

Modulation of Chicken Cecal Microbiota by a Phytogetic Feed Additive, Stodi®: A Metagenomic Analysis

Saravana Kumar Marimuthu, Brindhalakshmi Balasubramanian, Ramasamy Selvam, Prashanth D'Souza

Animal Health Science, R&D Centre, Natural Remedies Private Limited, Bengaluru, Karnataka, India

ABSTRACT

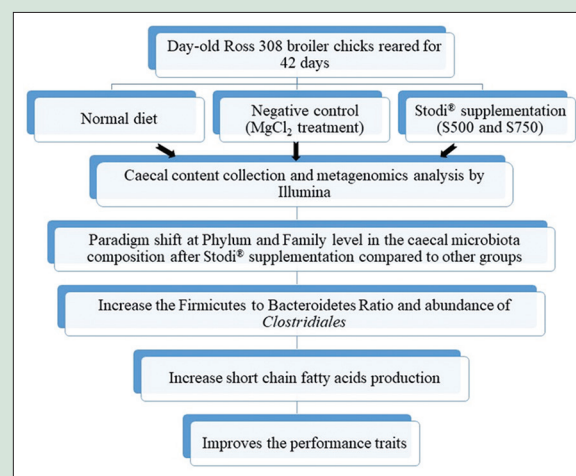
Background: The cecal microbiota plays a critical role in gut health and utilization of nutrients left undigested in the small intestine. **Objective:** The impact of Stodi® on the composition of cecal microbiota was evaluated in broilers using high-throughput sequencing of 16S rRNA gene amplicons.

Materials and Methods: A total of 960 1-day-old Ross 308 chicks were allocated to four groups, namely, normal diet (ND), negative control (NTC; challenged with 1.7% magnesium chloride [$MgCl_2$]), and Stodi® treatment groups which comprise NTC plus Stodi® (500 and 750 g/ton of feed). $MgCl_2$ was used to increase the cecal moisture content, and in turn, to disturb the cecal microbiota. **Results:** Birds challenged with $MgCl_2$ exhibited poor performance traits as compared to ND group, whereas the supplementation of Stodi® (500 and 750 g/ton) improved the performance of broilers. $MgCl_2$ did not produce a notable change in the microbiota, but supplementation of Stodi® (500 and 750 g/ton) produced a statistically significant shift in the microflora in comparison with ND. The *Firmicutes* to *Bacteroidetes* ratio was significantly elevated in comparison to ND. The abundance of energy harvesting bacteria belonging to specific families of *Lachnospiraceae* and *Ruminococcaceae* were increased by Stodi® supplementation, especially at 500 g/ton of feed. **Conclusion:** The supplementation of Stodi® was effective in modulating the cecal microbial population in a manner conducive for gut health and performance of broilers, as revealed by the increase in abundance of favorable microflora.

Key words: Cecal microbiota, gut health, magnesium chloride, metagenome, Stodi®

SUMMARY

- Supplementation of Stodi® produced a paradigm shift in cecal microbiota composition in broiler chickens challenged with $MgCl_2$ treatment
- Addition of Stodi® at 500 and 750 g/ton of feed increase the *Firmicutes* to *Bacteroidetes* ratio (FBR) and abundance of different members of order *Clostridiales* in cecal microbiota
- We speculate the observed improvement in body weight and feed conversion ratio in the Ross 308 broilers could be due to the augmentation of FBR.



Abbreviations Used: ANOVA: Analysis of variance; BW: Body weight; FBR: *Firmicutes* to *Bacteroidetes* ratio; FCR: Feed conversion ratio; ND: Normal diet; NGS: Next-Generation Sequencing techniques; NTC: Negative control; OTUs: Operational taxonomic units; PCoA: Principal coordinates analysis; S500: Stodi® (500 g/ton feed); S750: Stodi® (750 g/ton feed); SCFAs: Short-chain fatty acids; VFA: Volatile fatty acid.

Correspondence:

Mr. Prashanth D'Souza,
Animal Health Science, R&D Centre,
Natural Remedies Private Limited,
Plot 5B, Veerasandra Industrial Area,
Electronic City, Hosur Road, Bengaluru - 560 100,
Karnataka, India.
E-mail: prashanth@naturalremedy.com
DOI: 10.4103/pr.p_8_19

Access this article online

Website: www.phcogres.com

Quick Response Code:



INTRODUCTION

The ceca of the modern poultry bird have important implications on gut health and zootechnical performance.^[1] It is estimated that cecal microbial density reaches nearly 10^{11} – 10^{12} cells/g.^[2] The poultry cecal microflora is represented by three main phyla, the *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*.^[3] Several studies have shown the importance of the *Firmicutes* in the digestion of nutrients entering the ceca.^[4] It is estimated that 5%–10% of the energy from the poultry diet is extracted from cecal fermentation.^[5] The undigested nutrients and non-starch polysaccharides that reach ceca are fermented by cecal microflora, resulting in the generation of volatile fatty acids (VFAs) such as acetic acid, propionic acid, and butyric acid. These VFAs are absorbed in the gut and contribute to energy production by way of Krebs cycle.^[1]

Improvement in the cecal microflora to extract the undigested nutrients that reach the hind part of the intestine presents a great opportunity to improve the productivity of poultry birds. It creates an urgency to utilize the phytogetic feed additives (PFAs) which can optimize the gut health

and enhance the performance of poultry birds. PFA presents an unique opportunity to intervene the dynamics of cecal microflora since several herbal extracts and essential oils have been known to modulate the gut microflora. The blend of oregano, rosemary, and fennel volatile oils in diets was reported to augment the growth and to improve the intestinal microbial balance by a reduction of coliform bacteria and an increase in *Lactobacillus* spp. counts of broiler chickens.^[6] The researchers also observed a drastic decrease in genus *Bacteroides*, while certain members

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

Cite this article as: Marimuthu SK, Balasubramanian B, Selvam R, D'Souza P. Modulation of chicken cecal microbiota by a phytogetic feed additive, Stodi®: A metagenomic analysis. *Phcog Res* 2019;11:201-9.

of order *Clostridiales* were increased in the cecal microbiota of chickens supplemented diets with tannins.^[7] Members belonging to *Clostridia* have been associated with an improvement of intestinal health and feed efficiency in poultry.^[2] However, most of the published studies that evaluated the influence of PFAs on gut microflora of poultry birds have utilized culture methods of identification of few selective organisms by genetic methods. These traditional methods can only reveal a part of the microflora, and there is a paucity of literature indicating the utilization of the latest tools of metagenomics such as next-generation sequencing techniques (NGS). NGS will unravel the microbiota of the gut in a comprehensive way by identifying the various communities of bacteria, some of which are not culturable in the laboratory environment.

