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Microbial community diversity and structure in the cecum of laying hens with and without mannan-rich fraction supplementation

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Primary Audience: Nutritionists, Feed Mill Managers, Poultry researchers, Poultry producers

SUMMARY

The gastrointestinal microbiome in animals provides an attractive target for manipulation to improve animal health and production performance. A better understanding of the chicken intestinal microbiome and how nutritional interventions can be used to modulate the microbiota is needed. Most studies of the intestinal microbiome of chickens have examined broilers with few studies focusing on the layer microbiome. This study focused on examining the impact of mannan-rich fraction (**MRF**) supplementation on the cecal microbiota of layers during and post peak lay.

In a feeding trial, Shaver female laying hens were fed a control diet or a control diet supplemented with MRF in a randomized complete block design. Cecal content was collected from 10 randomly selected birds per treatment and subject to metagenome analysis at 4 timepoints (d 16, 32, 64, and 84 post-MRF introduction).

Alpha diversity analysis revealed that Chao1 was significantly greater at D 16, D 32, and D 64 post-MRF supplementation but was significantly lower at D 84 in the MRF supplemented layers compared with the control ($P < 0.005$). PCoA plots showed that the bacterial community composition at the species level differed significantly ($P < 0.001$) between control and MRF supplemented layers at each timepoint. Microbiome analysis showed that following 84-days supplementation with MRF the pathogenic bacteria *Listeria monocytogenes, Campylobacter jejuni, Enterococcus faecalis*, and *Clostridioides difficile* were significantly lower in the layer cecum.

In this study we observed greater alpha and beta diversity and lower bacterial pathogen detection over the 84-days following supplementation with MRF in laying hens. Increased bacterial diversity of the intestinal microbiota is one of the key determinants of colonization resistance against invading pathogens. With reference to the global challenge of antibiotic resistance and food security, reducing pathogenic bacterial species through the use of natural nonantibiotic alternatives is of particular importance for food chain integrity as well as flock health.

Key words: microbiome, laying hen, prebiotic, poultry, food safety

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DESCRIPTION OF PROBLEM

The domestic hen (*Gallus gallus* subsp. *domesticus*) is considered to be one of the main

Statistical nomenclature

Δ	difference
μ	mean
σ	standard deviation
σ^2	variance
η	median
$\sim$	approximal to
$\nsim$	not approximal to
FC	fold change
H_0	null hypothesis
H_A	alternative hypothesis
$N(\mu,\sigma^2)$	normal (Gaussian) distribution
P	P value
X	sample distribution

sources of food produced worldwide (Wang et al., 2017). Poultry meat and eggs provide high-quality animal protein for humans and play an important role in the health and nutrition of all individuals (Marangoni et al., 2015). From an economic perspective, the laying hen sector of poultry accounted for approximately 6.0 billion laying hens (in rearing and production) producing 86 million tons of eggs annually (FAOSTAT, 2019; Fan and Wu, 2022). For efficient and sustained meat and egg supply poultry health is closely linked to their gut microbiome profile and diversity (Aruwa et al., 2021). The digestive tracts of chickens are colonized by complex microbial communities, which are thought to play important roles in the overall health and performance of the birds. Microbiome functions include protection against pathogens, nutrients production, and host immune system maturation (Shang et al., 2018). The presence of a balanced and functional gut microbiome is crucial to poultry performance and health.

Historically antibiotics have been used subtherapeutically to promote growth, prevent diseases and protect bird health by modifying immune status. However due to the development of antibiotic resistance and the detection of drug residues in animal products, the use of antibiotics as growth promoters has been banned in many jurisdictions such as the European Union and the United States. Increases in poultry digestive diseases due to dysbiosis were observed, that is, an imbalance in gut microbiome, following a ban on the use of antimicrobials as growth promoters in European, American and other countries (Sood et al., 2020). A microbial imbalance in the gastrointestinal tract (**GIT**) may result in nutrient malabsorption and growth depression in the host (Sood et al., 2020). As such, the bacterial composition of the chicken GIT has become a prominent research focus and has been extensively studied in recent years to understand the numerous challenges associated with infectious diseases and suboptimal performance of flocks.

Studies of the commensal and pathogenic intestinal microbiome of chickens have mainly been focused on broilers, most likely due to their economic importance and short growth cycles (Ngunjiri et al., 2019). However, the economic importance of layers and the impact of the microbiome on performance cannot be underestimated (Bindari and Gerber, 2022). Broilers and layers compositionally differ in microbiota dynamics, mainly due to differences in the length of life cycles, flock management protocols such as caged housing and different dietary requirements, genetics and sex (Kers et al., 2018). Therefore, the composition of the gut microbiota in these 2 lines is likely very different and needs separate investigations.

Layer microbiota are observed to alter with bird maturation, where such changes are strongly associated with physiological changes as the bird enters the laying period of its life-cycle (Xing et al., 2019). Prolonging the laying cycle can only be achieved when the health of the bird is maintained. The poultry industry constantly strives to integrate novel strategies for improvements in production and animal health status, prioritizing those based on the holistic use of natural resources to control intestinal diversity and homeostasis.

As microbiome research continues to grow, it is becoming clear that poultry health and production performance are partly influenced by nonpathogenic, GIT bacterial symbionts. Several natural feed supplements focus on gut microbiome stabilization which aid intestinal health by reducing dysbiosis and mitigating disease, for example, prebiotics, probiotics and organic acids (Dittoe et al., 2018; Shehata et

al., 2022). Prebiotics are most commonly host-indigestible complex oligosaccharides that persist in the GIT until metabolized by the microbiota where they promote subpopulation growth (Gibson et al., 2017, Khan et al., 2020a). Prebiotics, such as mannan-rich fractions (**MRFs**) have been found to have beneficial effects in broilers in terms of decreasing pathogen load, improving bacterial diversity and modulating immunity (Corrigan et al., 2015, 2017, 2018; McCaffrey et al., 2021). A study by (Salami et al., 2022) showed that feeding mannan based prebiotics improves egg production, feed efficiency and reduces the mortality of laying hens. However, an important, yet comparatively unexplored, potential application of prebiotic MRF in layers is to improve bacterial diversity and impair pathobiont colonization in the GIT. Consequently, this study has investigated the effect of MRF supplementation on its ability to enhance bacterial diversity and to lower the abundance of bacteria associated with food safety concerns through the modulation of cecal microbiota in layers.

MATERIALS AND METHODS

Animal Trial, Sample Collection, and Preservation

This layer trial was performed at a research site in Scotland, United Kingdom and the accommodation and care of animals used in the study was in accordance with Directive 2010/63/EC (European Parliament Report, 2013) and European Commission Recommendation 2007/526/EC. A total of 344 Shaver female laying hens (*Gallus gallus* subsp. *domesticus*) were randomly allocated to 1 of 2 diets (1—standard commercial diet and 2—standard commercial diet + MRF) and identified by cage. Each diet was replicated 43 times with 4 birds per cage using a randomized complete block design. Birds were aged 16 wk on arrival, the study started when the birds were 28-wk old. The building was supplied with artificial, programmable lights, and forced ventilation. The temperature inside the building was as recommended by the breeder. The lighting program was 16-h light and 8-h dark during each 24-h period throughout the trial. Feed and water were available ad libitum throughout the trial and one feed hopper per cage was provided. General observations of health and temperature recording was carried out twice daily am and pm and feed and water supply was checked at least twice daily. The birds were fed a mash diet throughout the duration of the trial. Experimental diets were calculated to be isonutritive and to meet or exceed the nutrient requirements recommended by the national research council for laying hens (Dale, 1994). The composition and the calculated analyses of the basal diets are presented in Table 1. MRF (Alltech Biotechnology) was included in the experimental diet at 800 g/t until the birds were aged 34 wk and at 400 g/t from 34 wk of age until the end of the layer period.

Table 1. Composition and calculated analyses of standard commercial diet.

Ingredients	Commercial feed, %
Wheat	41.5139
Hipro soya	17.5
Maize	30
Soya oil	1.5
Sodium bicarbonate	0.2
Monocalcium phosphate	1.06
Lime flour	7
Salt	0.27
L-Lysine	0.0025
Methionine	0.173
Threonine	0.0295
DL-tryptophan	0.0011
Choline chloride	0.24
Vitamin E 50% adsorbate	0.01
Vitamin premix[1]	0.5
Calculated analyses[2]	
ME poultry, MJ/kg	11.679
Crude protein, %	15.029
Crude fat %	3.528
Crude fiber, %	2.48
Ash, %	9.962
Calcium, %	3.062
Total phosphorus, %	0.544
Sodium, %	0.18
Methionine	0.401
Lysine	0.603
M + C	0.616

Hipro soya is high protein soybean meal; M + C = methionine + cysteine.

