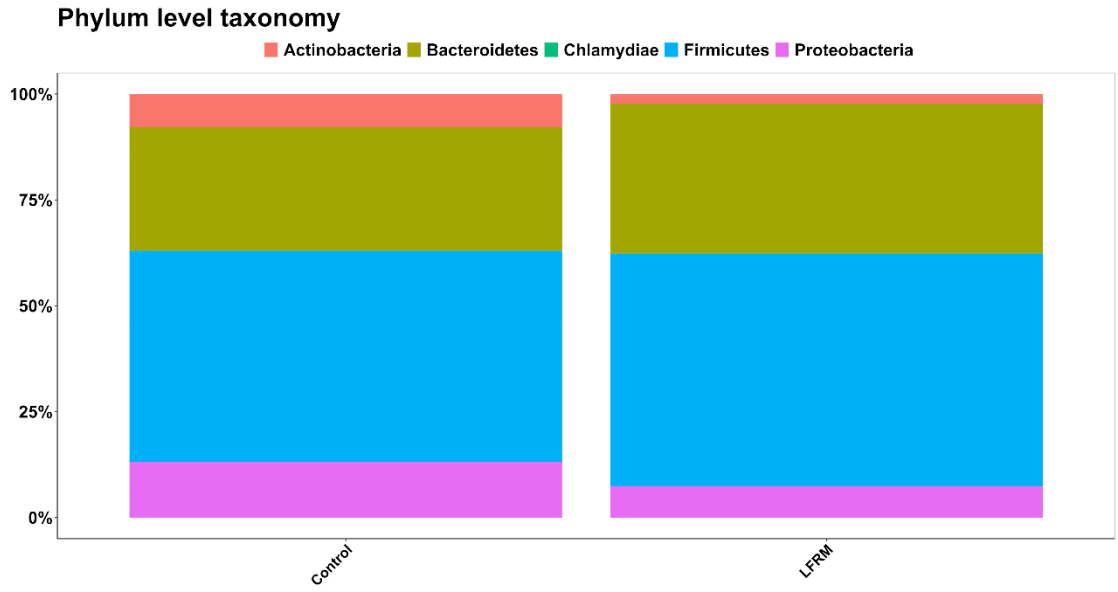

Figure 2. Phylum level taxonomy

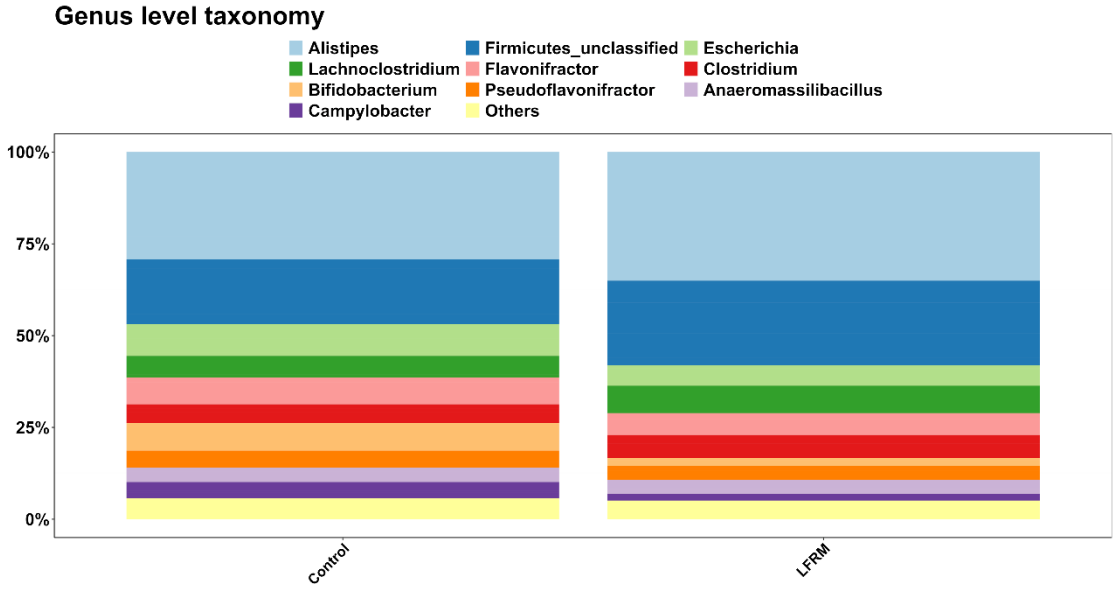