Stodi® is a blend of several Indian medicinal plants traditionally known and being used for gut health and contains fruit rinds of *Punica granatum*, aerial parts of *Andrographis paniculata*, bark of *Acacia nilotica*, fruits of *Terminalia bellirica*, and bark of *Holarrhena antidysenterica*. We hypothesize the Stodi® will impact the bacterial communities in the cecum favorably for gut health to improve the performance parameters in broilers. Hence, the present study was undertaken and designed to evaluate the effect of Stodi® on cecal microflora composition in broilers using high-throughput sequencing of 16S rRNA gene amplicons. We have employed a broiler model of challenging birds with magnesium chloride hexahydrate which is known to disturb the moisture level in the cecal contents.^[8]

MATERIALS AND METHODS

Stodi®

Stodi® is a proprietary polyherbal formulation developed by M/s. Natural Remedies Private Limited, Bengaluru, India, containing rinds of *P. granatum*, aerial parts of *A. paniculata*, bark of *A. nilotica*, fruits of *T. bellirica*, and bark of *H. antidysenterica*.

Study design

An experiment was conducted in Poultry Research Station, recognized by the Department of Scientific and Industrial Research, India; DSIR Reg No.: TU/IV-RD/2000/2016, located in Anniyalam, Tamil Nadu, India, for a period of 42 days. The chicks were housed in a semi-closed house divided into pens with floor space of 60 square feet. The approximate size of the individual pen was 6' × 10' × 5' (length × width × height). Each pen was equipped with a brooder, a bell drinker, a chick feeder, and/or jumbo feeder. The size and floor space of the pen were modified according to the number of chicks housed with the help of a polyvinyl chloride sheet. The chicks were provided with poultry mash feed *ad libitum*^[9] manufactured by Higain Feeds and Farms India Pvt. Ltd., Bengaluru (prestarter feed with 3000 kcal metabolizable energy/kg and 21% crude protein from days 1 to 10, starter feed with 3125 kcal metabolizable energy/kg and 20% crude protein from days 11 to 24 and finisher feed with 3150 kcal metabolizable energy/kg and 18.5% crude protein from days 25 to 42). A total of 960 one-day-old broiler chicks (Ross 308) purchased from Kavi Protein and Feed Pvt. Ltd., Bengaluru, were allocated equally into four groups (6 replicates/group; 40 birds/replicate) consisting of a normal diet (ND), negative control (NTC) (challenged with 1.7% magnesium chloride hexahydrate [$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$]), and two treatment groups of Stodi® (S500–500 g/ton and S750–750 g/ton). NTC and treatment groups were challenged with $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ to increase the cecal moisture content as magnesium chloride is known to decrease the reabsorption of water in the cecum and subsequently to disturb the cecal microbiota. All birds appeared healthy throughout the experimental period and were individually weighed on days 1 and 42. The body weight gain and the feed conversion ratio (FCR: ratio of feed intake/body weight) were calculated on day 42. Ten chickens per group were randomly selected on day 43 and sacrificed by jugular vein exsanguination. The cecal samples

were collected in Petri plates and the cecal consistency was assessed and scored as per Table 1.

Sample collection and DNA extraction

Ten chickens per group were randomly selected on day 43 and sacrificed by jugular vein exsanguination. The cecal contents were aseptically collected in the DNA/RNA Shield™ Fecal Storage Tube (Zymo Research, CA, USA) and stored at room temperature until DNA extraction. Total DNA was isolated from cecal contents using DNeasy® Blood and Tissue kit (Qiagen, Valencia, CA, USA) as per the manufacturer instructions. DNA concentration and quality were assessed in NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, DE, USA). DNA was stored at –80°C until further analysis.

16S rRNA gene library preparation and high-throughput sequencing

The 16S rRNA gene V3-V4 regions were amplified using Illumina primers (forward 5'-AATGATACGGCGACCACCGAGATCTACA C[i5]TCGTCGGCAGCGTC-3'; reverse 5'-CAAGCAGAAGACGGCAT ACGAGAT[i7]GTCTCGTGGGCTCGG-3') with standard adapter sequences attached for barcoding and multiplexing. Nextera DNA libraries were made for each of the five pooled samples per group (two samples per pool in *equimolar* concentrations) separately using Nextera™ DNA Library Preparation Kit and Nextera™ Index Kit (Illumina, San Diego, CA, USA) as per the manufacturer instructions. Paired-end sequencing (2 bp × 300 bp) was conducted on an Illumina MiSeq 500 sequencing platform.

Microbial community analysis

The Illumina paired-end V3-V4 reads (2 bp × 300 bp) were demultiplexed using bcl2fastq1 tool and quality checked using FastQC.^[10] The raw reads having primer sequence and high-quality bases were selected and further stitched using Fastq-join3. Microbial composition and diversity were analyzed in the stitched reads using Quantitative Insights into Microbial Ecology (QIIME)^[11] pipeline. Open-reference Operational Taxonomic Units (OTUs) picking was performed using UCLUST^[12] method against a curated chimera free 16S rRNA database (Greengenes^[13] v 13.8). The taxonomies were assigned using RDP^[14] classifier to these clusters at ≥ 97% sequence similarity against the reference database which resulted in the generation of a biom file. The biom was taken ahead for further advance analysis and visualization. To standardize sequence counts across samples, 130,000 sequences were selected per sample (rarefaction) and used as a basis to compare abundances of OTUs across samples. Alpha diversity was calculated by observed OTUs, Shannon Index, Simpson Index, and Chao Index. Principal coordinates analysis (PCoA) plot were computed based on weighted and unweighted UniFrac distances. This method is a beta diversity measure, termed as pair-wise analysis, that considers the phylogenetic divergence among OTUs to identify differences in the overall microbial community structure between the samples.

Statistical analysis

The relative abundances of bacterial populations were analyzed using GraphPad Prism Software, CA, USA.^[15] Relative abundances at each level

Table 1: Cecal consistency assessment score

Score	Severity of diarrhea
1	Normal (semi-solid and well formed)
2	Sticky droppings
3	Mild diarrhea (fluid feces and not well formed)
4	Severe diarrhea (watery and projectile)

of classification (phylum, class, order, family, and genus) were compared by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test and growth performance parameters was compared by one-way ANOVA followed by Least Significant Difference test, which were considered statistically significant when $P < 0.05$. Krona charts were generated using Krona tools^[16] which gives the quantitative phylogenetic information for all the samples. PCoA is constructed using QIIME^[11] pipeline.

RESULTS

Performance traits

All chicks were healthy before initiation of the experiment. The addition of magnesium chloride caused a significant reduction in body weight ($P < 0.05$) and worsened the FCR in NTC group on day 42. However, dietary addition of Stodi® (500 and 750 g/ton) improved the body weight and FCR on day 42 when compared to NTC group. The cecal consistency score was significantly increased in NTC as compared to ND, whereas S500 and S750 decreased the cecal consistency score as compared to NTC [Table 2].