[1]Provided per kilogram of diet.

[2]Based on institutional or published values for feed ingredients.

At each time point selected for metagenome analysis (d 16, 32, 64, and 84 post-MRF

introduction) the intact cecal pouch of 10 randomly selected birds per treatment was excised immediately after humane euthanization. Cecal content was aseptically transferred to tubes containing 20 mL of DNA/RNA shield (Zymo Research, Cambridge Bioscience, Cambridge, UK) transported at room temperature and stored at −80°C for downstream processing.

DNA Extraction and Sequencing

DNA was extracted from cecal contents using a DNeasy Powersoil Pro kit from (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA concentration, purity, and integrity were determined using an Agilent 5400 Fragment Analyzer System (Agilent Technologies, Santa-Clara, CA). Sequencing libraries were generated using NEBNext Ultra DNA Library Prep Kit for Illumina sequencing (NEB, Ipswich, MA). Whole DNA fractions were fragmented by sonication to the size of ~350 bp. The DNA fragments were then end-polished, A-tailed, and ligated using a full-length adaptor for Illumina sequencing with further PCR amplification. Each PCR product was purified (AMPure XP system) and library size distributions were established using an Agilent 2100 Bioanalyzer and quantified using a real-time PCR. Clustering of the index coded samples was performed on the Illumina cBot Cluster Generation System; then, the library preparations were sequenced on an Illumina HiSeq platform and paired-end reads were generated (Novogene, Cambridge, UK).

Dataset Quality Control

Each sample was quality controlled using TrimGalore! v.0.6.6 with the "−paired" and "−fastqc" flags (https://github.com/FelixKrueger/TrimGalore) and otherwise default settings. TrimGalore! was powered by Cutadapt v.3.4 (Martin, 2011) and FastQC v.0.11.9 (https://github.com/s-andrews/FastQC). For the control, this resulted in $3.442e^{+06} \pm 2.622e^{+05}$, $3.407e^{+06} \pm 2.123e^{+05}$, $3.232e^{+06} \pm 1.694e^{+05}$, and $3.260e^{+06} \pm 2.835e^{+05}$ metagenomic read segment pairs for D 16, D 32, D 64, and D 84, respectively. For MRF supplemented birds, this resulted in a total of $3.418e^{+06} \pm 3.283e^{+05}$, $3.571e^{+06} \pm 3.261e^{+05}$, $3.560e^{+06} \pm 4.074e^{+05}$ and $3.625e^{+06} \pm 3.107e^{+05}$ metagenomic read segment pairs for D 16, D 32, D 64, and D 84, respectively.

Marker Gene Extraction

A dataset of 16S rRNA marker genes were extracted from each sample using phyloFlash v.3.14 (Gruber-Vodicka et al., 2020) using a database of copy number weighted 16S rRNA sequences extracted from sequenced genomes as carried out by Leigh et al. (2022). For the control this resulted in 27,617 ± 3,125.91, 27,101.5 ± 2,120.09, 25,314 ± 2,614.06, and 24,954 ± 2,299.77 fully mapped 16S rRNA sequences for D 16, D 32, D 64, and D 84, respectively. For MRF supplemented birds this resulted in 26,506.7 ± 3,472.29, 29,515.2 ± 3,232.34, 29,016.8 ± 3,923.97, and 27,905.5 ± 2,277.47 fully mapped 16S rRNA sequences for D 16, D 32, D 64, and D 84, respectively.

Dataset Processing

The dataset was scaled to 100,000 16S rRNA sequences per sample and extreme outliers were processed using uniForest v.1 (Leigh et al., 2021b). For each taxa at each taxonomic rank, outliers were imputed with the median of the remaining inliers. The dataset was then rescaled to 100,000 sequences (Supplemental Tables: Reads).

Comparison of Treatments at Paired Timepoints

Each taxon (at each rank) in both treatment groups were assessed for Gaussianity using a Shapiro-Wilk test (H_0: $X \sim N(\mu,\sigma^2)$; H_A: $X \approx N(\mu,\sigma^2)$; Shapiro and Wilk, 1965) where $P < 0.05$ was used to determine if a given distribution was non-Gaussian. Each taxon distribution between neighboring timepoints or between datasets at the same timepoint was assessed for equivariance using a Levene's test (H_0: $\sigma^2_{(a)} = \sigma^2_{(b)}$; H_A: $\sigma^2_{(a)} \neq \sigma^2_{(b)}$; Levene, 1960). Gaussianity and equivariance were used to determine which statistical test to apply to the data and the most appropriate test that could be

used on all comparisons (as per Leigh et al., 2021a). As the data were not all Gaussian and as all comparisons were not all equivariant, a Brunner-Munzel test (H_0: $B = 0.5$; H_A: $B \neq 0.5$; Brunner and Munzel, 2000) (Supplemental Tables: Pairwise comparisons). To control potential spurious results, comparisons where the medians of both sample sets equated to 0 were not computed. A $P \leq 0.005$ (as per Benjamin et al., 2018) was used to determine significance and effect directionality was inferred by the difference between medians referred to as the median fold change (η_{FC}). An increased η_{FC} was observed when $\eta_{(b)} - \eta_{(a)} > 0$ and a decreased η_{FC} was observed when $\eta_{(b)} - \eta_{(a)} < 0$). A $P \leq 0.005$ was not used for Shapiro-Wilk or Levene's tests to prevent potential distribution or variance mischaracterization when selecting the most appropriate comparison (Leigh et al., 2021a).

Comparison of Treatments Over All Timepoints

Taxon abundance dynamics between treatment groups (across all timepoints) were assessed using an area-under-the-curve (**AUC**) model constructed from geometric mean for repeated measures at each timepoint. A constant (1) was added to each measure prior to calculation. This constant was added to allow for AUC calculations of sparse taxa (where a taxon was not observed at a given timepoint). Each AUC was quantified using Simpson's rule and compared between treatments using a 2-tailed Yates' corrected Pearson's chi-square test (H_0: $\pi_{(a)} = \pi_{(b)}$; H_A: $\pi_{(a)} \neq \pi_{(b)}$; Pearson, 1900; Yates, 1934) where the total population was the scaled sum of all species for a given timepoint ($\Sigma n_{K(\text{taxon})} = 100{,}000$ in all instances) (Supplemental Tables: Pairwise AUC).

Assessment of α-Diversity

For each sample, α-diversity was computed using the "alpha_diversity" driver functions in the skbio v.0.5.6 library (http://scikit-bio.org/). Taxon diversity was assigned using Chao1 (Chao, 1984), Simpson's *D'* (Simpson, 1949) and Shannon's *H'* (Shannon, 1948), and evenness was calculated using Simpson's *E* (Simpson, 1949). Each α-diversity metric between timepoints or treatment groups were compared using a Brunner-Munzel test (H_0: $B = 0.5$; H_A: $B \neq 0.5$) (Supplemental Table: alpha diversity). Again, a $P \leq 0.005$ was used to determine significance and effect directionality was inferred by the difference between medians.

Assessment of β-Diversity

A Bray-Curtis dissimilarity matrix (Bray and Curtis, 1957) was constructed to assess the divergence of each group (timepoint and treatment group). Divergences were statistically assessed using PERMANOVA (H_0: $G_{(\alpha)} - G_{(\beta)} = 0 \; \forall \; \{a, b, \ldots x\}$; H_0: $G_{(\alpha)} - G_{(\beta)} \neq 0 \; \forall \; \{a, b, \ldots x\}$; Anderson, 2001) using 999 permutations (Supplemental Table: Beta diversity). Again, $P \leq 0.005$ was used to determine significance.

RESULTS AND DISCUSSION

The gut microbiota is associated with the health and performance of chickens. The phylogenetic composition of microbiota typically found in various intestinal segments of broilers is well documented (Stanley et al., 2014; Shang et al., 2018; Feye et al., 2020). However, in layers, there is only limited literature available on the composition of microbiota in the gut. The commercial life span of layers is substantially longer than that of broilers. Hence, studies are required to understand the development and maturation of microbiota of laying hens during their commercial life span to develop practical applications for safer, sustainable and antibiotic free meat and egg production. This study set out to determine the bacterial community diversity and compositional changes in the cecum of laying hens at various timepoints throughout peak and post peak lay following dietary supplementation with MRF. During this lifestage stress can cause an imbalance in the gut microbiota resulting in production losses.