Figure 3. Genus level taxonomy

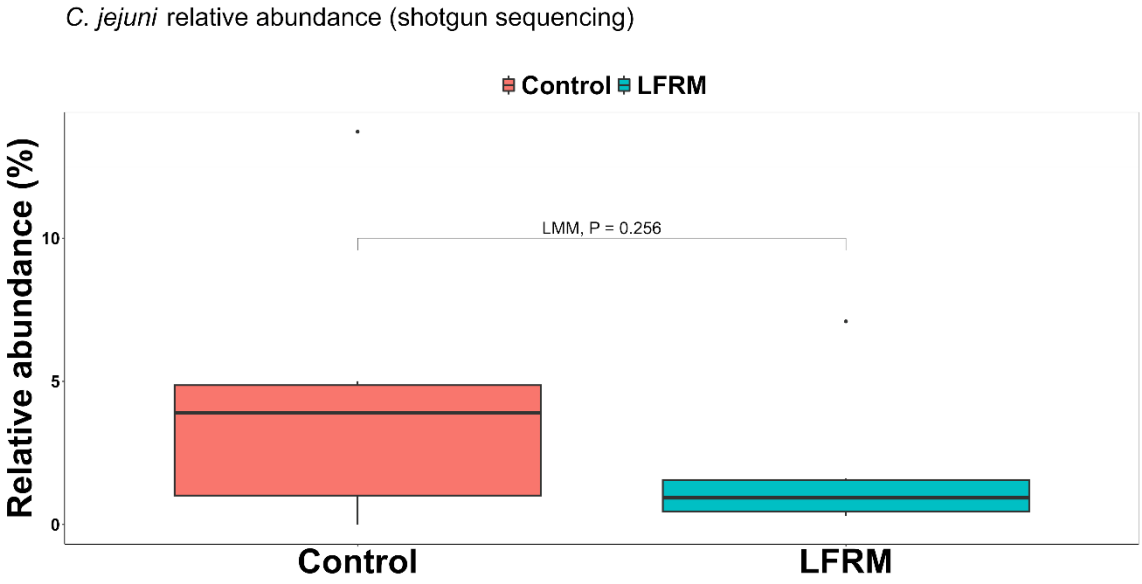

Figure 4. *Campylobacter jejuni* relative abundance (shotgun sequencing)