Sequencing data

A total of 8,690,663 paired-end reads were obtained from 20 pooled cecal samples. After quality filtering and removal of chimeric reads, the average clean tags obtained for the ND, NTC, S500, and S750 were 228606, 235133, 188960 and 297959 respectively. The average number of reads per cecal sample was 425836. An average of 952 OTUs with abundance $>0.005\%$ were obtained from all samples. At the phylum level, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* accounted for more than 85% of cecal microbiota, in which *Firmicutes* (40%–65%) and *Bacteroidetes* (20%–35%) were the predominant microbes followed by *Proteobacteria* (9%–14%) in all the sequences. *Firmicutes* alone was represented by eight genera, but only *Oscillospira*, *Faecalibacterium*, and two unclassified genera each represented more than 5% of all the sequences of this phylum. Other genera included *Megamonas*, *Ruminococcus* and two unclassified genera represented by more than 1% of the total bacterial sequences. *Bacteroides* was the most predominant genus, accounting for 50%–80% of the sequences in the phylum *Bacteroidetes*. Other relatively predominant genera in this phylum included *Parabacteroides* and *Alistipes*. *Campylobacter*, *Helicobacter*, *Sutterella* and *Desulfovibrio* were the most predominant genera in the phylum *Proteobacteria*. Furthermore, Krona chart is an interactive plot used to explore the relative abundances and confidences within the complex hierarchies of metagenomes classifications. In the present study, Stodi® treatment produces an impact on percentage of *Firmicutes* and *Bacteroidetes* ratio which was presented visually in one representative Krona chart [Figure 1]. In addition, stacked column plots were generated for the top 26 unique classified organisms identified at phylum, family, genus, and species taxonomic level using relative abundance values for all the samples [Figure 2].

Table 2: Effect of Stodi® on performance traits and cecal consistency (mean±standard error of mean)

Group	Body weight (g) (n=240)	FCR (n=6)	Cecal consistency score (n=6)
Normal diet	^{bc} 2330.25±24.16	^a 1.954±0.02	^a 1.00±0.00
Negative control	^a 2216.27±29.28	^a 1.994±0.03	^b 4.00±0.00
Stodi® (500 g/ton)	^{bc} 2303.43±29.10	^a 1.964±0.01	^b 3.50±0.34
Stodi® (750 g/ton)	^{bc} 2301.25±27.32	^a 1.957±0.02	^b 3.17±0.40

^{a-c}Means bearing different superscripts were significantly different ($P < 0.05$).
FCR: Feed conversion ratio

Alpha diversity

Internal sample alpha diversity was estimated through the number of observed species, Simpson, Chao, and Shannon indices (diversity). There was no statistical difference between the groups with respect to the number of observed species, estimated richness (Chao), or diversity (Simpson and Shannon indices) [Figure 3]. Rarefaction curves of observed species reached a plateau in all samples, indicating that the read depth was sufficient to cover the microbial diversity in poultry cecal samples [Figure 4].

Beta diversity

PCoA based on weighted and unweighted UniFrac distances was performed to determine the formation of clusters according to the treatments [Figures 5 and 6]. Although sample overlapping occurred between the four sample groups, clustering tendencies were observed, suggesting that the samples of each treatment inclined to form separate series, indicating the S500 and S750 differentially modulate the cecal microbiota in comparison with NTC and ND groups.

Effects of Stodi® on composition of cecal microbiota

In S500 and S750 groups, the *Firmicutes* in the cecum was more abundant ($P < 0.05$), while the *Bacteroidetes* was less abundant ($P < 0.05$) as compared to ND group. In addition, *Bacteroidetes* in the cecum was less abundant ($P < 0.05$) in S500 group as compared to NTC group. The ratio of the two-predominant phyla, *Firmicutes* and *Bacteroidetes* (FBR), did not differ significantly between the groups ($P > 0.05$). However, FBR was numerically improved in both S500 ($P < 0.05$ vs. ND only) and S750 group as compared to ND and NTC groups [Figure 7].

At the class level, *Clostridia* ($P < 0.05$ vs. ND only) and *Bacilli* were relatively more abundant in S500 and S750 as compared to ND and NTC groups, whereas *Erysipelotrichi* was relatively more abundant in S500 and S750 as compared to ND group [Figure 7].

At the family level, the abundance of *Ruminococcaceae* was increased in S500 ($P < 0.05$) and S750 as compared to ND group, whereas the abundance of *Lachnospiraceae* was increased in S500 ($P < 0.05$) and S750 as compared to ND and NTC groups. Similarly, the abundance of *Veillonellaceae* was greater in S500 and S750 than NTC group [Figure 8].

At the genus level, S500 and S750 showed a higher abundance of *Oscillospira* as compared to ND ($P < 0.05$) and NTC groups. Similarly, the abundance of *Megamonas* was numerically improved in S500 and S750 groups as compared to ND and NTC groups. At the species level, *Faecalibacterium prausnitzii* was relatively more abundant in S500 and S750 as compared to NTC group [Figure 8].

DISCUSSION

There is a constant pressure to upgrade the productivity of poultry birds to compensate for the increasing feed costs and bird management practices. The gastrointestinal tract of broiler chickens occupies a pivotal role in utilizing the nutrients effectively for growth and contributing to the productivity of the poultry. Specifically, the cecal microbiota of modern poultry birds plays a critical role by harvesting the energy from the diet. The traditional culture-based techniques enable the recovery of microbial isolates for characterization under both anaerobic and aerobic conditions. However, $<60\%$ of the bacterial population of the poultry intestine are estimated to be cultivable under standard laboratory conditions.^[2] Hence, the complexity of the gut microflora has been fairly understood by utilizing the latest techniques of molecular biology, namely, NGS techniques. In the present study, the NGS revealed a wide variety of microorganisms in the cecum of the Ross 308 broiler chickens and the impact of a polyherbal formulation, Stodi® on cecal microbiome was evaluated.