Microbial diversity in the layer cecum at each timepoint (D 16, D 32, D 64, D 84) was estimated using α-diversity indices (Shannon's *H*, ACE and Chao1). Shannon's *H*' index was

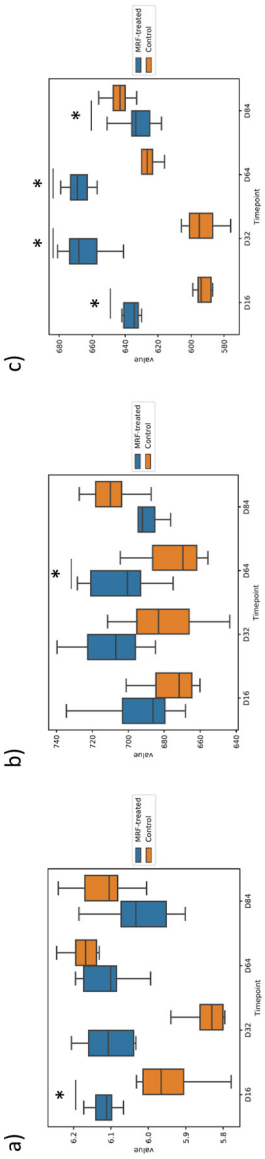

Figure 1. (A–C) Three α-diversity metrics (a = Shannon, b = ACE, c = Chao1) displayed for 4 timepoints explored in this study in both control and mannan-rich fraction supplemented groups. * Denotes statistical significance ($P_{BD} \leq 0.005$).

used to indicate species diversity (Figure 1A), while ACE and Chao1 were used to estimate species richness (Figure 1B and C). Alpha diversity analysis revealed that at D 16 Chao1 and Shannon's *H*' indices were significantly greater ($P < 0.005$) in the MRF supplemented layers compared with the control. Chao1 was also significantly greater at D 32 and D 64 but was significantly lower at D 84 in the MRF supplemented layers compared with the control. ACE was also significantly greater at D 64 in the MRF supplemented layers compared with the control layers but was not different at other timepoints.

Differences in β-diversity within the intestinal microbial population between groups at each timepoint was visualized using PCoA (Figure 2A–D). The PCoA plots show that the bacterial community composition at the species level differed significantly in the cecum ($P < 0.001$) between control and MRF supplemented layers with PC1 accounting for 30.90, 35.51, 31.03, and 29.09% of the total variation and PC2 accounting for 15.86, 9.54, 10.49, and 8.51% at D 16, D 32, D 64, and D 84, respectively.

The taxonomic composition of the layer cecal microbiome was determined by identifying sequences using the VSEARCH software to understand which bacterial taxa were contributing to separating the bacterial communities between control and MRF supplemented layers at each timepoint. The bacterial phyla Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria were the most prevalent phyla at all timepoints, accounting for ~90% of the cecal microbial populations (Table 2). Some of the main Phyla which were significantly impacted by MRF supplementation at D 16 included Bacteroidetes and Proteobacteria which were greater with MRF supplementation and Firmicutes which were lower in relative abundance in birds whose diet was supplemented with MRF. At D 32 and D 64, Actinobacteria were lower while Proteobacteria at D 32 were greater in the MRF supplemented ceca compared to the control. Firmicutes were lower and Proteobacteria greater in ceca at D 84 when MRF was included in the diet. At the species level, the 10 most abundant species in control and MRF supplemented layers are shown in Table 3. For both control and MRF supplemented layers *Megamonas hypermegale*

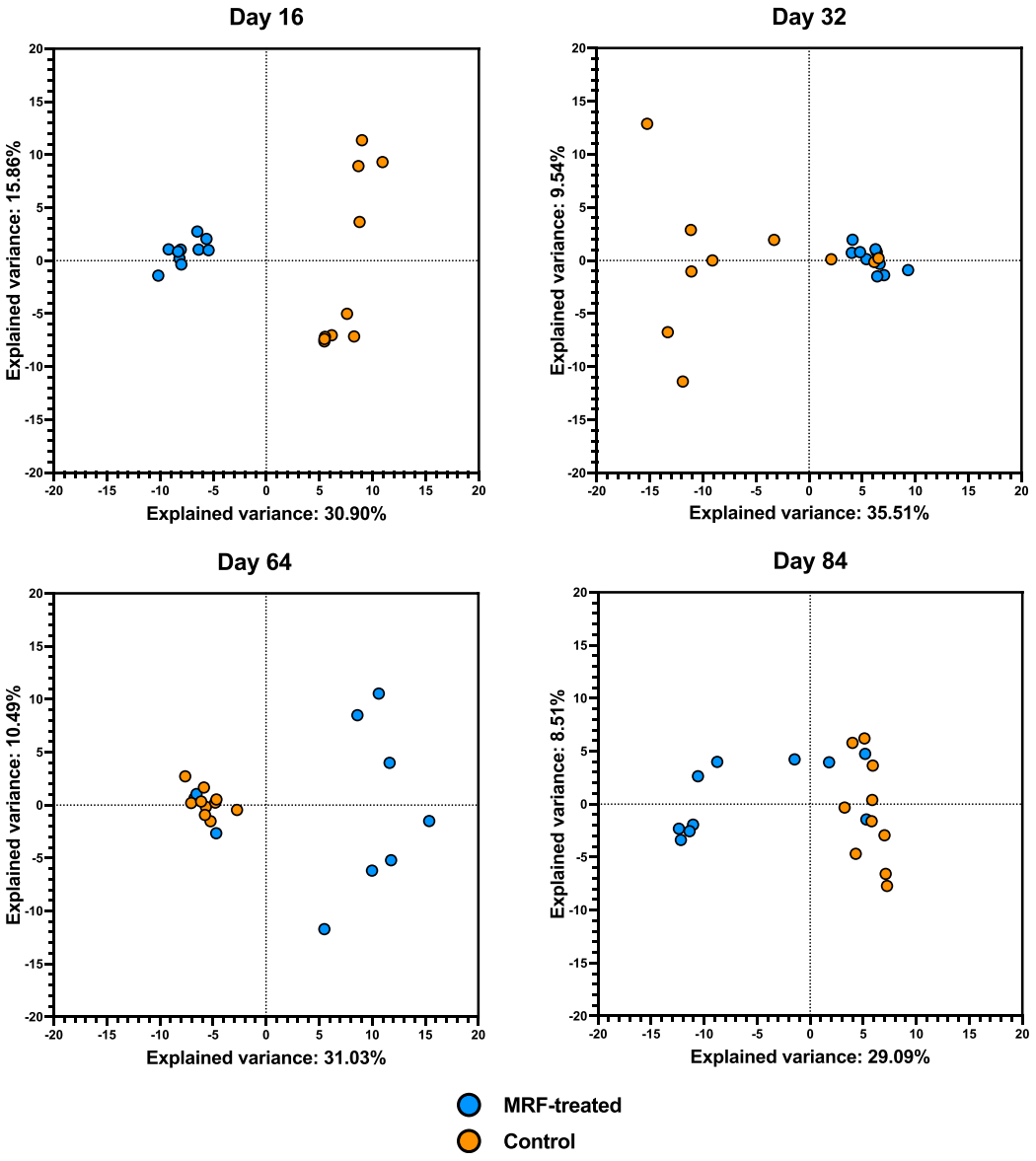

Figure 2. (A–D) Species-level Bray-Curtis distance matrix (β-diversity) expressed as PCoA between control (yellow) and MRF-supplemented (blue) layers at each timepoint.

and *Faecalibacterium prausnitzii* were the most abundant making up >30% of all species at each timepoint.

The relative abundance of several bacterial species was significantly different with MRF supplementation (Table 4). At the earlier timepoints (D 16, D 32) species from the genus Lactobacillus and Bifidobacterium tended to be lower while species from the genus Bacteroides, Ruminococcus, and Alistipes tended to be greater in the MRF supplemented birds compared with the control. Two pathogen-associated bacterial species with significant implications for food safety were identified to

Table 2. Median relative abundances (η%) of bacterial phyla observed at each timepoint in both control and mannan-rich fraction (MRF) supplemented layers.

