Nutraceuticals Induced Structural Changes in Broiler Gastrointestinal Tract Microbiota

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Research

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Abstract

Background The effects of nutraceuticals on the modulation of the intestinal microbiota are receiving increased attention however there are scarce number of studies investigating their effects in broiler meat production. The aim of this study was to implement feeding strategies and carry out a comprehensive trial on the interplay between carotenoids, anthocyanins, fructo-oligosaccharides, probiotics and gastrointestinal tract microbiota. Our feeding program was applied on an intensive production system with 1080 broiler Ross 308 flock.

Results We observed that nutraceuticals and synbiotics did not affect growth performance remarkably nevertheless, positive correlation was found between body weight and the beneficious *Bacteroidales*, *Corynebacteriales* and *Pseudomonadales*. Nutraceuticals were shown to boost broiler intestinal diversity and differentially enriched *Lactobacillus*, *Enterococcus*, *Streptococcus* and *Escherichia-Shigella* in core microbiome during the different stages of broiler rearing. Furthermore, diet supplemented with different nutraceuticals was shown to increase the number of unique beneficial bacteria (*Faecalibacterium, Akkermansia*).

Conclusion We concluded that nutraceutical-based feeding strategies can offer a promising, green approach for intensive poultry rearing by improving the health and production of livestock. We believe that the conversion of food industrial waste would result more sustainable bioactive component rich forages which invention could be implemented by other commercial antibiotic-free animal production system providing safe and quality meat.

Background

During the past two decades, the poultry industry has become one of the most efficient protein production systems, that forms the basis of global protein production [1]. An intensive breed selection was invented to develop chicken, which converts feed into muscle mass more efficiently [2]. Modern chicken breeds such as Ross 308 require less forage to achieve their desired drastic increase (about 70-80x) in weight (35 g - ~ 3 kg) throughout the production period (35–42 days) [3]. This extreme growth rate can be associated with a range of pathological conditions [3–7], including hypertension [6], heart failure [6–9], insulin resistance [10] and increased susceptibility to infections [11].

In 2000, antibiotic resistance was identified by the World Health Organization (WHO) as one of the most significant global threats to public health [12, 13, 14]. For extremely growing animals, the application of sub-therapeutic dose of antibiotics was generally acquired to improve health and productivity [15]. The use of such additives is associated with unwanted consequences, such as depletion of beneficial intestinal microbiota and increased occurrence of antibiotic-resistant microbial pathogens [16], deteriorating public health [15–19].

Gastrointestinal tract (GIT) microbiota plays an important role in overall health and function of the host [20–23]. GIT microbiota is in the focus of major research efforts in meat production animals [24], since it

has a positive impact on the immune system [24–27], GIT physiology [21, 27, 28], nutrition [23, 29], detoxification of certain compounds [30], and productivity [29, 31, 32]. It has also an important role in poultry industry, requiring animals capable of growing rapidly within a short period of time [33, 34].

There are growing number of evidences that alterations in poultry GIT microbiota composition have a pivotal role in the development of metabolic disorders [28, 35–44]. The diversity of the microbiota is one of the key determinants against invading pathogens [45]. A higher microbial community diversity is related to a healthier host status, whereas a significant loss in the complexity can be associated with various diseases and susceptibility to pathogen colonization [29, 46–49]. The shift of the GIT microbiota community towards benefitial bacteria could improve health conditions of the host.

Health-promoting probiotic bacteria can ferment prebiotics which are undigestible and non-absorbable for the host and convert them to lactic acid and short-chain fatty acids (SCFAs) [50–60]. SCFA producing bacteria may directly enhance the absorption of some nutrients [61–66].

It was already proved that the deteriorations of the community diversity and the associated alterations in SCFAs can be restored by alternative treatment strategies both in humans and animals [67]. Some of them promote the use of prebiotic-rich diet combined with probiotics which may alleviate disease symptoms [54]. Several functional medicine have already been explored thoroughly and demonstrated to regenerate dysbiotic intestinal flora [68].

With this trail we focused on the therapeutic potential of natural, bioactive components (carotenoids, anthocyanins, fructooligosacharides and synbiotics) obtained from plant-based food industrial waste. By enriching diet of 1080 broiler flock in Hungary with nutraceuticals we investigated their effect on community diversity, and alterations in baseline symbiotic microbiota. We also managed to unravel compositional shifts in GIT microbiota and investigated how these might relate to the growth performance of broiler Ross 308. Based on our observations nutraceuticals did not deteriorate chicken development and delivered promising results in stimulating GIT health.

Results

General description of sequencing results

16S rRNA gene based (V3-V4 region) amplicon sequencing was carried out on Illumina MiSeq platform generating a total of 11 million reads by processing 96 broiler fecal samples with the mean count of 86 470 ± 24 361 reads per sample. Quality filtering with the DADA2 software resulted an average denoised read count of 42 763 ± 13 425 per sample and after the merging process the read count dropped to an average of 41 085 ± 12 991 reads per sample. At the end, the average number of non-chimeric reads was 27 778 ± 7622 per sample.

Growth Performance And Food Intake

To investigate the effects of dietary supplements on broiler growth performance average body weight (BW), average daily feed intake (ADFI), and average daily gain (ADG) have been monitored throughout the feeding trial (Fig. 1). At the beginning of the feeding experiment, the average BWs for male and female meat chicken were; ♂: 38.6 ± 2 g, ♀: 38.3 ± 1.2 g, while by the end of this trial broiler chicken reached 2693 ± 64.82 g (69.7x increase in ADG) on average, with the total weight gain of 2654.23 ± 60 g. There were no significant differences noticed in weight due to diet when comparing treatment groups (TS3, TS4, TS5, TS6) to controls (CS1, CS2). However, by the end of broiler productive lifespan, a moderate but not significant decrease in body weight was registered due to anthocyanins based dietary supplementations in comparison to controls (TS6 BW: 2590 ± 264 g, CS1, CS2 BW:2742 ± 222 g). On day 32–42 animals treated with anthocyanins and β-glucan (CS2, TS6: 132 g/day/bird) resulted significantly higher ADFI in comparison to control (CS1: 114 g/day/bird).