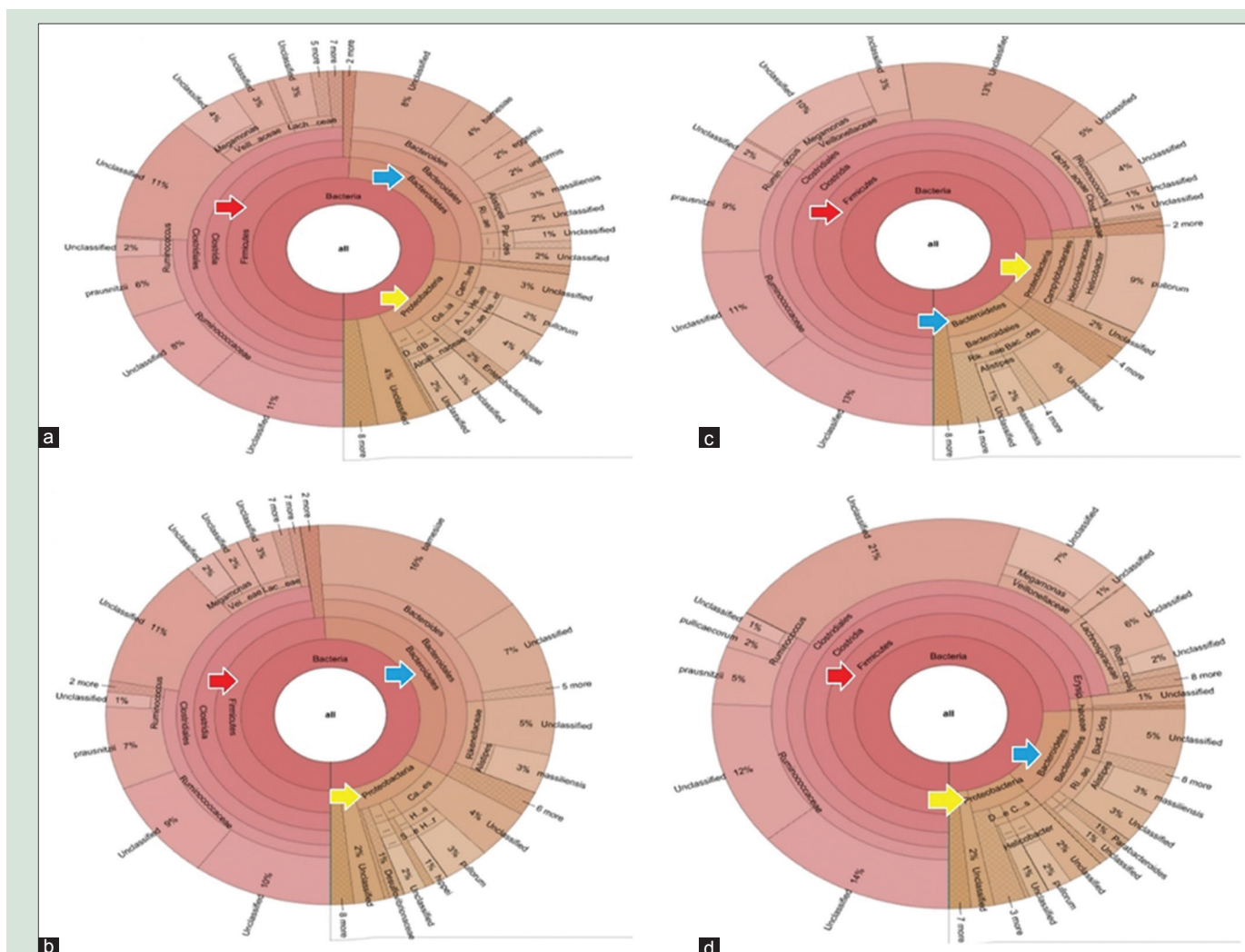


Figure 1: Krona chart of one representative sample from each group. (a) Normal diet, (b) Negative control, (c) Stodi® (S500), (d) Stodi® (S750). Red arrow indicates *Firmicutes*, blue indicates *Bacteroidetes*, and yellow indicates *Proteobacteria*

The results of the present study revealed three important phyla in the ceca, namely, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* in the broiler birds and were in accordance with the previous studies.^[4,17] Moreover, the FBR was elevated upon supplementation with Stodi® at both doses but significantly at S500 (500 g/ton of feed) and is positively correlating with improved body weight and FCR of broiler birds. A wide variety of growth promoters increase the FBR, for example, antibiotic growth promoters,^[3] plant extracts,^[18] and prebiotics.^[19] The effect of polyphenolic compounds from blueberry and blackberry have been shown to improve the performance and FBR in the ceca of broilers.^[18] The Stodi® contains phytoconstituents such as polyphenols (gallic acid, catechins, punicalagins from *P. granatum*, *T. bellirica*, *A. nilotica*, and *H. antidysenterica*) and diterpenoid lactone (andrographolide from *A. paniculata*). The observed improvement in FBR and performance could be attributed to the polyphenolic constituents of Stodi®.

The predominant phylum increased upon Stodi® supplementation was *Firmicutes*. These organisms play a vital role in fermentation of dietary substrates, transporting many substances across cellular membranes. It has been suggested that *Firmicutes* dense ceca increases the capacity of the host to extract energy from the diet, with carbohydrate utilization being the important key element.^[4] The cecal *Firmicutes* are known to be specifically involved in the production of butyrate as well.^[20] The

Firmicutes are predominantly represented by three classes, namely, *Clostridia*, *Erysipelotrichi*, and *Bacilli*. The most abundant class that was represented by all treatments were *Clostridia* which was significantly increased by both doses of Stodi®. The genus *Clostridium* is reputed in the poultry industry, mainly because of a harmful species, *Clostridium perfringens*. It is important to note that *Clostridium* is just one of the 123 genera belonging to the order *Clostridiales* under the family *Clostridia*. Furthermore, the species *C. perfringens* is only remotely related to the 129 species within the genus *Clostridium*. Most of the *Clostridia* are very beneficial to gut health and are associated with economic impact.^[2]

The family *Ruminococcaceae* under the order *Clostridiales* was significantly increased by Stodi® at 500 g/ton of feed. This family is involved in fostering the performance of the broiler birds mainly by the way of production of short-chain fatty acids (SCFAs) and degradation of plant materials.^[21] Similarly, the family *Lachnospiraceae* was increased by Stodi® at a dose of 500 g/ton. The members of *Lachnospiraceae* are correlated with increased energy production by releasing SCFAs and improving gut health.^[21]

Stodi® had an interesting impact on the genus *Oscillospira*. Both doses of Stodi®, dose dependently increased the abundance of this genus in a statistically significant manner. *Oscillospira*, a genus under the family