	D 16			D 32			D 64			D 84		
	Control (η%[1])	MRF (η%)	FC[2]	Control (η%)	MRF (η%)	FC	Control (η%)	MRF (η%)	FC	Control (η%)	MRF (η%)	FC
Firmicutes	**51.72**	**45.72**	**−0.116***	42.35	43.55	0.028	46.19	45.13	−0.023	**50.83**	**46.92**	**−0.077***
Bacteroidetes	**28.17**	**35.30**	**0.253***	33.77	39.51	0.170	32.74	36.51	0.115	31.29	32.26	0.031
Actinobacteria	10.09	8.07	−0.200	**14.98**	**5.48**	**−0.634***	**10.01**	**6.90**	**−0.311***	8.54	9.63	0.127
Proteobacteria	**1.05**	**1.38**	**0.313***	**0.72**	**1.29**	**0.804***	1.45	1.31	−0.097	**0.81**	**1.46**	**0.809***
Others	8.97	9.53	0.838	8.19	10.17	0.198	9.60	10.15	−0.124	8.53	9.73	0.240

*Denotes significant differences ($P \leq 0.05$) and emboldened for each row at each timepoint.
[1]η% = median relative abundance.
[2]FC = fold change.

be significantly lower in birds who received MRF in the diet compared with the control (Table 4): *Campylobacter jejuni* and *Clostridioides difficile*. At D 16, *C. jejuni* was noted to be absent in MRF supplemented ($P < 0.005$) and not detected at any timepoint thereafter and at D 64 *Cl. difficile* was significantly lower in MRF supplemented layers when compared with the control group. *Lactobacillus agilis* was noted to be greater in the MRF group with a 21.40 η_{FC} compared to the control at D 16. *Bacteroides vulgatus* was also noted to be significantly greater in MRF supplemented birds at D 16 with a 6.87 η_{FC} increase. At D 32 *Oscillibacter valericigenes* was greater by 5.47 η_{FC} along with 2 Alistipes species, *A. senegalensis* (7.91 η_{FC}) and *A. inops* (7.23 η_{FC}) in the MRF group compared with the control. At D 64 *Coprococcus catus* was greater in MRF supplemented layers with a 20.09 η_{FC} difference.

The impact of supplementation with MRF on bacterial populations over time using an area under the curve method was also studied. Across the 84-days approximately 29 different bacterial phyla were detected between control and MRF supplemented groups. The most abundant phyla detected were the Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria for both the control and MRF supplemented layers (Table 5). These bacterial phyla were also significantly different between control and MRF supplemented layers with Firmicutes and Actinobacteria significantly lower and Bacteroidetes and Proteobacteria significantly greater in MRF supplemented birds over the 84-days supplementation.

At the species level 268 bacterial species level OTUs (99%) were detected across the 84 d. *Megamonas hypermegale, Faecalibacterium prausnitzii*, and *Lactobacillus johnsonii* were the top 3 most abundant OTUs in the control supplemented layers and *Megamonas hypermegale, Faecalibacterium prausnitzii*, and *Bacteroides plebeius* were the most abundant in the MRF supplemented layers. Differential enrichment of bacterial taxa at the species level (99%) according to diet was calculated and the results are shown in Table 6. Of the bacterial species detected 200 were noted to be significantly differentially abundant over the 84-day period with 85 species level OTUs significantly

Table 3. The (10) most prevalent bacterial species observed at each timepoint in both control and mannan-rich fraction (MRF) supplemented.

Timepoint	Rank	Control (species)	η%[1]	MRF (species)	η%
D 16	1	Megamonas hypermegale	23.266	Megamonas hypermegale	23.688
	2	Faecalibacterium prausnitzii	12.583	Faecalibacterium prausnitzii	12.340
	3	Bifidobacterium pullorum	9.513	Bacteroides plebeius	6.363
	4	Lactobacillus johnsonii	8.542	Lactobacillus johnsonii	5.727
	5	Lactobacillus crispatus	8.170	Megamonas funiformis	5.009
	6	Lactobacillus amylovorus	3.235	Lactobacillus crispatus	4.786
	7	Lactobacillus reuteri	2.202	Bifidobacterium pullorum	3.591
	8	Lactobacillus salivarius	2.015	Lactobacillus salivarius	3.352
	9	Collinsella tanakaei	1.606	Bacteroides coprocola	2.329
	10	Lactobacillus helveticus	1.530	Lactobacillus amylovorus	1.739
D 32	1	Megamonas hypermegale	18.486	Megamonas hypermegale	26.455
	2	Faecalibacterium prausnitzii	15.851	Faecalibacterium prausnitzii	12.439
	3	Lactobacillus johnsonii	13.931	Bacteroides plebeius	8.137
	4	Bifidobacterium pullorum	7.639	Lactobacillus johnsonii	6.455
	5	Lactobacillus amylovorus	5.490	Megamonas funiformis	3.393
	6	Lactobacillus helveticus	4.004	Bacteroides coprocola	3.094
	7	Lactobacillus reuteri	3.019	Bifidobacterium pullorum	2.436
	8	Prevotella rara	1.441	Lactobacillus amylovorus	2.280
	9	Bacteroides clarus	1.328	Bacteroides salanitronis	2.140
	10	Lactobacillus salivarius	1.288	Bacteroides clarus	1.640
D 64	1	Megamonas hypermegale	22.634	Megamonas hypermegale	38.341
	2	Faecalibacterium prausnitzii	14.612	Faecalibacterium prausnitzii	14.583
	3	Bacteroides plebeius	8.946	Bacteroides plebeius	4.214
	4	Lactobacillus johnsonii	4.460	Lactobacillus crispatus	3.716
	5	Lactobacillus crispatus	4.291	Lactobacillus johnsonii	2.789
	6	Megamonas funiformis	3.548	Lactobacillus amylovorus	2.348
	7	Bacteroides coprocola	3.546	Megamonas funiformis	1.892
	8	Lactobacillus amylovorus	1.835	Bacteroides coprocola	1.582
	9	Bacteroides salanitronis	1.566	Bacteroides salanitronis	1.356
	10	Butyricicoccus pullicaecorum	1.493	Lactobacillus salivarius	1.161
D 84	1	Megamonas hypermegale	28.467	Megamonas hypermegale	33.760
	2	Faecalibacterium prausnitzii	12.805	Faecalibacterium prausnitzii	14.438
	3	Megamonas funiformis	6.486	Lactobacillus crispatus	4.513
	4	Lactobacillus johnsonii	5.677	Megamonas funiformis	4.453
	5	Lactobacillus crispatus	5.442	Bifidobacterium pullorum	4.143
	6	Bifidobacterium pullorum	4.319	Lactobacillus johnsonii	3.294
	7	Bacteroides coprocola	2.888	Bacteroides coprocola	2.967
	8	Lactobacillus amylovorus	2.575	Bacteroides salanitronis	2.083
	9	Bacteroides salanitronis	1.922	Prevotella rara	1.412
	10	Lactobacillus salivarius	1.301	Lactobacillus amylovorus	1.258

[1] η% = median relative abundance.

lower and 115 OTUs significantly greater in the ceca of birds with MRF in the diet. 29 OTUs were noted to be only present in the MRF supplemented group and 19 OTUs were noted to be only present in the control group. Of note those bacteria whose abundance were promoted with MRF supplementation included *Coprococcus catus, Roseburia hominis, Roseburia intestinalis*, and *Christensenella minuta* as well as 4 *Prevotella* species (*P. albensis, P. bryantii, P pectinovora*, and *P. pleuritidis*). Those bacteria that were not present with MRF supplementation included the noteworthy bacteria from a food safety perspective *Listeria monocytogenes* and *Campylobacter jejuni. Enterococcus faecalis* and *Clostridioides difficile* were also significantly depleted in the MRF supplemented layers. Bacterial species which were most significantly depleted in MRF supplemented layers included *Kineothrix alysoides, Collinsella stercoris, Ruminococcus albus, Bifidobacterium pseudolongum, Lactobacillus mucosae*. Those

Table 4. Significantly altered (increased or decreased) species observed at each timepoint ($P \leq 0.005$) in both control and mannan-rich fraction (MRF) supplemented datasets.