Significant associations between broiler body weight gain and GIT microbiota

We managed to unravel nutraceuticals induced GIT community variations associated with broiler ADG. Alterations in strength- and direction of correlations were obtained at the order level (Fig. 2). The order *Bacteroidales* showed positive correlations with ADG throughout all of the experimental groups in comparison to controls (CS1 R: 0.62, CS2 R: 0.50) whilst the correlations were the strongest in nutraceutical and synbiotics treated samples (TS3 R: 0.70, TS4 R: 0.86, TS5 R: 0.80, TS6 R: 0.79). Further consistent positive correlations were observed between ADG and the order *Campylobacteriales* (R: 0.75 ± 0.03), *Corynebacteriales* (R: 0.23 ± 0.17), *Micrococcales* (R: 0.25 ± 0.03) and *Pseudomonadales* (R: 0.56 ± 0.20). The application of synbiotics based supplementation has been shown to express the highest correlations with ADG for the above-mentioned taxa. Positive correlations were detected for *Betaproteobacteriales* due to anthocyanins TS6 R: 0.17 vs. other R: -0.23 ± 0.17. Further discrepancies were seen in the case of *Enterobacteriales* (CS1, TS3, TS6 R: -0.17 ± 0.15 vs. other R: 0.26 ± 0.21), *Clostridiales* (TS3 R: -0.066 vs. other R: 0.28 ± 0.14). Interestingly, in the case of β-glucan (CS2 R: 0.02) and carotenoid (TS3 R: 0.03) treated samples the order *Lactobacillales* showed weak positive correlations with ADG, while negative correlations were measured due to FOSs (TS4 R: -0.3), synbiotics (TS5 R: -0.45) and anthocyanins (TS6 R: -0.38).

Profound alterations in alpha and beta diversities due to age and treatment

Alpha diversity indices were calculated to track remarkable conversions in community diversity of controls (CS1, CS2) and treatment groups (TS3, TS4, TS5, TS6) (Fig. 3). Chao-1 (Fig. 3a), Faith's phylogenetic (Fig. 3b), Shannon (Fig. 3c) and Simpson (Fig. 3d) diversity indices were applied to evaluate species abundance, richness and evenness of the broiler GIT microbiota. Chao-1 and Faith's PD indicated a significant increase in chicken GIT community diversity by the end of the productive lifespan in the case of FOSs (TS4 Chao-1: 276 ± 76 Faith's PD: 20.3 ± 4.6), synbiotics (TS5 Chao-1: 319 ± 14, Faith's PD: 22.54 ± 0.8), anthocyanins (TS6 Chao-1: 333 ± 10, Faith's PD: 21.8 ± 2.92) based dietary supplementations in comparison to negative control (CS1 Chao-1: 61.75 ± 24, Faith's PD: 78 ± 0.9) fed with basal diet (Fig. 3a, Fig. 3b). During grower (day 22–31) and finisher (day 32–42) feeding periods, FOSs, synbiotics and

anthocyanins exerted significant increase on Faith's PD indices. At day 31 carotenoids whilst at day 42 anthocyanins improved the Shannon diversity significantly compared to 7-day old negative control (CS1) chicks (Fig. 3c). Simpson diversity indices did not alter significantly during the experiment (Fig. 3d). In general, remarkable differences in pattern dynamics were observed in alpha diversity indices (Fig. 3e). A regular increase was detected in alpha diversity due to β-glucan, FOSs, synbiotics and anthocyanins as animals aged. Faith's PD, Chao-1, Shannon and Simpson indices of basal dietary controls and carotenoid treated samples improved steadily with animal growth whilst a deterioration was observed in these parameters after 31 days. Broadly, during grower phase (day 22–31) the highest community diversity was associated with carotenoid tretaed birds, while by the end of the finisher period (day 40) the community diversity proved to be the lowest in the case of animals receiving basal diet.

Four beta diversity heatmaps were generated by measuring Bray-Curtis, Jaccard, Weighted- and Unweighted Unifrac distances (Fig. 3f) between the different experimental groups in relation to age and diet. Distance-based dissimilarity matrices showed that flock development exerted remarkable influence on overall community variations thus a gradual increase in community diversity was accompanied with increased heterogeneity of the GIT microbiota.

Baseline Git Microbiota Reflects Dynamic Equilibrium Of Livestock

Estimations about the healthy core microbiota have been made for all experimental groups (CS1-TS6) at the phylum, order and genus taxonomic ranks, by considering taxa represented in at least 50% of the samples (Fig. 4). Characteristically, FOSs, synbiotics and anthocyanins exerted the most emphasized community shifts in the core microbiota of chickens younger than 19 days. The two core phyla; *Firmicutes* (93%±6.9) and *Proteobacteria* (6.9%±0.9) expressed deviating proportions with dietary supplementations and host development. The existence of *Proteobacteria* was most pronounced on day 19 (31%±3.4), and day 42 (2.2%±0.1). In 19-day old animals an appreciable depletion of *Firmicutes* was observed in both control groups CS1, CS2 (75%±9). Between day 32–42 observable gains in the proportion of *Proteobacteria* were incurred for FOSs (TS4 90%±8) and synbiotics (TS5 87%±7.2). *Lactobacillales* was the most abundant order during the entire growth period (82%±0.22) followed by *Clostridiales* (9.1 ± 0.6), *Enterobacteriales* (6%±0.6) and *Erysipelotrichales* (1%±0.1). In the case of *Lactobacillales*, the highest relative abundances were accounted (97.51%±2.23) to the pre-starter feeding period. In the case of the control group a pronounced ageing related remission was observed in the abundance data from day 7 to day 31 (CS1; from 97%±0.2 to 61%±0.6). A significant decrease in *Clostridiales* (7.3%±0.3) was shown on day 31 in animals receiving immunostimulants in the form of β-glucan (CS2 7%±0.3), FOSs (TS4 7% ±5.9) and anthocyanins (TS6 7%±5) in comparison to negative control animals (CS1 23%±4). By the end of the grower phase (day 32–42) this difference completely disappeared representing similar relative proportions for *Lactobacillales* (75%±5.28), *Clostridiales* (16% ±1.77), *Enterobacteriales* (5.26%±4.4) and *Erysipelotrichales* (2.87%±2.11) in all of our experimental groups. We found eight genera representing the 50% core microbiota; *Lactobacillus* (55.69%±19),