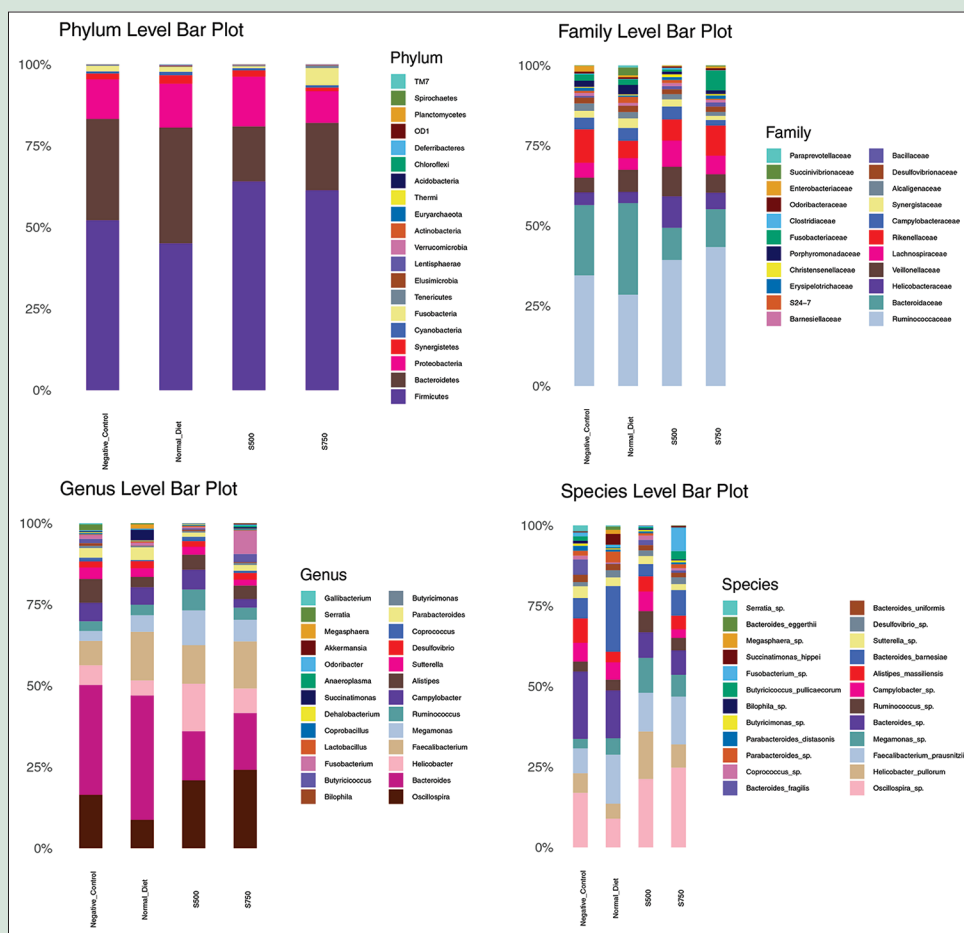


Figure 2: Stacked column plot showing the relative abundance of Phyla and family distribution

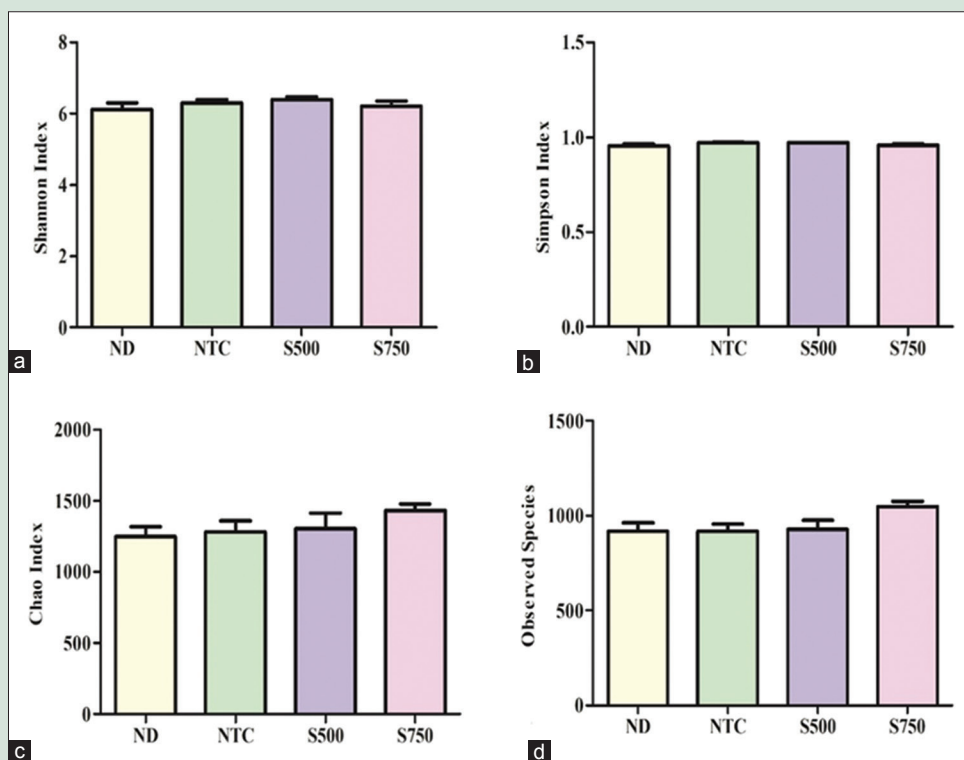


Figure 3: Effect of Stodi® supplementation on (a) Shannon Index, (b) Simpson Index, (c) Chao Index, (d) number of observed species (mean $\pm$ standard error of the mean)

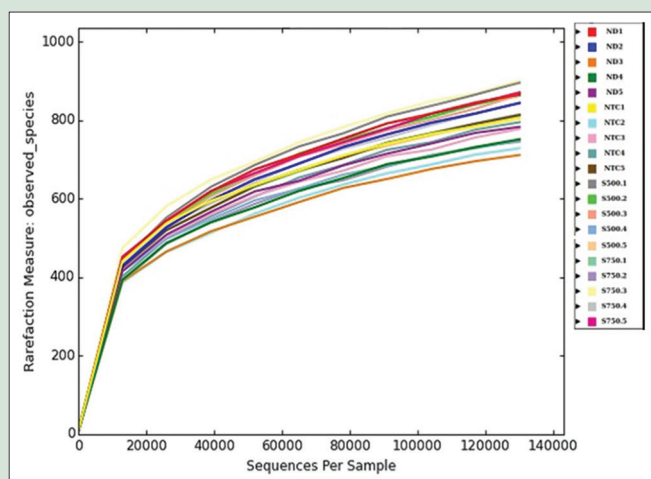


Figure 4: Rarefaction curves of number of observed species obtained based on 16S rRNA gene V3-V4 sequences. Each curve corresponds to a single pooled of two cecal samples

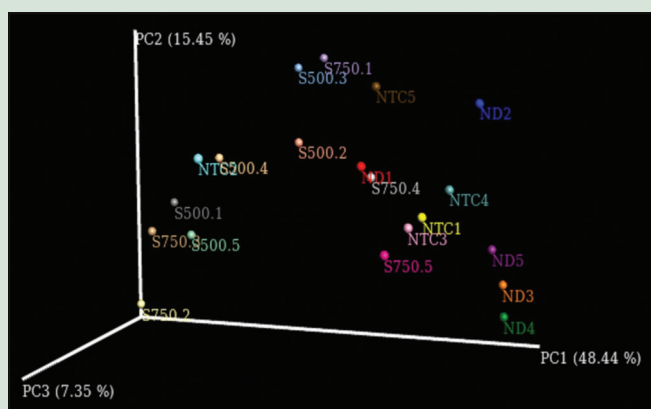


Figure 5: Principal coordinates analysis based on weighted UniFrac distance matrix of 4 groups. The axes represent the percentage of data explained by each coordinate dimension. ND: Normal diet; NTC: Negative control group; S500: Stodi® (500 g/ton); S750: Stodi® (750 g/ton)