Day	Taxon	Control (Ƞ[1])	MRF (Ƞ)	Change	FC[2]
D 16	Bifidobacterium biavatii	9.662	1.761	Decrease	−0.818
	Bifidobacterium pseudolongum	25.402	4.582	Decrease	−0.820
	Butyricimonas virosa	3.113	1.877	Decrease	−0.397
	Lactobacillus aviarius	29.893	3.318	Decrease	−0.889
	Lactobacillus hamsteri	26.800	7.237	Decrease	−0.730
	Lactobacillus ultunensis	11.877	3.368	Decrease	−0.716
	Campylobacter jejuni	6.060	0.000	Decrease	Depletion
	Prevotella buccae	36.732	14.275	Decrease	−0.611
	Eubacterium brachy	4.810	0.000	Decrease	Depletion
	Slackia piriformis	7.669	3.217	Decrease	−0.581
	Bifidobacterium longum	19.636	10.204	Decrease	−0.480
	Porphyromonas gingivalis	32.780	23.009	Decrease	−0.298
	Lactobacillus equigenerosi	6.526	2.713	Decrease	−0.584
	Lactobacillus frumenti	10.183	1.818	Decrease	−0.821
	Lactobacillus pontis	37.851	16.092	Decrease	−0.575
	Collinsella tanakaei	163.441	87.556	Decrease	−0.464
	Bacteroides vulgatus	1.596	12.563	Increase	6.871
	Muribaculum intestinale	20.291	30.797	Increase	0.518
	Prevotella multiformis	1.632	5.454	Increase	2.343
	Prevotella scopos	1.557	6.320	Increase	3.061
	Prevotella shahii	6.243	16.245	Increase	1.602
	Alistipes senegalensis	2.377	8.704	Increase	2.663
	Pseudoclostridium thermosuccinogenes	6.060	9.610	Increase	0.586
	Ruminiclostridium cellulolyticum	1.077	1.318	Increase	0.224
	Ruminococcus gnavus	104.425	167.163	Increase	0.601
	Dorea formicigenerans	23.357	27.272	Increase	0.168
	Desulfitobacterium dehalogenans	0.310	0.352	Increase	0.136
	Anaerotruncus colihominis	3.092	9.317	Increase	2.013
	Sporomusa sphaeroides	0.517	0.589	Increase	0.138
	Oscillibacter valericigenes	2.377	14.447	Increase	5.079
	Bacteroides coprophilus	31.686	89.849	Increase	1.836
	Bacteroides gallinarum	0.000	6.626	Increase	Introduction
	Bacteroides coprocola	40.561	243.977	Increase	5.015
	Bacteroides plebeius	70.208	666.703	Increase	8.496
	Alistipes inops	3.092	8.348	Increase	1.700
	Brevibacillus thermoruber	0.481	0.511	Increase	0.060
	Roseburia faecis	4.103	10.186	Increase	1.482
	Bacteroides sartorii	1.061	4.726	Increase	3.453
	Anaerostipes hadrus	3.122	3.215	Increase	0.030
	Bacteroides zoogleoformans	3.036	6.211	Increase	1.046
	Odoribacter splanchnicus	43.686	72.374	Increase	0.657
	Prevotella loescheii	4.845	22.640	Increase	3.672
	Lactobacillus agilis	1.615	36.188	Increase	21.405
	Candidatus Borkfalkia ceftriaxoniphila	10.046	33.225	Increase	2.307
	Coprococcus catus	0.000	1.607	Increase	Introduction
	Oscillibacter ruminantium	1.546	5.937	Increase	2.840
D 32	Bifidobacterium choerinum	51.008	15.836	Decrease	−0.690
	Bifidobacterium pullorum	789.918	217.422	Decrease	−0.725
	Bifidobacterium scaligerum	6.725	2.038	Decrease	−0.697
	Gardnerella vaginalis	40.335	8.858	Decrease	−0.780
	Collinsella bouchesdurhonensis	27.570	18.470	Decrease	−0.330
	Lactobacillus aviarius	6.015	4.881	Decrease	−0.188
	Thermoanaerobacter ethanolicus	3.259	2.104	Decrease	−0.355
	Lactobacillus frumenti	17.034	4.768	Decrease	−0.720
	Lactobacillus reuteri	312.190	130.824	Decrease	−0.581

continued

Table 4. Continued

Day	Taxon	Control (η[1])	MRF (η)	Change	FC[2]
	Lactobacillus helveticus	414.014	41.267	Decrease	−0.900
	Coriobacterium glomerans	2.634	0.000	Decrease	Depletion
	Bifidobacterium animalis	44.151	17.127	Decrease	−0.612
	Bifidobacterium margollesii	11.169	2.237	Decrease	−0.800
	Bifidobacterium pseudolongum	13.540	3.966	Decrease	−0.707
	Lactobacillus antri	86.967	20.827	Decrease	−0.761
	Lactobacillus salivarius	133.198	42.818	Decrease	−0.679
	Dialister invisus	53.503	34.821	Decrease	−0.349
	Dialister micraerophilus	10.866	6.921	Decrease	−0.363
	Olsenella profusa	2.339	0.000	Decrease	Depletion
	Bifidobacterium magnum	5.920	0.000	Decrease	Depletion
	Lactobacillus ultunensis	21.843	6.910	Decrease	−0.684
	Bacteroides coprocola	28.988	276.174	Increase	8.527
	Bacteroides coprophilus	39.273	102.374	Increase	1.607
	Bacteroides plebeius	52.153	726.374	Increase	12.928
	Barnesiella intestinihominis	3.908	5.706	Increase	0.460
	Barnesiella viscericola	4.182	18.917	Increase	3.523
	Ruminiclostridium cellulolyticum	1.008	2.168	Increase	1.151
	Blautia hominis	1.628	7.285	Increase	3.475
	Pseudomonas stutzeri	0.000	8.414	Increase	Introduction
	Escherichia coli	6.429	8.564	Increase	0.332
	Parapedobacter indicus	0.000	5.960	Increase	Introduction
	Bacteroides gallinarum	0.000	4.249	Increase	Introduction
	Lachnospira eligens	1.756	3.889	Increase	1.215
	Alistipes senegalensis	0.915	8.152	Increase	7.909
	Alistipes inops	1.607	13.225	Increase	7.228
	Mucilaginibacter paludis	0.000	0.667	Increase	Introduction
	Roseburia faecis	4.354	9.646	Increase	1.216
	Oscillibacter valericigenes	1.970	12.747	Increase	5.470
	Desulfitobacterium hafniense	1.301	2.768	Increase	1.128
	Bacteroides ovatus	2.003	4.165	Increase	1.080
	Bacteroides salanitronis	104.866	191.057	Increase	0.822
D 64	Candidatus Amulumruptor caecigallinarius	23.038	15.528	Decrease	−0.326
	Prevotella fusca	2.846	2.311	Decrease	−0.188
	Prevotella maculosa	3.809	1.793	Decrease	−0.529
	Lactobacillus frumenti	5.893	3.116	Decrease	−0.471
	Moorella glycerini	8.683	3.599	Decrease	−0.585
	Anaerococcus prevotii	2.001	1.025	Decrease	−0.488
	Alistipes inops	28.508	10.577	Decrease	−0.629
	Bacteroides coprocola	318.147	163.107	Decrease	−0.487
	Bacteroides massiliensis	5.828	2.441	Decrease	−0.581
	Bacteroides salanitronis	140.492	139.719	Decrease	−0.006
	Prevotella scopos	3.518	3.407	Decrease	−0.032
	Clostridioides difficile	33.424	6.523	Decrease	−0.805
	Prevotella denticola	37.276	42.833	Increase	0.149
	Prevotella loescheii	3.809	16.770	Increase	3.402
	Lactobacillus agilis	11.039	22.145	Increase	1.006
	Lactobacillus hamsteri	8.541	14.052	Increase	0.645
	Desulfitobacterium dichloroeliminans	0.320	0.559	Increase	0.749
	Escherichia coli	2.534	27.117	Increase	9.699
	Coprococcus catus	0.599	12.641	Increase	20.089
	Hallella seregens	8.576	9.409	Increase	0.097
	Flavonifractor plautii	20.389	31.221	Increase	0.531
D 84	Gardnerella vaginalis	14.957	4.820	Decrease	−0.678
	Bacteroides gallinarum	10.466	4.858	Decrease	−0.536
	Prevotella bergensis	6.857	1.869	Decrease	−0.727

continued

Table 4. Continued

Day	Taxon	Control (η[1])	MRF (η)	Change	FC[2]
	Lactobacillus ingluviei	7.404	0.000	Decrease	Depletion
	Flavonifractor plautii	23.726	16.595	Decrease	−0.301
	Azospira oryzae	4.158	2.415	Decrease	−0.419
	Lactobacillus antri	37.378	5.196	Decrease	−0.861
	Lactobacillus mucosae	16.138	0.000	Decrease	Depletion
	Lachnobacterium bovis	0.279	0.000	Decrease	Depletion
	Alistipes onderdonkii	4.127	1.623	Decrease	−0.607
	Alistipes putredinis	7.984	1.607	Decrease	−0.799
	Clostridium saudiense	2.058	0.000	Decrease	Depletion
	Parapedobacter indicus	4.441	1.694	Decrease	−0.618
	Aeriscardovia aeriphila	5.895	0.000	Decrease	Depletion
	Collinsella bouchesdurhonensis	19.747	22.040	Increase	0.116
	Collinsella intestinalis	16.633	25.881	Increase	0.556
	Enorma timonensis	8.296	14.460	Increase	0.743
	Prevotella colorans	2.002	4.999	Increase	1.497
	Pontibacter ramchanderi	2.089	3.950	Increase	0.890
	Bifidobacterium pseudocatenulatum	4.247	11.350	Increase	1.673
	Pontibacter actiniarum	0.674	4.309	Increase	5.397
	Desulfofarcimen acetoxidans	2.102	2.748	Increase	0.307
	Selenomonas ruminantium	0.895	1.275	Increase	0.424
	Oxalobacter formigenes	2.089	5.372	Increase	1.571
	Chitinophaga caeni	0.000	0.719	Increase	Introduction

Data discussed in the main text are in bold.
[1]η = standardized median read counts.
[2]FC = fold change.

that were significantly enriched with MRF supplementation included *Prevotella colorans, Mucispirillum schaedleri, Oxobacter pfennigii, Clostridium hylemonae, Pontibacter ramchanderi*, and *Anaerobutyricum hallii*.