Enterococcus (19%±18), *Streptococcus* (7.7%±6.6), *Escherichia-Shigella* (6.9%±6.9), *Faecalibacterium* (3.5%±3.9), *Turicibacter* (1.1%±1.5), *Rombutsia* (1.7%±1.6) and *Aerococcus* (48%±0.6). Again, the genus *Lactobacillus* showed a clear dominance during the experiment except in day 19 samples (27.4 ± 3.66) where its abundance shifted significantly in favour of *Enterococcus* (36%±11.54). On the genus level chicken development exerted the most explicit effect on the relative occurrence of *Enterococcus*. In chicken younger than 19 days this genus seemed to be the second most abundant (34%±1.3), whereas in older chicks a drastic fall (3.3 ± 1.87) was observable. The effect of nutraceuticals remarkably decreased the proportion of the genus *Escherichia-Shigella* in 19-day old chicken (control groups 31%±3.4 vs. treatment groups 11.6%±6.9). It was also observable that herbal extracts boosted *Faecalibacterium* (CS1 9.9%±1.7 vs other 5.7%±0.9) and *Rombutsia* (CS1 1.5%±0.1 vs. other 2.1%±0.18) in 31-day old animals. By the end of the broiler rearing period variations in the 50% core alleviated with the exception of two genera; FOSs and anthocyanins increased the relative proportions of *Enterococcus*; 6.8%±0.2 (TS4), 12% ±0.2 (TS6) vs. 2.5%±1.8 (CS1, CS2, TS3, TS5), while nutraceutical treatment generally increased *Faecalibacterium* 13%±0.8 (TS3), 6.8%±0.2 (TS4), 6.8%±0.2 (TS5), 12%±0.2 (TS6) in comparison to controls: 3.18%±0.84.

Significant shifts in community taxonomy were revealed due to age and diet

To further discover key taxa representing significant shifts among study parameters and experimental settings, differentially abundant linear discriminant analysis (LDA) effect size (LEfSe) method was used to perform class comparisons among study groups. We found 22 bacterial clades which were significantly enriched with respect to age and diet (Fig. 5). LDA scores estimate the effect size of each differentially abundant features. Great extensions were seen in the main phyla *Proteobacteria* and *Gammaproteobacteria* (day 19; LDA 5.43 ± 0.0004) associated with negative controls, in *Firmicutes* (day 7; LDA 5.99), *Bacteroidetes* (day 31; LDA 4.85) owed to carotenoids, and in *Alphaproteobacteria* (day 7; LDA 4.3) due to β-glucan dietary supplementation. During the pre-starter period (day 7) notable growths in *Erysipelatoclostridium* (LDA 4.66) were observed in the presence of synbiotics. Further accessions were measured in the order *Bacillales* (day 19; LDA 4.96) and *Pseudomonadales* (day 31; LDA 5.2) and in the family *Burkholderiace* (day 7; LDA 4.33) due to anthocyanin treatment. FOSs boosted *Moraxellacea* (day 40; LDA 4.2), while *Enterobacteriales* and *Enterobacteriacea* were significantly accessed in controls (day 19; LDA 5.42). Further compelling rises were seen as per β-glucan in the clades *Streptococcacea* (day 19; LDA 5.2) and the genus *Delftia* (day 7; LDA 4.05).

Diet significantly impacts the composition of the broiler GIT microbiota

We unravelled diet induced and age-related compositional differences at phylum, class, genus and species taxonomic ranks through the broiler production by cataloguing the GIT microbiota of the six experimental groups. Under our experimental settings we identified in total 7 phyla, 12 classes, 20 orders, 31 families and 60 genera. The phyla *Firmicutes* (89.53%±2.94), *Proteobacteria* (7.39%±2.90) and *Bacteroidetes* (1.44%±0.73) were the most predominant accounting for the 32.79%±42.62 of the sequence reads followed by, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* (Fig. 6a). Diet related

differences in the *Firmicutes* to *Bacteroides* ratios (F/B ratio) may reflect alterations in (poly)saccharide utilisation of flocks. The highest log2 F/B ratio was detected in CS2 birds 7.14 (92% vs 0.65%) by the inclusion of β-glucan, while it proved to be the lowest in anthocyanin treated samples 4.89 (83.6% vs 2.8%). *Epsilonbacteraeota*, *Tenericutes* and *Verrucomicrobia* were also detectable, but with very low abundances (≤ 1%). Anthocyanins were shown to increase the proportion of *Proteobacteria*. Furthermore, anthocyanin treatment was also associated with the highest proportion of *Verrucomicrobia* (0.48% ±0.002). Appreciable age and diet related alterations were found in the proportions of the dominant classes; *Clostridia* (24.36%±5.9), *Bacteroidia* (1.49%±0.73), *Gammaproteobacteria* (6.95%±2.82) (Fig. 6b). As it is shown, diet exerted a strong effect on the distribution of the 6 most abundant classes. *Bacilli* (62% ±6.2) dominated in all experimental groups with the highest relative frequency in animals receiving β-glucan (CS2 71.1%±2.2) whilst the lowest frequencies were measured due to anthocyanin treatment (TS6 53.8%±0.82). Apparently, we observed low variations in the relative abundances of *Bacilli* among group CS2 (71.1%±2.2), TS3 (58.42%±1.1), TS4 (67%±0.8) and TS5 (61.63%±1.8). *Clostridia* and *Gammaproteobacteria* were represented with high proportions (C: 24%±5.9 and G: 7%±2.8). Diet did not impact *Bacteroidia* (1.4%±0.7). Effective levels of nutraceuticals and probiotics as feed supplements are important resource providers of SCFA production. In the present study the effects of nutraceuticals on SCFA producing genera were also investigated. As shown in Fig. 6c anthocyanins exerted a positive effect on the abundance of *Faecalibacterium* during finisher feeding period. Significantly lower abundances were observed in 31-day old birds receiving FOSs (TS4 0.88%±0.299) in comparison to birds fed with basal forage (CS1 9.26%±1.8). In general, FOSs, synbiotics and carotenoids were able to exert positive effect on the abundance of the genus *Faecalibacterium* in chickens older than 31 days.