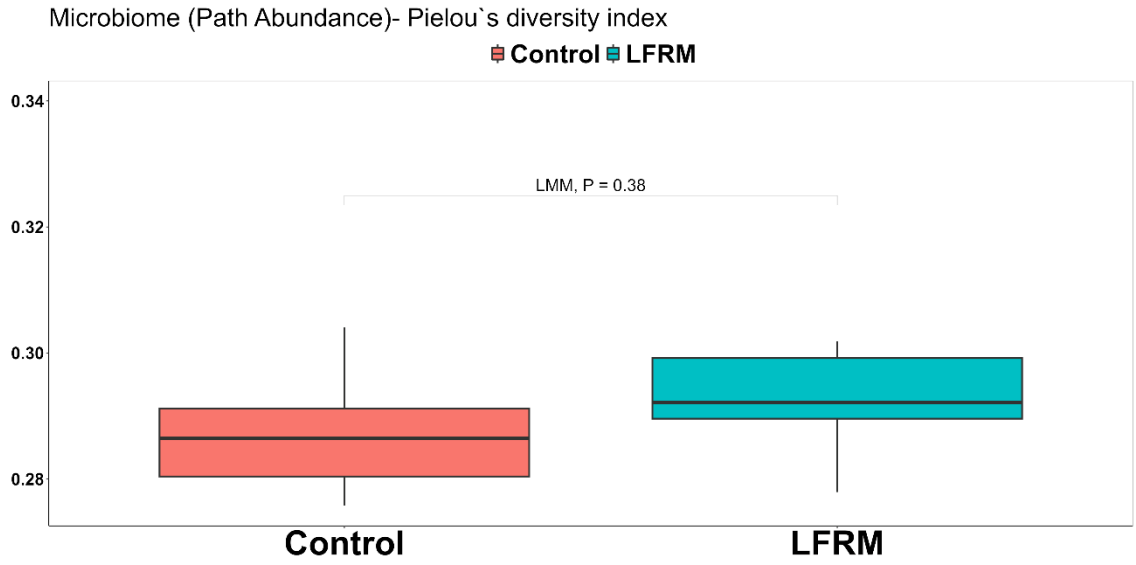

Figure 5. Microbiome Alpha-diversity on path abundance (Pielou's diversity index)

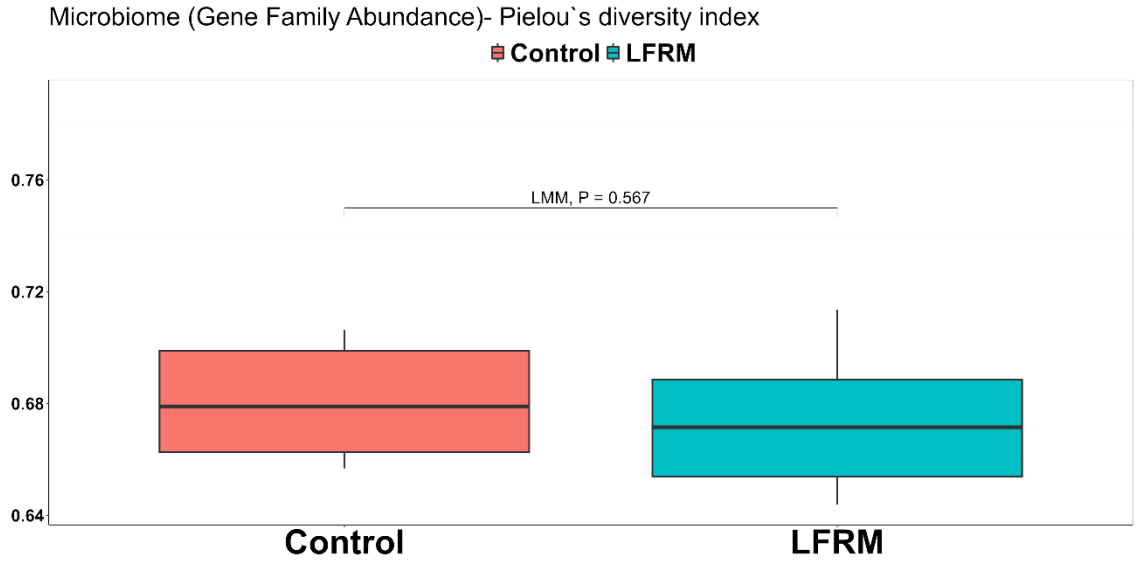

Figure 6: Microbiome Alpha-diversity gene family abundance (Pielou's diversity index)