In this study dietary MRF resulted in significantly greater bacterial alpha diversity richness indices at D 16, D 32, and D 64 compared to the control. The diversity of intestinal microbiota is one of the key determinants of colonization resistance against invading pathogens and higher diversity is negatively correlated with dysbiosis (Ducatelle et al., 2015; Valdes et al., 2018; Kogut, 2019). Abiotic stressors or infection can reduce α-diversity, leading to dysbiosis (Diaz Carrasco et al., 2019; He et al., 2021). Comparatively, β-diversity metrics are also measures of health and significant differences between treatment groups at each timepoint were identified in this study indicating differences in the heterogeneity of the bacterial community compositions (Corrigan et al., 2015, 2018). The dysbiosis amelioration effect of MRF via community composition alteration and increased α-diversity observed here are in agreement with previously published studies (Corrigan et al., 2015, 2018). Increased α-diversity and lower β-diversity in broilers can be achieved using pre- and probiotics, and such strategies positively correlate with improved FCR and feed efficiency (Hooge et al., 2011; Spring et al., 2015; Al-Khalaifa et al., 2019; Jha et al., 2020). A study by Wang et al. (2020) also showed that high yield laying hens had a significantly greater alpha diversity than low yield laying hens and that the greater alpha

Table 5. Total area of bacterial phyla observed over 84-days supplementation in both control and mannan-rich fraction (MRF) supplemented layers calculated using an area-under-the-curve (AUC) model constructed from geometric mean for repeated measures at each timepoint.

Taxon	Control area	MRF area	FC[1]
Firmicutes	**388055.104**	**373588.350**	**−0.037***
Bacteroidetes	**263017.431**	**306135.598**	**0.164***
Actinobacteria	**87589.742**	**57616.859**	**−0.342***
Proteobacteria	**10570.855**	**11760.435**	**0.113***
Others	78284.533	85230.869	

*Denotes significant differences ($P \leq 0.005$) and data are emboldened for each row.
[1]FC = fold change.

Table 6. Significantly altered (increased or decreased relative to control) species ($P \leq 0.005$) observed over 84-days supplementation in both control and mannan-rich fraction (MRF) supplemented layers calculated using an area-under-the-curve (AUC) model constructed from geometric mean for repeated measures at each timepoint.

Taxon	Control area	MRF area	Change	FC[1]
Bacteroides acidifaciens	0.000	38.787	Increase	Introduction
Prevotella albensis	0.000	97.480	Increase	Introduction
Prevotella bryantii	0.000	22.030	Increase	Introduction
Prevotella pectinovora	0.000	361.937	Increase	Introduction
Prevotella pleuritidis	0.000	128.468	Increase	Introduction
Alistipes finegoldii	0.000	30.861	Increase	Introduction
Pontibacter mucosus	0.000	57.021	Increase	Introduction
Mucilaginibacter paludis	0.000	11.542	Increase	Introduction
Parapedobacter indicus	0.000	93.077	Increase	Introduction
Sphingobacterium haloxyli	0.000	53.728	Increase	Introduction
Paenibacillus polymyxa	0.000	46.965	Increase	Introduction
Enterococcus cecorum	0.000	9.782	Increase	Introduction
Lactobacillus kefiranofaciens	0.000	114.041	Increase	Introduction
Christensenella minuta	0.000	371.770	Increase	Introduction
Lactonifactor longoviformis	0.000	67.398	Increase	Introduction
Hungateiclostridium saccincola	0.000	71.458	Increase	Introduction
Thermoclostridium stercorarium	0.000	36.208	Increase	Introduction
Coprococcus catus	0.000	34.964	Increase	Introduction
Faecalicatena contorta	0.000	36.561	Increase	Introduction
Lachnospira eligens	0.000	70.895	Increase	Introduction
Murimonas intestini	0.000	215.101	Increase	Introduction
Roseburia hominis	0.000	159.821	Increase	Introduction
Roseburia intestinalis	0.000	155.842	Increase	Introduction
Tyzzerella nexilis	0.000	65.807	Increase	Introduction
Desulfitobacterium hafniense	0.000	47.753	Increase	Introduction
Desulfotomaculum hydrothermale	0.000	61.413	Increase	Introduction
Caproiciproducens galactitolivorans	0.000	94.149	Increase	Introduction
Sutterella wadsworthensis	0.000	20.852	Increase	Introduction
Candidatus Desulfovibrio trichonymphae	0.000	38.598	Increase	Introduction
Bacteroides massiliensis	213.382	0.000	Decrease	Depletion
Lentimicrobium saccharophilum	213.342	0.000	Decrease	Depletion
Prevotella amnii	34.806	0.000	Decrease	Depletion
Alistipes shahii	14.554	0.000	Decrease	Depletion
Pedobacter indicus	71.702	0.000	Decrease	Depletion
Candidatus Saccharibacteria genomosp. TM7-H1	134.551	0.000	Decrease	Depletion
Listeria monocytogenes	34.758	0.000	Decrease	Depletion
Paenibacillus lutimineralis	14.553	0.000	Decrease	Depletion
Lactobacillus equi	128.822	0.000	Decrease	Depletion
Lactobacillus equigenerosi	139.223	0.000	Decrease	Depletion
Lactobacillus ingluviei	55.886	0.000	Decrease	Depletion
Eubacterium brachy	86.211	0.000	Decrease	Depletion
Anaerofustis stercorihominis	162.340	0.000	Decrease	Depletion
Cuneatibacter caecimuris	19.781	0.000	Decrease	Depletion
Lachnotalea glycerini	31.709	0.000	Decrease	Depletion
Ethanoligenens harbinense	70.192	0.000	Decrease	Depletion
Succinispira mobilis	108.465	0.000	Decrease	Depletion
Veillonella caviae	35.709	0.000	Decrease	Depletion
Campylobacter jejuni	84.245	0.000	Decrease	Depletion
Kineothrix alysoides	107.911	7.363	Decrease	−0.932
Collinsella stercoris	193.980	13.962	Decrease	−0.928
Ruminococcus albus	223.588	18.042	Decrease	−0.919
Bifidobacterium pseudolongum	774.310	84.909	Decrease	−0.890
Lactobacillus mucosae	387.911	52.679	Decrease	−0.864
Desulfosporosinus orientis	42.370	5.988	Decrease	−0.859