The drastic alterations in relative frequencies of *Lactobacillus* (55%±19) were rather age- than diet related. Similarities in abundance patterns were observed between animals fed with basal forage and those receiving β-glucan, carotenoids and synbiotics representing fluctuating trends in relative frequencies. On the contrarily, FOSs and anthocyanins represented an increasing tendency by reaching day 31. Elevated levels of *Lactobacillus* during the starter (day 1–9) and finisher (day 32–42) phases of production might be associated with the anti-pathogenic characteristics of the members of this genus. *Lactobacillus alvi* represented increments in anthocyanins treated samples (0.11%±0.2 vs. other groups 0.015%±0.09). The levels of *Lactobacillus salivarus* and *L. aviarius* proved to be relatively high in all experimental groups. *L. salivarius* which is known to exhibit a protection against *Salmonella* and other pathogen colonization showed enrichment in the control animals (CS1, CS2; 15%±1.2 vs. treatment groups; 7.5%±2.2), whereas *L. aviarius* showed remarkable increments due to synbiotics (TS5; 3%±0.2) and anthocyanins (TS6; 7%±0.9). Noticeably, a rise in the genus *Campylobacter* and the bacterial diarrheal gastroenteristis-causing *C. jejuni* (TS6 0.4%±0.022 vs others 0.06%±0.02) was shown in anthocyanin fed animals without affecting chicken welfare. In the case of the butyrate producing genera *Subdoligranumlum* and *Butyricoccus* similarly to *Lactobacillus* alternating (ascending and descending) frequencies were observed presenting higher proportions during the first quarter of broiler rearing. Furthermore, anthocyanin treatment (TS6 2.89%±1.05) significantly increased the abundance of *Subdologranulum*. The genera *Streptococcus, Bacteroides*, *Blautia* and *Ruminococcus* were not prevalent

in chicks younger than 7 days. Anthocyanins and synbiotics were able to elevate the levels of *Bacteroides* notably in both 7-day and 19-day old chicken. Noticeably, in the case of *Streptococcus* and *Blautia* the highest proportions were registered on day 31. By the end of the meat production anthocyanin treatment significantly increased the abundance of *Blautia* (TS6 1.32%±0.4 vs CS1 0.1%±0.18) and *Ruminococcus* (TS6 0.1%±0.05 vs CS1 0%±0) in comparison to controls.

With regard to genera representing predominantly potential pathogenic organisms, diet did not exert considerable effect on their scarce abundances (Fig. 6d). *Eggerthella, Fusobacterium* and *Helicobacter* were represented in low number. However, as our data indicated a noticeably rise in *Campylobacter* (TS6 0.43%±0.16 vs. others 0.06%±0.02) was shown due to anthocyanin treated samples. We also investigated the effect of nutraceuticals and probiotics on certain genera associated with enhanced metabolism. We found, that *Alistipes* showed traceable abundances only in CS1 controls (0.09%±0.02) and carotenoid treated samples (0.14%±0.03). *Eubacterium* (TS6 1.7%±0.4 vs. other 0.5%±0.2) and *Bacillus* (TS6 0.1 ± 0.09 vs. other 0.018 ± 0.01) showed the highest increments in anthocyanin treated animals.

Attention was also payed to the estimated relative proportions of relevant species listed in Fig. 6e. We detected the anaerobic *Anaeromassilibacillus senegalensis* having short exposition time under aerobe conditions [69] in all of our experimental groups with similar frequency (0.15%±0.1) which can reflect adequate sample handling and processing. *Bacteroides gallinaceum* which was previously isolated from the caeca of a healthy broiler seems to play an important role in the digestive system [70] however, it was only traceable in carotenoid, (TS3 0.14%), and anthocyanin (TS6 0.12%) treated samples. Butyrate-producing *Butyricicoccus desmolans* was only traceable in very low proportions in sample sets; CS1 (0.0035%), TS3 (0.0065%) and TS6 (0.00402%). Simlarily, *Lactobacillus alvi* (0.01%±0.03) which is frequently obtained from chicken feces and intestine [71] was represented uniformally. Benefitial *Lactobacillus salivarius* and *Lactobacillus aviarius* were observed in all experimental groups representing outlier ratios in both the control groups (CS1 and CS2 15%±2.2 vs. other 7.5%±2.2) and due to synbiotics and anthocyanins (TS5 15.53%±0.2 and TS6 7.7%±0.9 vs. other 6%±4). Noticeably, β-glucan treated samples showed the highest species diversity for lactic acid bacteria recovering eight *Lactobacillus* strains out of which *Lactobacillus aviarius*, *L. salivarius* and *L. alvi* were universally represented. The lowest level of the newly described anaerobic, non-spore forming, fatty acid producing *Traorella massiliensis* [69] was observed in birds treated with anthocyanins (TS6 0.04%±0.015 vs. other 0.1% ±0.17). Also, the short-chain fatty acid producer *Pseudomonas fragi* [72] showed relative high abundance in TS6 (0.3%±0.2) samples.

Comparison Of Diet Induced Structural Modulations

We explored remarkable alterations in family taxonomic data due to carotenoids, FOSs, synbiotics and anthocyanins. A composite heat map was created to pronounce distortions in the relative frequencies normalized to the data of control animals fed with non-supplemented basal forage (Fig. 7). During the

pre-starter phase, we observed remarkable increments in *Bifidobacteriacea*, *Ruminococcacea* and *Erysipelotrichacea* due to synbiotics and anthocanins. Also, greater abundances were seen in *Ruminococcacea* and *Erysipelotrichaceae*, *Clostridiacea* and *Lachnospiracea* in anthocyanin (TS6) challenged animals. Nutraceuticals uniformly decreased the level of *Staphylococcacea* and *Leuconostocacea* in comparison to controls. In FOSs challenged 19-day old birds remarkable increments were shown in *Barnesiellace, Brevibacteriaceae, Bacteroidaceae* and *Clostridiacea* accompanied by decrements in *Bifidobacteriacea*, *Burkholderiacea*. During the grower phase (day 22–31) of meat production appreciable shifts were manifested due to carotenoids (TS3) representing large increments in *Bifidobacteriacea, Barnesiellacea* and decrements in *Aerococcacea, Enterococcacea, Clostridiacea, Peptostreptococcacea* and *Moraxellacea*. Further remarkable declines were evidenced in *Dermabacteriacea, Planococcacea, Staphylococcacea, Leuconostocacea, Clostridiacea* and *Pseudomonadacea* due to β-glucan (CS2). In 31-day old animals solid increment in *Campylobacteriacea, Planococcaceae* and *Pseudomonadacea* and cutbacks in *Bacteroidacea, Helicobacteriacea* were registered due to anthocyanins. By the finisher phase of meat production impressive diminutions were encountered in *Brevibacteriacea* in all of our treatment groups. Enrichments in *Helicobacteriacea* through FOSs, synbiotics and anthocyanins were detected. Also, during finisher (day 32–42) feeding period a rise was detected in *Akkermansia* due to β-glucan, synbiotics and anthocyanins.