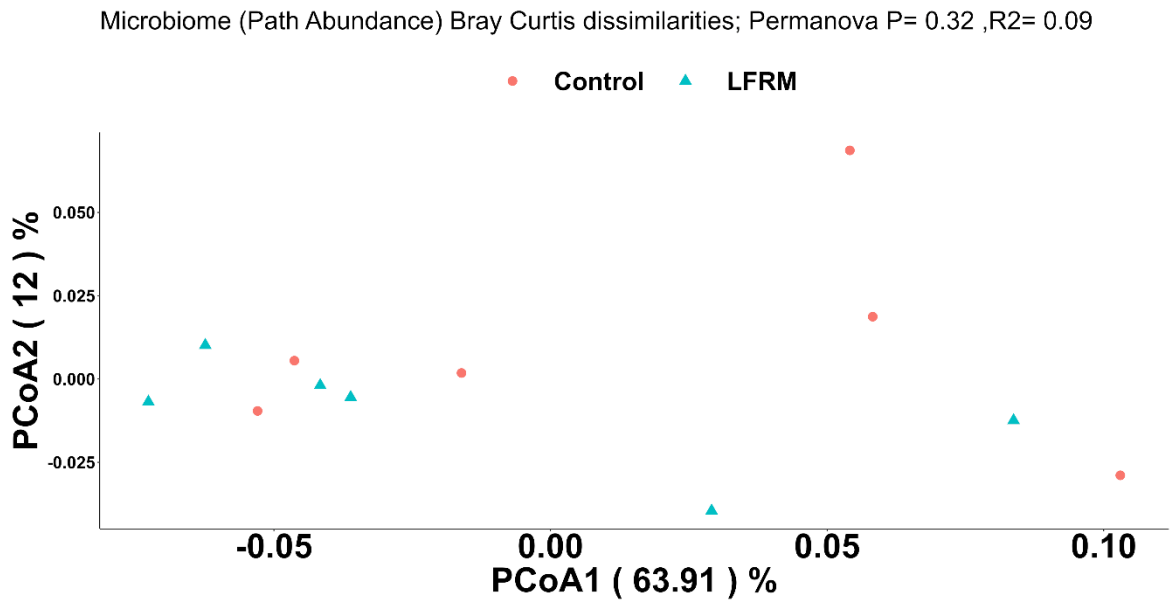

Figure 7: Microbiome: Beta-diversity of pathways abundance (Bray Curtis dissimillarities: Permanova P = 0.32, R2 = 0.09

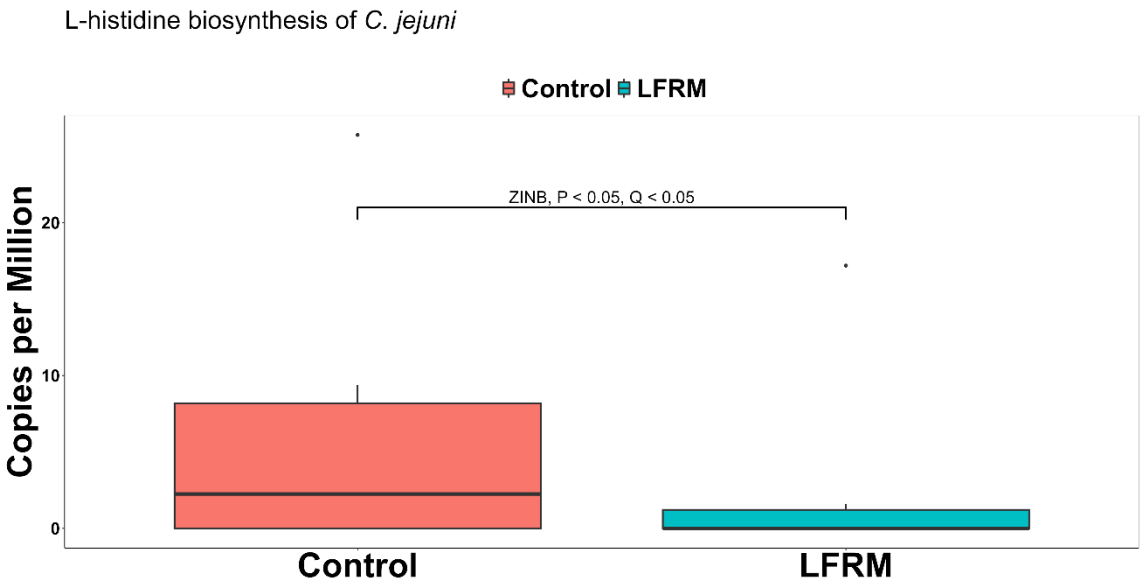

Figure 8. L-histidine biosynthesis pathway of *Campylobacter jejuni*.