continued

Table 6. Continued

Taxon	Control area	MRF area	Change	FC[1]
Paeniclostridium sordellii	1088.225	209.077	Decrease	−0.808
Anaeromassilibacillus senegalensis	288.212	63.300	Decrease	−0.780
Bifidobacterium biavatii	206.646	48.690	Decrease	−0.764
Succinatimonas hippei	1429.890	347.847	Decrease	−0.757
Lactobacillus pontis	2008.618	503.896	Decrease	−0.749
Intestinimonas massiliensis	187.830	48.181	Decrease	−0.743
Prevotella veroralis	40.915	11.178	Decrease	−0.727
Ruminococcus flavefaciens	265.118	74.986	Decrease	−0.717
Anaerococcus prevotii	79.279	25.257	Decrease	−0.681
Lactobacillus frumenti	478.919	152.829	Decrease	−0.681
Gardnerella vaginalis	1213.277	402.308	Decrease	−0.668
Enterococcus faecalis	188.908	64.154	Decrease	−0.660
Lactobacillus helveticus	8814.205	3034.996	Decrease	−0.656
Ruminiclostridium hungatei	363.104	147.891	Decrease	−0.593
Emergencia timonensis	201.303	84.048	Decrease	−0.582
Bifidobacterium margollesii	481.775	226.348	Decrease	−0.530
Pseudoflavonifractor capillosus	316.231	149.081	Decrease	−0.529
Prevotella buccae	2331.617	1165.003	Decrease	−0.500
Faecalitalea cylindroides	3060.161	1540.155	Decrease	−0.497
Prevotella copri	214.530	113.459	Decrease	−0.471
Monoglobus pectinilyticus	136.431	78.495	Decrease	−0.425
Butyricimonas virosa	138.103	80.396	Decrease	−0.418
Blautia hydrogenotrophica	481.471	284.081	Decrease	−0.410
Dialister micraerophilus	617.272	368.761	Decrease	−0.403
Candidatus Amulumruptor caecigallinarius	2225.730	1333.304	Decrease	−0.401
Bifidobacterium choerinum	3002.801	1813.406	Decrease	−0.396
Faecalicoccus pleomorphus	226.185	140.152	Decrease	−0.380
Bifidobacterium pseudocatenulatum	474.765	297.614	Decrease	−0.373
Moorella glycerini	315.932	203.857	Decrease	−0.355
Bacteroides pyogenes	112.484	72.781	Decrease	−0.353
Bifidobacterium pullorum	45870.881	30067.938	Decrease	−0.345
Bifidobacterium animalis	1769.367	1177.916	Decrease	−0.334
Lactobacillus coleohominis	231.511	155.505	Decrease	−0.328
Collinsella tanakaei	8669.180	5872.864	Decrease	−0.323
Lactobacillus reuteri	14012.440	9589.249	Decrease	−0.316
Butyricicoccus pullicaecorum	7895.742	5418.655	Decrease	−0.314
Lactobacillus johnsonii	47177.766	32657.874	Decrease	−0.308
Lactobacillus aviarius	2855.725	1988.538	Decrease	−0.304
Clostridioides difficile	1154.462	825.324	Decrease	−0.285
Lactobacillus oris	233.971	167.297	Decrease	−0.285
Bacteroides xylanisolvens	831.501	596.706	Decrease	−0.282
Lactobacillus antri	2086.944	1504.834	Decrease	−0.279
Candidatus Borkfalkia ceftriaxoniphila	1998.287	1443.216	Decrease	−0.278
Lactobacillus crispatus	46039.988	33339.786	Decrease	−0.276
Parabacteroides merdae	925.279	675.748	Decrease	−0.270
Prevotella maculosa	281.879	205.949	Decrease	−0.269
Lactobacillus hamsteri	1303.205	958.879	Decrease	−0.264
Ruminococcus bromii	1515.317	1165.744	Decrease	−0.231
Clostridium dakarense	677.417	525.252	Decrease	−0.225
Ruminococcus torques	1988.524	1633.737	Decrease	−0.178
Bacteroides uniformis	2346.372	1933.177	Decrease	−0.176
Prevotella nigrescens	631.068	530.932	Decrease	−0.159
Lactobacillus amylovorus	20791.629	17874.170	Decrease	−0.140
Bacteroides fragilis	2362.924	2039.377	Decrease	−0.137
Collinsella intestinalis	1786.705	1548.696	Decrease	−0.133
Ruminococcus lactaris	7841.722	6959.990	Decrease	−0.112

continued

Table 6. Continued

Taxon	Control area	MRF area	Change	FC[1]
Dialister pneumosintes	2758.892	2464.333	Decrease	−0.107
Collinsella bouchesdurhonensis	1896.884	1702.279	Decrease	−0.103
Prevotella rara	9558.590	8788.125	Decrease	−0.081
Lactobacillus salivarius	10312.114	9760.670	Decrease	−0.053
Faecalibacterium prausnitzii	101217.883	109838.189	Increase	0.085
Bacteroides coprophilus	6935.215	7576.654	Increase	0.092
Odoribacter splanchnicus	6068.866	6704.230	Increase	0.105
Parabacteroides distasonis	2443.770	2745.221	Increase	0.123
Prevotella baroniae	2245.221	2531.238	Increase	0.127
Blautia obeum	6328.675	7198.406	Increase	0.137
Bacteroides plebeius	40928.562	47345.356	Increase	0.157
Dorea longicatena	3292.228	3819.372	Increase	0.160
Bacteroides clarus	8518.361	10078.645	Increase	0.183
Bacteroides coprocola	17209.998	20573.462	Increase	0.195
Harryflintia acetispora	1858.817	2232.143	Increase	0.201
Megamonas hypermegale	165116.289	198521.498	Increase	0.202
Pseudoclostridium thermosuccinogenes	971.430	1178.021	Increase	0.213
Bacteroides salanitronis	10657.483	13253.030	Increase	0.244
Acetobacteroides hydrogenigenes	707.653	881.394	Increase	0.246
Muribaculum intestinale	2681.514	3413.963	Increase	0.273
Alistipes communis	944.892	1207.573	Increase	0.278
Coprococcus comes	1182.216	1533.658	Increase	0.297
Prevotella koreensis	360.063	467.897	Increase	0.299
Barnesiella viscericola	1012.062	1326.321	Increase	0.311
Clostridium leptum	479.036	653.058	Increase	0.363
Anaerotruncus colihominis	506.368	691.814	Increase	0.366
Prevotella oris	231.573	316.919	Increase	0.369
Collinsella aerofaciens	1250.062	1722.504	Increase	0.378
Enorma massiliensis	1077.727	1495.235	Increase	0.387
Prevotella bergensis	400.143	563.704	Increase	0.409
Bifidobacterium longum	730.836	1040.115	Increase	0.423
Prevotella denticola	3342.492	5060.290	Increase	0.514
Escherichia coli	664.995	1040.447	Increase	0.565
Roseburia faecis	253.208	402.314	Increase	0.589
Hallella seregens	478.446	768.807	Increase	0.607
Bacteroides thetaiotaomicron	435.082	709.597	Increase	0.631
Ilumatobacter fluminis	58.237	95.493	Increase	0.640
Lactobacillus agilis	546.011	898.110	Increase	0.645
Megamonas funiformis	13216.892	21971.690	Increase	0.662
Prevotella multiformis	140.342	233.335	Increase	0.663
Staphylococcus saprophyticus	676.283	1150.489	Increase	0.701
Faecalicatena fissicatena	553.300	961.174	Increase	0.737
Intestinimonas butyriciproducens	349.263	607.067	Increase	0.738
Alloprevotella tannerae	327.779	576.618	Increase	0.759
Bacteroides vulgatus	375.165	672.355	Increase	0.792
Prevotella dentasini	121.387	219.343	Increase	0.807
Pseudomonas stutzeri	200.732	364.268	Increase	0.815
Bacteroides sartorii	46.497	85.082	Increase	0.830
Candidatus Soleaferrea massiliensis	182.676	337.142	Increase	0.846
Clostridium methylpentosum	239.335	443.028	Increase	0.851
Petroclostridium xylanilyticum	150.217	280.980	Increase	0.870
Drancourtella massiliensis	271.598	519.029	Increase	0.911
Bifidobacterium scaligerum	125.958	247.154	Increase	0.962
Bifidobacterium thermacidophilum	32.319	64.312	Increase	0.990
Bacteroides helcogenes	59.786	121.083	Increase	1.025
Slackia equolifaciens	120.229	244.535	Increase	1.034

continued

Table 6. Continued

Taxon	Control area	MRF area	Change	FC[1]
Lactobacillus ultunensis	253.369	520.061	Increase	1.053
Blautia producta	446.790	938.508	Increase	1.101
Candidatus Cibiobacter qucibialis	557.654	1177.743	Increase	1.112
Dorea formicigenerans	853.675	1826.220	Increase	1.139
Tepidibacillus fermentans	37.504	80.452	Increase	1.145
Selenomonas ruminantium	36.127	77.886	Increase	1.156
Flavonifractor plautii	1000.128	2205.067	Increase	1.205
Bacteroides fluxus	18.931	42.965	Increase	1.270
Prevotella shahii	420.689	998.863	Increase	1.374
Coprobacter fastidiosus	159.895	380.238	Increase	1.378
Barnesiella intestinihominis	208.904	514.637	Increase	1.464
Prevotella scopos	161.126	412.800	Increase	1.562
Oscillibacter valericigenes	302.678	838.060	Increase	1.769
Hungatella hathewayi	121.173	342.010	Increase	1.822
Azospira oryzae	45.210	131.175	Increase	1.901
Alistipes senegalensis	85.171	256.174	Increase	2.008
Prevotella fusca	106.884	343.436	Increase	2.213
Prevotella loescheii	391.867	1280.695	Increase	2.268
Bacteroides zoogleoformans	99.048	366.885	Increase	2.704
Blautia coccoides	69.475	276.205	Increase	2.976
Anaerostipes hadrus	92.387	374.797	Increase	3.057
Bacteroides gallinarum	36.186	152.563	Increase	3.216
Methylohalobius crimeensis	37.510	162.258	Increase	3.326
Alistipes putredinis	21.177	101.666	Increase	3.801
Enterocloster clostridioformis	6.887	33.699	Increase	3.893
Pontibacter actiniarum	35.636	190.721	Increase	4.352
Schleiferia thermophila	119.155	647.883	Increase	4.437
Clostridium phoceensis	13.993	81.436	Increase	4.820
Anaerobutyricum hallii	51.802	316.042	Increase	5.101
Pontibacter ramchanderi	7.243	61.594	Increase	7.504
Clostridium hylemonae	29.441	274.678	Increase	8.330
Oxobacter pfennigii	22.050	228.498	Increase	9.363
Mucispirillum schaedleri	191.502	2564.850	Increase	12.393
Prevotella colorans	6.939	123.967	Increase	16.866

Data discussed in the main text are highlighted in bold.