Nutraceuticals induced structural shifts in comparison to controls

Taxonomic heat trees have been made to generate comprehensive microbial community profiles to represent nutraceuticals induced community shifts in relation to both of the control groups. (Fig. 8). FOSs and synbiotics did not shift the abundance of *Proteobacteria* and *Gammaproteobacteria*. On the contrary, carotenoids (TS3) decreased, while anthocyanins (TS6) increased remarkably their proportions. Noticeably, anthocyanins decreased while carotenoids, FOSs and synbiotics aggregated taxa of the class *Bacteroidia*. We observed a rise in the phylum *Tenericutes* as per carotenoids (TS3) and synbiotics (TS5). Depletion was found in the class *Alphaproteobacteria* in relation to β-glucan (CS2) supplemented diet. Carotenoids (TS3) and anthocyanins (TS6) decreased the relative abundance of the family *Lactobacillacea*. In the case of the treatment groups a slight increment was observed in *Enterococcacea* frequencies. Appreciable increase was detected in *Clostridium* due to FOSs (TS4), synbiotics (TS5) and anthocyanins (TS6).

Comparative Metagenomics Provide Insights Into Interplay Of Taxa

To identify diet influenced mutual interconnections within broiler intestinal microbiota comparative metagenomic analysis was performed which involved the comparison of the family and genus frequency data. We estimated the extent to which genera tend to change together. Relative proportions of taxa were

correlated in terms of Spearman's method (Fig. 9). We managed to identify families and genera which were notoriously present among all of our experimental groups with highly similar correlations according to the coefficient values and the directions of associations. The strongest negative correlations between possible opportunistic genera such as *Enterococcus* (R: -0.71, -0.72), *Streptococcus* (R: -0.55, -0.54), *Escherichia-Shigella* (R: -0.57, 0.43) and the major butyrate producers; *Butyricoccus* and *Ruminococcus* were revealed due to anthocyanins. The dominant *Lactobacillus* genus represented very discordant correlations with the other genera according to dietary supplementations. Noteworthy *Enterococcus* and *Lactobacillus* negatively correlated ($p < 0.05 – 0.001$) with each other in all of our experimental groups except the anthocyanin (TS6) treated samples where we observed strong positive correlations ($p < 0.01$, R:0.8) between their relative abundances. Furthermore, the values of correlations and the direction of associations were also remarkable. Similarities were recognized between basic controls and synbiotics treated samples where *Lactobacillus* represented negative correlations with the other genera. Hence, in the other treatment groups positive correlations (TS3; $p < 0.04$, R: 0.25; TS4; $p < 0.0009$, R: 0.33; TS6; $p < 0.22$, R: 0.1) were detected between *Lactobacillus* and *Bacteroides*. In CS1 control samples *Helicobacter* correlated strongly with most of the genera except *Escherichia-Shigella* ($p < 0.002$, R: -0.31), *Enterococcus* ($p < 0.0016$, R: -0.28) and *Lactobacillus* ($p < 0.094$, R: -0.31). In anthocyanin treated samples *Lactobacillus* represented significant correlations ($p < 0.01 – 0.001$) with *Streptococcus* (R: 0.6), *Corynebacterium* (R: 0.1), *Staphylococcus* (R: 0.26), *Escherichia-Shigella* (R: 0.2), *Aerococcus* (R: 0.44*), Rombutsia* (R: 0.22) and *Faecalibacterium* (R: 0.18). In carotenoids treated samples members of the genus *Escherichia-Shgiella* were posititvely correlated with *Streptococcus* (R: 0.28), *Staphylococcus* (R: 0.45) and *Rombutsia* (R: 0.28). Apparently, in the case of synbiotics based dietary supplementation *Bifidobactrium* was shown to correlate positively with *Lachnoclostridium* (R: 0.65), *Ruminoclostridium* (R: 0.65), *Ruminococcacea* (R: 0.36), *Fournierella* (R: 0.56*), Sellimonas* (R: 0.55) and *Buytiricoccus* (R: 0.69) ($p < 0.001$).

Discussion

The routine administration of antibiotics of meat producing animals as growth promoters has been banned by the EU on January 1st, 2006 [14]. A great number of commensal organisms inhabiting the broiler gastrointestinal tract contribute to the proper maintenance of the host and may improve the quality of meat [23, 28, 33, 73]. Data about their immunostimulatory effects conferring beneficial role against infections are ancient and not doubted [27, 74, 75]. Herbal medicines are receiving widespread attentions especially in developing countries because of their antibacterial behaviour and effect to improve performance and product safety in meat production systems [21, 54, 61, 76–80]. There are growing number of evidences, that complex, bioactive component rich plant extracts increase digestive enzyme secretion, nutrient absorption and decrease feed-to-gain ratio in meat-type chickens [28, 33, 40, 51, 73, 81–90].

Our prior aim was to develop and apply natural feed additives which can stimulate broiler GIT health, without deteriorating the meat production parameters. The feeding program of this trial was applied according to the normatives widely used in Ross 308 chicken production in Hungary [52]. This technology was designed to achieve high weight gain while producing quality meat. We obtained, that under our

experimental conditions diet enriched in FOSs, anthocyanins and synbiotics did not alter growth performance during the production stages supporting the estimations of other data [57, 91, 92]. Furthermore, based on our findings plant derived nutraceuticals have been shown to strengthen the positive correlations between body weight gain and the beneficious *Bacteroidales*, *Campylobacteriales*, *Corynebacteriales* and *Pseudomonadales* associated with increased absorption of nutrients through the improvement of the integrity of the intestinal epithelia [78, 93–95].