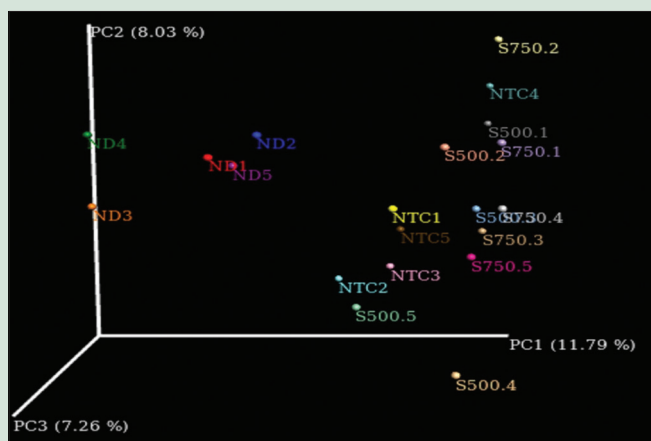


Figure 6: Principal coordinates analysis based on unweighted UniFrac distance matrix of four groups. The axes represent the percentage of data explained by each coordinate dimension. ND: Normal diet; NTC: Negative control group; S500: Stodi® (500 g/ton); S750: Stodi® (750 g/ton)

Ruminococcaceae, is an important organism in the ceca for energy metabolism and is involved in the fermentation of various mono- and oligo-saccharides, which results in the generation of SCFAs that contributes to the energy production^[22] and promotion of gut health as well. Stodi® did not produce significant impact on *Proteobacteria* although there was nonsignificant reduction in abundance of this phylum at the dose of 750 g/ton of feed. There was also a reduction in family *Enterobacteriaceae* upon Stodi® supplementation at both doses, although this was not statistically significant.

The beta-diversity analysis revealed that the Stodi® treatment produced a distinct microbiota shift in comparison with ND and NTC. However, the beta-diversity between the treatments of two doses of Stodi® was small, indicating that both doses produce a shift in the microflora in analogous manner. In general, the digestion in the fore and midgut influences the microflora of the hindgut.^[2] Furthermore, the type and quantity of nutrients reaching the hindgut and ceca would have been influenced by Stodi® supplementation, as the different ingredients of Stodi® have been reported to have antibacterial effects [Table 3]. Andrographolide, being one of the active constituents of Stodi®, has been reported to improve the activity of brush border enzymes such as maltase and sucrose.^[32] These effects would have impacted the metagenomic profile of ceca of Stodi®-treated groups.

The findings of the present study also indicate, for the first time, the PFA Stodi® produces a shift in the cecal microbiota favorable for better gut health and improves the FBR and subsequently the body weight and FCR in broiler chickens. The improvement in the *Firmicutes* abundance caused by Stodi® could have resulted in better energy extraction from the utilization of carbohydrates and butyrate production. The improvement in *Ruminococcaceae* and *Lachnospiraceae* by Stodi® could have resulted in better fermentation of various mono- and oligo-saccharides, resulting in the generation of SCFAs and contributing to the energy production and promotion of gut health and better performance traits in broiler chickens.

Table 3: Effect of Stodi® ingredients on antibacterial activity

Ingredients	Organism	References
<i>A. paniculata</i>	<i>E. coli</i> , <i>S. sonnei</i> , <i>S. typhi</i> , <i>V. cholera</i> , <i>S. aureus</i> , <i>S. boydii</i> , <i>B. licheniformis</i> , <i>S. typhimurium</i> , <i>V. alginolyteus</i> , <i>B. subtilis</i> , <i>P. aeruginosa</i> , <i>C. albicans</i>	[23,24]
<i>T. bellirica</i>	<i>E. coli</i> , <i>S. typhi</i> , <i>S. aureus</i> , <i>S. typhimurium</i> , <i>P. aeruginosa</i> , <i>C. albicans</i> , <i>S. pneumoniae</i> , <i>Y. enterocolitica</i>	[25]
<i>H. antidysenterica</i>	<i>E. coli</i> , <i>S. flexneri</i> , <i>V. cholera</i> , <i>S. aureus</i> , <i>S. boydii</i> , <i>B. licheniformis</i> , <i>S. typhimurium</i> , <i>P. aeruginosa</i> , <i>S. enteritidis</i>	[26,27]
<i>P. granatum</i>	<i>L. monocytogenes</i> , <i>S. aureus</i> , <i>E. coli</i> and <i>Y. enterocolitica</i> , <i>P. vulgaris</i> , <i>B. subtilis</i>	[28,29]
<i>A. nilotica</i>	<i>S. aureus</i> , <i>E. coli</i> , <i>P. vulgaris</i> , <i>K. pneumoniae</i> , <i>P. aeruginosa</i> , <i>C. albicans</i> , <i>S. pneumoniae</i> , <i>H. influenzae</i>	[30,31]

E. coli: *Escherichia coli*; *S. sonnei*: *Shigella sonnei*; *S. typhi*: *Salmonella typhi*; *V. cholera*: *Vibrio cholera*; *S. aureus*: *Staphylococcus aureus*; *S. boydii*: *Shigella boydii*; *B. licheniformis*: *Bacillus licheniformis*; *S. typhimurium*: *Salmonella typhimurium*; *V. alginolyteus*: *Vibrio alginolyteus*; *B. subtilis*: *Bacillus subtilis*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *C. albicans*: *Candida albicans*; *S. pneumoniae*: *Streptococcus pneumoniae*; *Y. enterocolitica*: *Yersinia enterocolitica*; *S. flexneri*: *Shigella flexneri*; *S. enteritidis*: *Salmonella enteritidis*; *L. monocytogenes*: *Listeria monocytogenes*; *Proteus vulgaris*; *K. pneumoniae*: *Klebsiella pneumoniae*; *S. pneumoniae*: *Salmonella pneumoniae*; *H. influenzae*: *Hemophilus influenzae*; *A. paniculata*: *Andrographis paniculata*; *T. bellirica*: *Terminalia bellirica*; *H. antidysenterica*: *Holarrhena antidysenterica*; *P. granatum*: *Punica granatum*; *A. nilotica*: *Acacia nilotica*