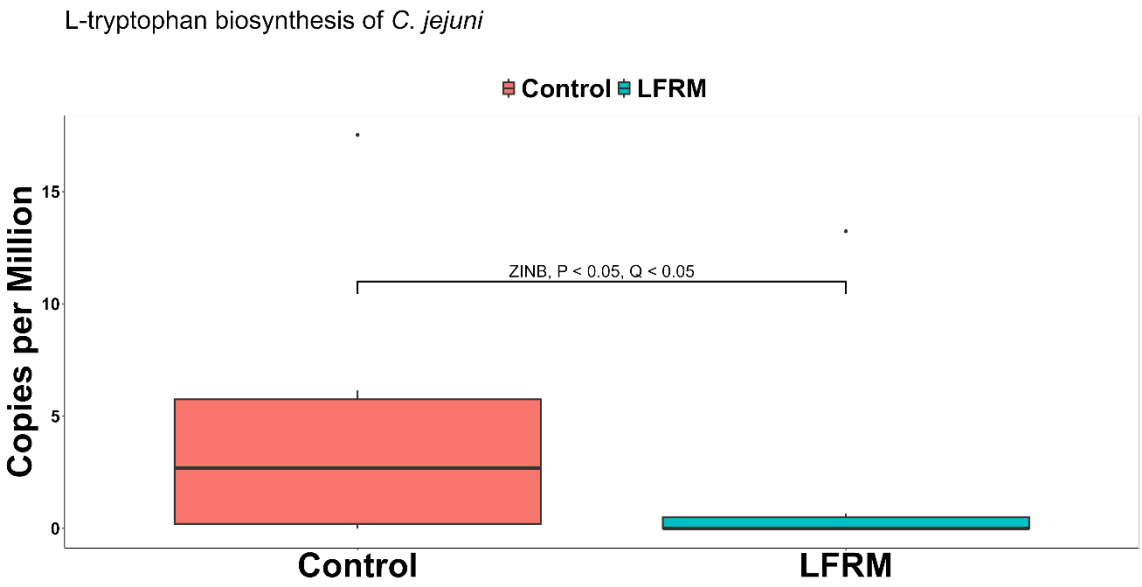

Figure 9. L- tryptophan biosynthesis pathway of Campylobacter jejuni.

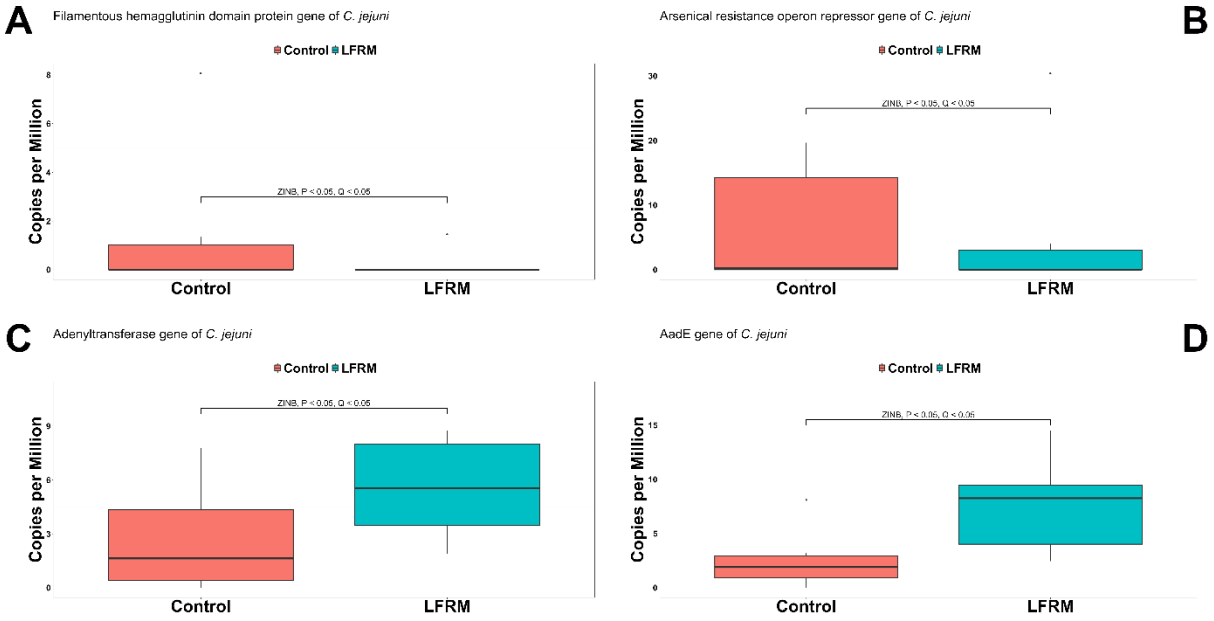

Figure 10. Differential abundance of *Campylobacter jejuni* gene families