[1]FC = fold change.

diversity indicated a stable microbiota which had an ability to recover more quickly and that lower diversity in the low yield hens indicated that the microbiome is fragile and susceptible to perturbations. Taken together these results indicate that MRF supplementation in layer diets could be beneficial to gastrointestinal health.

At the phylum level, the composition of gut microbiota was dominated by Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria, which is consistent with previous findings and show the important role of these bacteria in gut health (Khan et al., 2020; Sun et al., 2021). A significant increase at D 16 (+7.13%) and numerical increase at D 32 (+5.80%), D 64 (+3.77%), and D 84 (+0.97%) in the overall population of Bacteroidetes in the MRF supplemented flock suggests the potential beneficial impact of MRF on gut health. Phylum Bacteroidetes is comprised of numerous bacteria that primarily produce propionate and succinate in the GIT which are involved in intestinal gluconeogenesis. This phylum can also digest complex substrates, such as xylan and cellulose (Dodd et al., 2011; De Vadder et al., 2014, 2016; de León et al., 2020). Interestingly, MRF effects on Bacteroidetes have been demonstrated previously in the broiler cecum (Corrigan et al., 2015, 2018). Proteobacteria, although making up a relatively small percentage of the overall relative abundance, was also significantly affected by MRF supplementation with increases at D 16 (0.33%), D 32 (0.57%),

and D 84 (0.65%) compared to the control. Proteobacteria contain genera of opportunistic pathogens such as Campylobacter, Escherichia, Shigella, Salmonella, and Helicobacter which are of concern from a food safety perspective (Fung et al., 2018; Bantawa et al., 2019; Franz et al., 2019). However, in the current study, at the genus level the abundance levels of these bacteria were numerically lower in the MRF supplemented group at these time points when compared to the control. Further examination of changes in bacterial community composition at the species level as a result of MRF supplementation showed that for both the control and supplemented groups the most abundant bacterial species are related to *Megamonas hypermegale* and *Faecalibacterium prausnitzii*, both of which are known SCFA producers. Short-chain fatty acids that are absorbed in the cecum and colon can be used for energy and as substrates for gluconeogenesis and lipogenesis suggesting the presence of these bacteria could be beneficial for GIT health (Cui et al., 2022). Previous studies also identified these 2 genera as some of the most abundant in the cecum of layer and broiler chickens (Dong et al., 2017; Zhang et al., 2021; Aruwa et al., 2021). The impact of MRF supplementation on specific bacterial species also showed that some pathogen associated bacterial species were significantly lower compared to the control. *Campylobacter jejuni* was undetectable in the layer cecum of MRF supplemented birds at D 16 and was significantly lower compared to the control. At D 64 *Cl. difficile* was also significantly lower in the MRF supplemented layers when compared to the control. Interestingly, *Oscillibacter valerigenes*, a valerate producer, was significantly higher at D 32 (5.47 η_{FC}) and valerate has been shown to inhibit *Cl. difficile* growth (McDonald et al., 2018). When looking at the overall effect of MRF supplementation on bacterial species over the 84-days supplementation, these bacteria (*C. jejuni* and *Cl. difficile*) were again shown to be significantly lower along with *Listeria monocytogenes* and *Enterococcus faecalis*. MRF supplementation has been previously shown to have an impact on lowering the levels of *Campylobacter* spp., in the chicken cecum (Corrigan et al., 2017) while the effects of MRF on *L. monocytogenes, E. faecalis*, and *Cl. difficile* have not previously been reported. A baseline measurement of the microbiota prior to MRF supplementation in this study (Supplemental Table: Reads—Species, Abundances—Species, D 0) showed that *C. jejuni* was present in one sample at very low levels, *L. monocytogenes* was not detected, while *E. faecalis* and *C. difficile* were present in all samples tested. Given the randomized nature of the trial these results suggest that differences observed are due to MRF supplementation. These 4 bacteria are known chicken zoonotic and opportunistic pathogens and reducing their presence in or on poultry products using nonantibiotic alternatives would be beneficial from an economic and food safety standpoint of both the producer and the consumer. Due to the emergence of widespread multidrug resistance in these bacterial pathogens and difficulty in treating infectious cases it is important to identify natural nonantibiotic biological methods to limit their spread through the food chain (Łojewska and Sakowicz, 2021). As noted already, bacterial diversity of the intestinal microbiota is one of the key determinants of colonization resistance against invading pathogens. It is possible that the improved alpha and beta diversity as a result of MRF noted in this study has ameliorated dysbiosis and pathogen colonization.

Other bacteria which were affected by MRF supplementation at specific time points included *Lactobacillus agilis* which was noted to be greater at D 16 and is noted for its probiotic and anticampylobacter effects in chickens (Kobierecka et al., 2017). *Bacteroides vulgatus* was also noted to be significantly greater in MRF supplemented birds at D 16. Some strains of *B. vulgatus* are correlated with lower LPS levels and can have a protective effect on the GIT, via modulation of cytokine production and regulation of the structure of the gut microbiota (Wang et al., 2021). At D 32 two species of *Alistipes* were shown to be greater in the MRF supplemented group. *A. senegalensis* has been shown to be mannose fermenter and its increase may be related to the prebiotic effects of MRF in the diet while *A. inops* is a SCFA producer producing succinate and acetate as metabolic end products (Mishra et al., 2012; Shkoporov et al., 2015;

Parker et al., 2020). At D 64 *Coprococcus catus* was increased in MRF supplemented layers with a 20.09 η_{FC} difference and is noted for the production of propionate and butyrate (Reichardt et al., 2014; Maki et al., 2019). Further examination of bacterial species affected over the 84-day supplementation period identified numerous bacteria to be differentially abundant. Of note a number of bacteria which were only present with MRF supplementation included the SCFA producer *C. catus* along with *R. hominis, R. intestinalis*, and *C. minuta* all of which are noted as beneficial intestinal bacteria with strong SCFA producing and anti-inflammatory capabilities (Patterson et al., 2017; Kropp et al., 2021; Nie et al., 2021). Previous studies in pigs and poultry have demonstrated the ability of MRF in the diet to improve GIT health, increase SCFA production and attenuate inflammation. These results support the idea that MRF in the diet of layers could have beneficial effects on gastrointestinal health through the production of SCFA (Silva et al., 2020; Liu et al., 2021). Numerous species of the genus *Prevotella* were also noted to be unique to the MRF supplemented group compared with the control. *Prevotella* species have been correlated with plant-rich diets, abundant in carbohydrates and fibers. Many correlative studies have associated members of the genus *Prevotella* with positive outcomes in pig production, including growth performance and immune response and with lower methane emissions in ruminants (Aguilar-Marin et al., 2020).

CONCLUSIONS AND APPLICATIONS

1. This study highlighted that dietary MRF supplementation yielded significantly different bacterial diversity and composition. Increased bacterial diversity is linked strongly with microbiome resilience.
2. In this study MRF supplemented birds were noted to have lower levels of the zoonotic pathogens *Cl. difficile, C. jejuni, L. monocytogenes*, and *E. faecalis* as measured using a metagenomic sequencing approach. With reference to the global challenge of antibiotic resistance and food security, lowering pathogenic bacterial species is of particular importance for food chain integrity as well as flock health.
3. Higher relative abundances of known SCFA producing bacteria were also associated with MRF supplementation and have been noted previously in other species. Increased SCFA can be beneficial to GIT health as they are known attenuate inflammation and improve mucosal barrier integrity.
4. Nutritional interventions that can lower the levels of harmful bacteria will have a positive impact on animal health and food safety since antibiotic use has been prohibited. These results highlight a role for MRF supplementation in laying hen production.

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Ethics Approval and Consent to Participate: The accommodation and care of animals used in this study will be in accordance with Directive 2010/63/EC (EC, 2010) and European Commission Recommendation 2007/526/EC.

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DISCLOSURES

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SUPPLEMENTARY MATERIALS

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