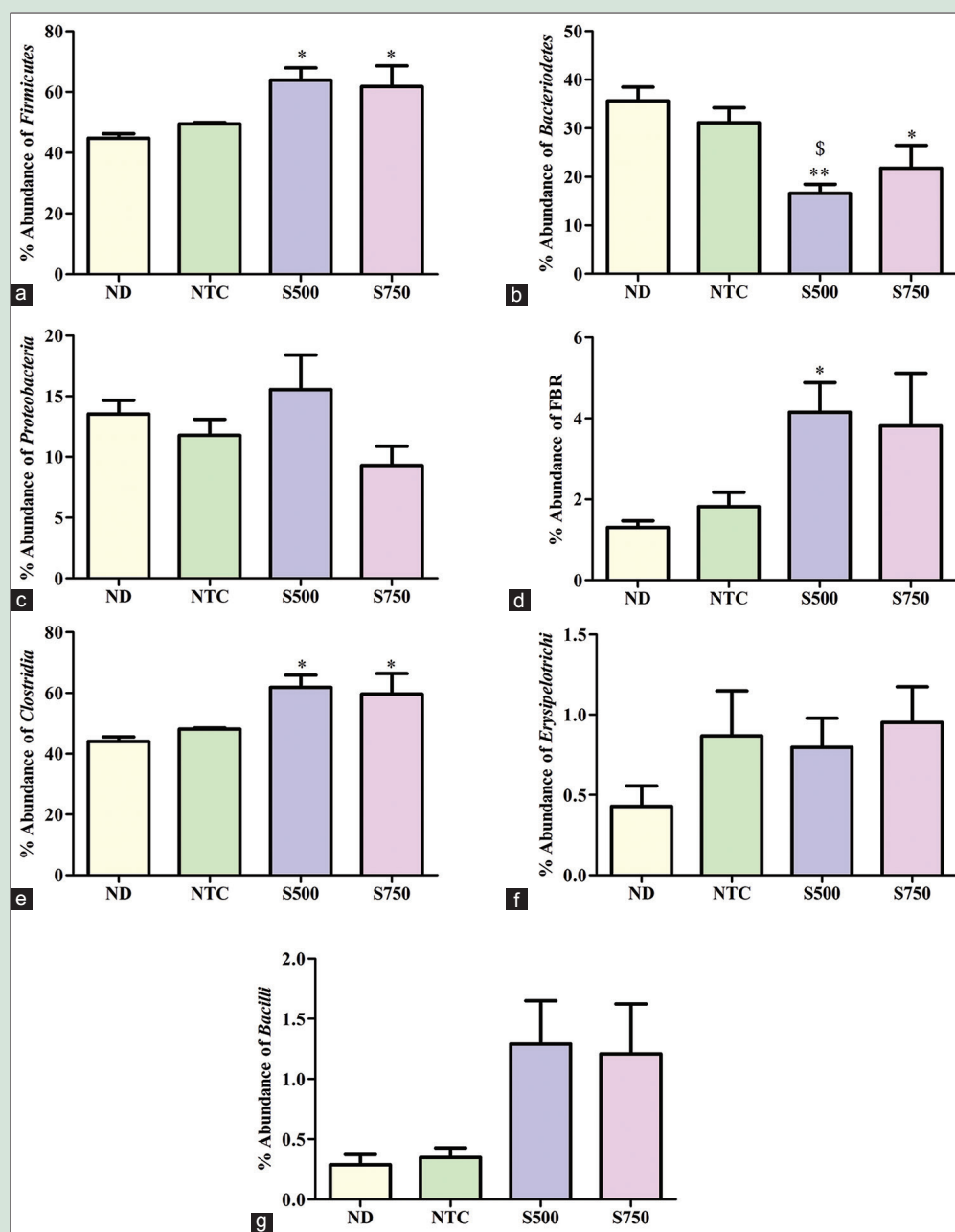


Figure 7: Effect of Stodi® on caecal microbiota (mean±SEM) ($n = 5$). ND: Normal diet; NTC: Negative control group; S500: Stodi® (500 g/ton); S750 - Stodi® (750 g/ton). * $P < 0.05$ and ** $P < 0.01$, and $^{\$}P < 0.05$ indicates significant different from ND and NTC respectively. (a) Firmicutes, (b) Bacteroidetes, (c) Proteobacteria, (d) Firmicutes to Bacteroidetes ratio (FBR), (e) Clostridia, (f) Erysipelotrichi, (g) Bacilli

CONCLUSION

The supplementation of Stodi® in the diet of broilers produced a shift in the cecal microbiota conducive for the gut health and better performance characteristics. Stodi® caused a favorable improvement in *Firmicutes*, specifically *Clostridiales* and more specifically *Ruminococcaceae* and *Lachnospiraceae*, and subsequent better utilization of carbohydrates and production of SCFA's contributing to energy production and gut health.

Acknowledgements

The authors are thankful to the Managing Director, M/s. Natural Remedies Private Limited, Bengaluru, India, for providing facilities and infrastructure to carry out the study. The authors gratefully thank all of the staff at the Genotypic Technology Pvt. Ltd., for metagenomic analysis.

Financial support and sponsorship

Nil.

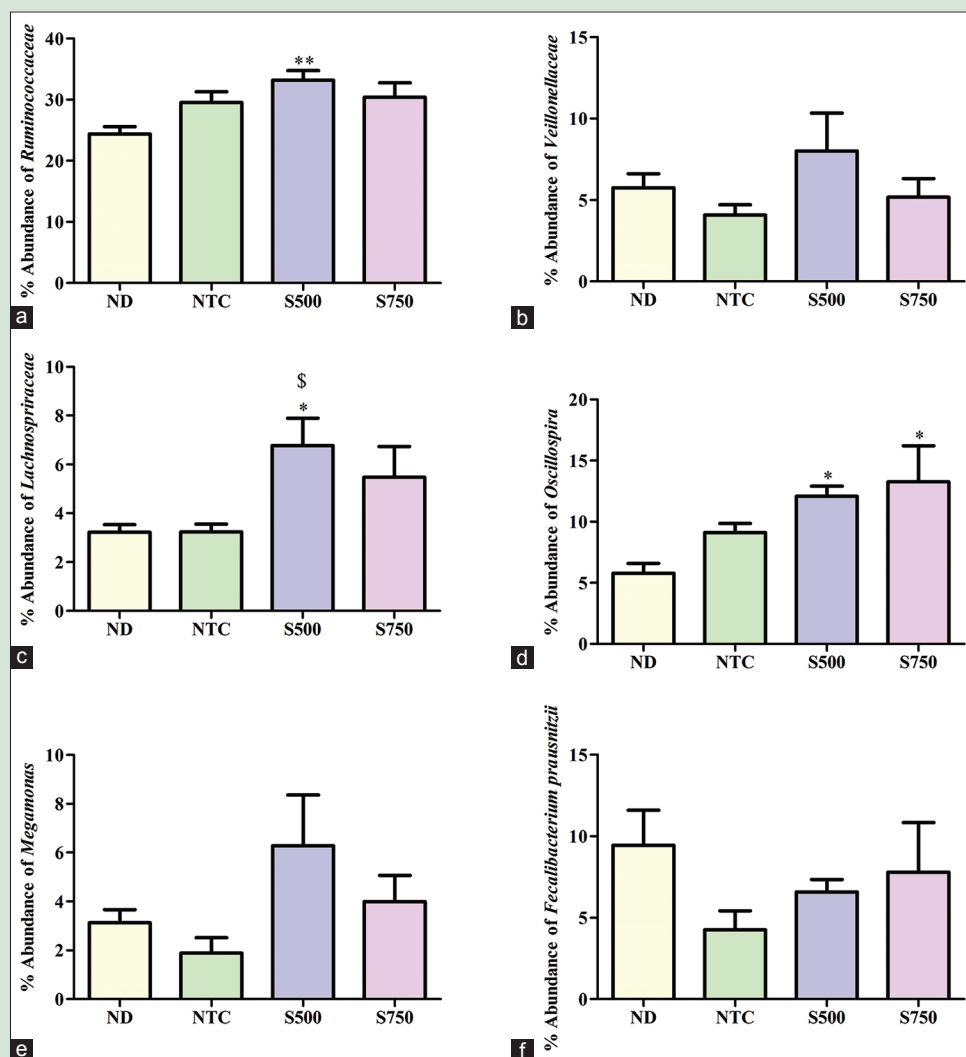


Figure 8: Effect of Stodi® on caecal microbiota (mean $\pm$ SEM) ($n = 5$). ND: Normal diet; NTC: Negative control group; S500 - Stodi® (500 g/ton); S750 - Stodi® (750 g/ton). ** $P < 0.05$ and * $P < 0.01$, and $^{\$}P < 0.05$ indicates significant different from ND and NTC respectively. (a) *Ruminococcaceae*, (b) *Veillonellaceae*, (c) *Lachnospiraceae*, (d) *Oscillospira*, (e) *Megamonas*, (f) *Faecalibacterium prausnitzii*

Conflicts of interest

There are no conflicts of interest.