Spore forming probiotic *Bacillus* species associated with increased body weight, were only attainable in broiler feces receiving β-glucan and anthocyanins (data not shown). It is worth to mention, that by the beginning (pre-starter) and by the end (finisher) of the feeding periods of broiler meat production anthocyanins increased significantly the levels of the beneficial bacteria such as *Lachnospiraceae*, *Ruminococcaceae* associated with improvements in feed conversion [81], (FCRs; day 7 TS6: 0.26 ± 0.04 vs CS1 0.19 ± 0.04, day 40 TS6: 1.87 ± 0.3 vs. CS1 1.26 ± 0.3). We did not capture gains in frequencies of *Lactobacillacea* and *Bifidobacteriacea* which were previously reported to enhance the utilization of fructooligosaccharides in FOSs challenged chicken [47, 96–101]. Furthermore, our data did not confirm that the implementation of probiotics in poultry diet correlates with enhanced growth during production period which might be explained by a number of different environmental and genetic factors [54, 58].

The intricate interconnection of the genera *Lactobacillus* [102–106] *Enterococcus* [107, 108], *Bifidobacterium* [109, 110], *Clostridium* [111], *Bacteroides* [112], *Peptostreptococcus* [113] regulates primary bile salt synthesis and secondary bile salt metabolism of the host [114]. Growth-promoting mechanism of most subtherapeutic dose antibiotics can be related to decreased activity of the bile salt hydrolase (BSH) enzyme that catalyses deconjugation of bile salts [115]. Certain *Lactobacillus* species (such as *L. salivarius*) are the main suppliers of the enzyme BSH [116]. The noticeable decrease in the Gram-positive intestinal *Lactobacillales* and *Clostridium* and Gram-negative *Bacteroides* due to anthocyanins might also be associated with alterations in bile biotransformation due to the decreased level of deconjugated bile salts through which microbiota exerts a negative impact on host fat digestion and utilization. Based on our data however, we did not observe significant remission in the gain rate of the anthocyanins treated broiler which can be explained by considering the intricate metabolic potential of the GIT microbiota (TS6 day 40: 2590 g vs. CS1: 2758 g). Furthermore, in certain concentrations bile salts are associated with antimicrobial effects trough disrupting bacterial membranes, denaturing proteins, causing oxidative damage to DNA, and controlling the expression of certain eukaryotic genes involved in host defence [117]. *Corynebacteriaceae* is known to correlate with elevated triglyceride level and weight gain [118]. Nutraceuticals significantly increased *Corinebacteriaceae* in 40-day old chicken, without important changes in growth performances in comparison to control groups.

The intestinal epithelial layer forms the major barrier between host and environment. Especially, species belonging to the phylum *Proteobacteria* are reported to increase epithelial cell death and mucus degradations [119]. Only some specialized bacteria such as; *Clostridiaceae*, *Lactobacillaceae*, *Helicobacteraceae* and *Enterococcaceae* are capable to adhere to mucus layer suggesting that these bacteria have a pivotal role in maintaining the gut intestinal barrier integrity [120–122]. Mucin degrading

Akkermansia which have been previously shown to lower visceral fat deposits are associated with decreased body weight gain rate [123]. Furthermore, the presence of mucin-degrading bacteria is associated with intestinal health, due to competitive exclusion of other bacteria which adhere less effectively to the mucosal surface [121, 123]. Based on our data, the increase in the abundance of *Akkermansia* due to anthocyanins treatment decreased body weight moderately (TS6 2590 ± 280 g vs CS1 2758 ± 264 g) and FCR (TS6 1.36 ± 0.19 vs CS1 1.06 ± 0.3) in comparison to controls.

Bacterial saccharolytic fermentation can transform non-digestible dietary carbohydrates into bioactive molecules associated with positive health outcomes and regulation of the appetite [124]. Among a variety of metabolites produced by the beneficious gastrointestinal tract microbiota short-chain fatty acids (SCFAs) received increased attention because of their important role in disease prevention and recovery [125]. Both *Bacteroides* and *Firmicutes* are associated with SCFA synthesis. According to data, increments in *Firmicutes* can be associated with an increase in nutrient absorption, whereas an elevation in *Bacteroidetes* correlates usually with enhanced hydrolysis of glycogen, starch and polysaccharides [1, 21, 43, 75, 126–128]. *Firmicutes* to *Bacteroidetes* ratio (F/B ratio) is important for the optimal nutrition of the host. In this study F/B ratio was the lowest in anthocyanins challenged animals resulting into lower body weight in comparison to controls. There was an increase in *Bacteroides* in 19-day old flock due to synbiotics that might also correlate with enhanced activity of polysaccharide metabolism since members of this genus are generally associated with degradation of starch and glucan [69]. Acetate and propionate are mainly produced by *Bacteroidetes* while *Firmicutes* are the main butyrate supplier [55].

Members of the *Bacteroidetes* are associated with alpha-amylase, alpha-1,2 mannosidase and endo-1,4-beta-mannosidase production being involved in the metabolism of starch and other polymeric substances [129]. Synbiotics based diet was shown to favor of the occurrence of the important propionate producer *Bacteroides* [130, 131]. The highest ratios for *Bacteroides dorei, B. gallinaceum* were detected in samples receiving carotenoids, synbiotics and anthocyanins. By the end of the production period, anthocyanins ameliorated the levels of *Bacteroideacea* and *Barnesiellacea* usually linked to more efficient intestinal absorption of components as described previously [83] that might be suggestive of improvements in growth parameters, however, this was also not strengthened by our data. Effects of nutraceuticals manifested in gains in the proportion of the butyrate producer *Lachnosiracea* and *Ruminococcacea* in 40-day old chicken. For the colonocytes butyrate is an important energy source which is largely metabolized in the epithelial mucosa [132]. Anthocyanins were shown to favor for the relative enrichment of important butyrate producers; *Eubacterium* and *Faecalibacterium* [62, 64, 124, 133] while FOSs and synbiotics proved to be propulsive for the increment of the genus *Clostridium* associated with beneficiary effects on chicken GIT health [134] in broilers especially during the finisher feeding period.

We also investigated the effects of different dietary supplements on the community complexity through the production of Ross 308 *Gallus gallus forma domestica*. Therefore, alpha diversity indices (Chao-1, Faith's PD, Shannon and Simpson) were monitored during the four feeding periods (pre-starter, starter, grower, finisher). Based on our results, distinctive differences were observed in GIT microbiome richness among our experimental groups. In general, a tendency representing a gradual increase in richness and

evenness was captured by reaching a plateau around day 31. By the end of the broiler productive lifespan a steep decrease was seen in alpha diversity indices in broilers receiving basal diet nevertheless, anthocyanins significantly increased the Chao-1, Shannon and Faith's phylogenetic diversity indexes in comparison to controls. To the best of our knowledge this is the first study investigating the effect of anthocyanins on poultry GIT community diversity. Based on our estimations, FOSs supplemented diet increased alpha diversity indexes (Chao-1 and Faith's phylogenetic diversity indexes) which were consistent with the results reported by Shang *et al.* [53]. Furthermore, in accordance to a previous study [135], we found that carotenoids did not exert significant effects on community complexity. Probiotics are also increasingly applied to animals especially in poultry industries [61, 136]. In agreement to our findings, Baldvin *et. al* also reported that probiotics based dietary supplementations exerted a positive effect on community diversity [137]. According to our findings, β-glucan supplementation did not have remarkable influence on community diversity. Similarly, to previous reports our data indicated that the composition of the broiler GIT microbiota diversifies remarkably as the GIT microbial population becomes more complex in ageing broiler [21, 61, 62, 138]. Increase in the community alpha diversity made symbiotic communities more discordant which was also supported by Bray-Curtis, Jaccard, Weighted- and Unweighted Unifrac distances. Noteworthy, the present study revealed that appreciable beneficial effects of nutraceuticals manifested mostly by the end of the broiler productive lifespan, as the diversity started to decrease. This may suggest that dietary supplementation has a lesser impact on a more diverse symbiotic microbiota. A higher microbial diversity is commonly related to a healthier host status, whereas the lack of sufficient diversity in a microbial community structure has been associated with different intestinal diseases [22, 139–145]. Furthermore, imbalance of the gut microbiome composition often leads to the elimination of subset of beneficial bacteria, while the abundance of pathogenic bacteria increases, in conjunction with significant loss of diversity [146].

One of our paramount purposes was the portrayal of GIT core microbiota of livestock. A combined age-related view was achieved at the phylum, order and genus taxonomic ranks to unravel the intricate interconnections of core bacteria at different stages of broiler Ross 308 production. This showed that broiler GIT microbiota was dominated by the two core phyla; *Firmicutes* (93%±6.9) and *Proteobacteria* (6.9%±0.9). The order *Clostridiales* being concordant with a substantial amount of beneficious SCFAs represented conflicting abundances among the control and treatment groups. Interestingly, during the grower period of the broiler production, a remarkable decline (7.3%±0.3) in its presence was observed as per administration of plant derived nutraceuticals. Notorious members of the potential pathogen genera *Clostridium, Campylobacter, Staphylococcus, Fusobacterium* and *Helicobacter* have also beneficial physiological effects on various biological responses by synthesizing essential vitamins and micronutrients; thiamine pyrophosphate, riboflavin, nicotinamide, pantothenic acid, biotin, tetrahydrofolate, neurotransmitters; biogenic amines (TMAO), secondary bile acids, lipopolysaccharides for the host [83, 147, 148]. Furthermore, certain members of the genus *Clostridium* are known polyphenol producers, possessing antioxidant activity and decreasing inflammation [149]. As such, in the case of *Clostridium* the lowest ratios were observed in birds treated with carotenoids and anthocyanins.

Lipoglycans of *Clostridium* and *Enterococcus* spp. are known to trigger inflammatory responses and insulin resistance [150].

One can note, that identifying symbiotic and dysbiotic taxa is not a straightforward task and there are no obvious “good or bad guys” in the complex microbial communities. However, it is essential to consider the problem of contamination of livestock both for sanitational and economic reasons [97]. In our experimental rearing system with 1080 animals the mortality proved to be very low, 0.56% nonetheless no significant differences in lethality patterns were observed between our experimental settings. We aimed to estimate the effect of the nutraceuticals on the susceptibility of the host to pathogen colonization. Therefore, we managed to investigate how nutraceuticals can shift the abundance of *Campylobacter, Fusobacterium, Enterococcus, Eggerthella, Helicobacter* and *Clostridium* associated with potential zoonotic strains such as *Salmonella enterica, Clostridium difficile, Campylobacter jejuni*, and *Helicobacter* pylori causing enteric diseases and subsequent contamination of poultry products.

Previous studies reported about decreased *Campylobacter* and *Clostridium* colonisation measured in broiler fed with fructans [58]. According to our data, the proportion of *Campylobacteriaceae* was significantly decreased in 40-day old animals receiving immunostimulants in comparison to controls. Furthermore, with the exception of the finisher feeding period, we measured increasing *Clostridiacea* concentrations to control group (log2 abundance difference 3.12 ± 1.36) in FOSs treated animals. We did not find appreciable differences in the abundance of the genera *Fusobacterium* ($P < 0.99$), *Eggerthella* ($P < 0.99$) due to different dietary settings, whereas in the case of the *Campylobacter* spp. a notable increment was registered in birds fed with synbiotics, while carotenoids and anthocyanins were able to decline *Helicobacter* spp. in comparison to basic controls (TS3 0.69%±0.02, TS6 0.189 ± 0.01 vs. CS1 1.1%±0.05). *Campylobacter jejuni* was traced in all of our experimental groups. Interestingly, in anthocyanins fed chicken a noticeably increment was registered for the bacterial diarrheal gastroenteritis-causing *C. jejuni* (TS6 0.009%±0.022 vs others 0.001%±0.06 $P < 0.673$) without affecting chicken welfare. Of note, *C. jejuni* can also be involved in the maintenance of intestinal epithelial integrity and the modulation of anti-inflammatory and antitumor effects [53, 87, 151].

The final two weeks of the growing period of the broiler production systems are associated with elevated mortality and production losses due to localized or systemic bacterial infections. In the poultry industry, besides being commensal and playing important role in the digestion of carbohydrates and proteins some of the members of the genus *Clostridium* are important pathogens [99] that colonize the gastrointestinal (GI) tract of chicken causing necrotic enteritis [47–49]. Infections caused by avian pathogenic *Clostridium perfringens* and *Escherichia coli* are among the relevant economically significant problems appreciably deteriorating poultry industry worldwide [98, 99]. Although the specific mechanisms have not been fully elucidated, by supporting host immunity, phytonutrients rich in antioxidants can reduce pathogenic stress [152]. The Gram-negative, rod-shaped, opportunistic pathogen *Alcaligenes faecalis* which can trigger infections by colonizing the respiratory tract [153] was not traceable in broiler receiving either β-glucan or nutraceuticals. Being potential pathogens the genus *Bacteroides* also encode a high number of proteins involved in polysaccharide and monosaccharide metabolism, decrease colonic

pH, improve the function of the epithelial cells [60]. In maximizing flock productivity, beside the gastrointestinal tract microflora, water quality, feed, temperature, ventilation and humidity are relevant external factors that influence success of meat production performance.