REFERENCES

- Svihus B, Choct M, Classen HL. Function and nutritional roles of the avian caeca: A review. *Worlds Poult Sci J* 2013;69:249-64.
- Rinttilä T, Apajalahti J. Intestinal microbiota and metabolites – Implications for broiler chicken health and performance. *J Appl Poult Res* 2013;22:647-58.
- Costa MC, Bessegatto JA, Alfieri AA, Weese JS, Filho JA, Oba A. Different antibiotic growth promoters induce specific changes in the cecal microbiota membership of broiler chicken. *PLoS One* 2017;12:e0171642.
- Corrigan A, de Leeuw M, Penaud-Frézet S, Dimova D, Murphy RA. Phylogenetic and functional alterations in bacterial community compositions in broiler ceca as a result of mannan oligosaccharide supplementation. *Appl Environ Microbiol* 2015;81:3460-70.
- Sergeant MJ, Constantinidou C, Cogan TA, Bedford MR, Penn CW, Pallen MJ. Extensive microbial and functional diversity within the chicken cecal microbiome. *PLoS One* 2014;9:e91941.
- Cetin E, Yibar A, Yesilbag D, Cetin I, Cengiz SS. The effect of volatile oil mixtures on the performance and ileo-caecal microflora of broiler chickens. *Br Poult Sci* 2016;57:780-7.
- Díaz Carrasco JM, Redondo EA, Pin Viso ND, Redondo LM, Farber MD, Fernández Miyakawa ME, *et al.* Tannins and bacitracin differentially modulate gut microbiota of broiler chickens. *Biomed Res Int* 2018;2018:1879168.
- van der Hoeven-Hangoor E, van der Vossen JM, Schuren FH, Verstegen MW, de Oliveira JE, Montijn RC, *et al.* Ileal microbiota composition of broilers fed various commercial diet compositions. *Poult Sci* 2013;92:2713-23.
- National Research Council. *Nutrient Requirement of Poultry*. 9th revised ed. Washington, DC, USA: National Academy Press; 1994.
- Andrews S. FastQC: a Quality Control Tool for High Throughput Sequence Data; 2010. Available from: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>. [Last accessed on 2018 Sep 04].
- Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010;7:335-6.
- Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010;26:2460-1.
- DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, *et al.* Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* 2006;72:5069-72.
- Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 2007;73:5261-7.
- GraphPad Software. *GraphPad Prism Version 5.00 for Windows*. San Diego, CA, USA: GraphPad Software; 2003.
- Ondov BD, Bergman NH, Phillippy AM. Interactive metagenomic visualization in a web browser. *BMC Bioinformatics* 2011;12:385.
- Torok VA, Hughes RJ, Mikkelsen LL, Perez-Maldonado R, Balding K, MacAlpine R,

- et al.* Identification and characterization of potential performance-related gut microbiotas in broiler chickens across various feeding trials. *Appl Environ Microbiol* 2011;77:5868-78.
18. Salaheen S, Kim SW, Haley BJ, Van Kessel JA, Biswas D. Alternative growth promoters modulate broiler gut microbiome and enhance body weight gain. *Front Microbiol* 2017;8:2088.
 19. Choi KY, Lee TK, Sul WJ. Metagenomic analysis of chicken gut microbiota for improving metabolism and health of chickens – A review. *Asian-Australas J Anim Sci* 2015;28:1217-25.
 20. Onrust L, Ducatelle R, Van Driessche K, De Maesschalck C, Vermeulen K, Haesebrouck F, *et al.* Steering endogenous butyrate production in the intestinal tract of broilers as a tool to improve gut health. *Front Vet Sci* 2015;2:75.
 21. Stanley D, Hughes RJ, Geier MS, Moore RJ. Bacteria within the gastrointestinal tract microbiota correlated with improved growth and feed conversion: Challenges presented for the identification of performance enhancing probiotic bacteria. *Front Microbiol* 2016;7:187.
 22. Jozefiak D, Rutkowski A, Fratzak M, Boros D. The effect of dietary fibre fractions from different cereals and microbial enzyme supplementation on performance, ileal viscosity and short-chain fatty acid concentrations in the caeca of broiler chickens. *J Anim Feed Sci* 2004;13:487-96.
 23. Singha PK, Roy S, Dey S. Antimicrobial activity of *Andrographis paniculata*. *Fitoterapia* 2003;74:692-4.
 24. Mishra US, Mishra A, Kumari R, Murthy PN, Naik BS. Antibacterial activity of ethanol extract of *Andrographis paniculata*. *Indian J Pharm Sci* 2009;71:436-8.
 25. Elizabeth KM. Antimicrobial activity of *Terminalia bellerica*. *Indian J Clin Biochem* 2005;20:150-3.
 26. Kavitha D, Shilpa PN, Devaraj SN. Antibacterial and antidiarrhoeal effects of alkaloids of *Holarrhena antidysenterica* WALL. *Indian J Exp Biol* 2004;42:589-94.
 27. Ballal M, Srujan D, Bhat KK, Shirwaikar A, Shivananda PG. Antibacterial activity of *Holarrhena antidysenterica* [Kurchi] against the enteric pathogens. *Indian J Pharmacol* 2000;32:392-3.
 28. Al-Zoreky NS. Antimicrobial activity of pomegranate (*Punica granatum* L.) fruit peels. *Int J Food Microbiol* 2009;134:244-8.
 29. Prashanth D, Asha MK, Amit A. Antibacterial activity of *Punica granatum*. *Fitoterapia* 2001;72:171-3.
 30. Abeer MH, Sanaa OY. Anti-microbial activity of *Acacia nilotica* extracts against some bacteria isolated from clinical specimens. *Res J Med Plants* 2007;1:25-8.
 31. Mustafa NK, Tanira MO, Dar FK, Nsanze H. Antimicrobial activity of *Acacia nilotica* subsp. *nilotica* fruit extracts. *Pharm Pharmacol Commun* 1999;5:583-6.
 32. Choudhury BR, Poddar MK. Andrographolide and kalmegh (*Andrographis paniculata*) extract: Effect on intestinal brush-border membrane-bound hydrolases. *Methods Find Exp Clin Pharmacol* 1985;7:617-21.