The most widely used probiotics are members of the relevant acetate producer genus *Lactobacillus* [45, 154] which have also been reported to affect gut health of poultry positively by reducing inflammation, directly modifying intestinal morphology and controlling enteric bacterial infections through regulating mucin composition [29, 32, 71, 106, 136, 155]. In this trial remarkable enrichments in lactic acid bacteria were identified in phase grower to finisher (day 31 - day 40) due to carotenoids. During the whole experiment, robust relative abundances were observed in FOSs supplemented animals which can be related to the specific enzymatic activities associated with the oligosaccharide transport system of *Lactobacilli* [80, 156]. These data are consistent with the results of other studies reporting *Lactobacillus* as a major beneficial bacterium that showing increments in broilers fed with fructans [55, 59]. In the case of the control samples remarkably elevated levels were measured for *Lactobacillus salivarius* which can be associated with enhanced induction of anti-inflammatory responses in chicken in comparison to treatment groups receiving immune modulators in the form of herbal extracts such as; carotenoids, anthocyanins, and health promoting prebiotics (fructo-oligosaccharides) and synbiotics providing synergistic effect on the gut health [154]. Furthermore, the age-related oscillating patterns in the genus *Lactobacillus* might be congruent with the deconjugated bile acid concentrations in broiler chicken [107, 157]. Both human and animal studies found an association between the accumulation of lactic acids and different disease states such as colitis and gut resection [158, 159]. In our study, taxonomy heat trees represented, that anthocyanins remarkably decreased the relative abundance of the family *Lactobacillacea*.

Apparently, β-glucan, FOSs and anthocyanins based dietary supplementations represented highly similar mutual interconnections among the most relevant genera. The most pronounced negative correlations between butyrate-producer genera such as *Butyricoccus, Ruminococcus* and lactic acid-producing *Staphylococcus, Streptococcus, Enterococcus* and *Lactobacillus* have been revealed in anthocyanins treated animals. This might be associated with improvements in epithelial intestinal barrier functions by decreasing lactic-acid build-up and increasing osmotic load [160]. In synbiotics fed animals the strong negative correlations between the lactate and acetate producing *Bifidobacterium*, and other lactic acid producing genera such as *Lactobacillus* ($P < 0.003$), *Streptococcus* ($P < 0.004$) and *Staphylococcus* ($P < 0.003$) allude to the intricate interconnections of synbiotics microbiota. Furthermore, evidences showed that some butyrate producers depend on exogenous acetate to butyrate conversion, which implies that a reduction in acetate producer bacteria can be associated with a decrease in intestinal butyrate levels [160]. In the case of animals fed with non-supplemented diet the genus *Lactobacillus* which was previously identified with poor feed conversion showed strong negative correlations with *Bacteroides*, *Faecalibacterium* improving metabolic efficiency and reducing colonization by undesirable microbes [54, 80, 154, 158, 161].

Conclusions

This feeding trial was devoted to improve our knowledge about the interplay between carotenoids, fructo-oligosaccharides, anthocyanins, synbiotics and the broiler gastrointestinal tract microbiota. Based on our scientific data the following main conclusions can be drawn: i) A tendential increase was measured in broiler GIT community diversity as chicken aged, by reaching a plateau around day 31 of the grower period followed by a sharp decline in alpha diversity metrices. Noticeably, these deteriorating parameters were ameliorated by treating birds with FOSs, synbiotics and anthocyanins. ii) Great emphasis was also placed how the taxonomy data correlate with enhanced bird performance. Based on our observations synbiotics expressed the strongest positive correlations between body-weigt gain and the order *Campylobacteriales*, *Corynebacteriales*, *Micrococcales* and *Pseudomonadales*. iii) The symbiotic broiler Ross 308 microbiota was also deciphered. Considering the 50% core taxonomy data FOSs, synbiotics and anthocyanins were shown to exert the most emphasized community shifts in comparison to controls by registering the most drastic community shifts especially during the pre-starter and starter periods. iv) In general, *Firmicutes, Bacteroidetes, Proteobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria*, *Erysipelatoclostridium*, *Enterobacteriacea*, *Burkholderiace*, *Moraxellacea*, *Streptococcacea* and *Delftia* were identified as key taxa representing significant shifts in community taxonomy due to nutraceuticals. v) We sorely investigated the alterations in relative frequencies of commensal beneficial, short-chain fatty acid producer bacteria and conditioned pathogens. The *Firmicutes* to *Bacteroides* ratio proved to be the highest in β-glucan, and the lowest in anthocyanin treated animals. Coincidently, anthocyanins were shown to increase *Proteobacteria*, *Verrucomicrobia*, *Ruminococcus*, *Blautia* whilst *Bacilli* which dominated across all experimental groups represented remarkable increments due to β-glucans. Generally, FOSs, synbiotics and anthocyanins exerted a positive impact on *Faecalibacterium*, which difference was more pronounced by the end of broiler rearing. Impressive alterations in *Lactobacillus* were mostly age related with carotenoids and anthocyanins representing abatements in frequency data. Carotenoids were shown to increase *Alistipes, Bifidobacteriacea, Barnesiellacea* while reduce *Enterococcacea* and *Clostridiacea*. In general, nutraceuticals consistently decreased the level of *Staphylococcacea* and *Leuconostocacea.* vi) Finally, comparative metagenomics was performed to identify mutual interconnections among taxa. *Streptococcus, Staphylococcus, Escherichia-Shigella* and *Rombutsia* showed strong positive correlations with each other, whereas exerted strong negative correlations with *Ruminococcus, Subdoligranulum* and *Ruminococcacea*. Also, thorought all of our experimental groups, strong positive correlations were observed among relevant SCFA producers, such as *Blautia, Faecalibacterium, Bacteroides* and *Christenellacea* by noticing that in anthocyanain treated samples, *Blautia* whilst in β-glucan treated samples *Bacteroides* showed no correlations with any of these genera. Given its complexity, this trial is particular, which underpins the health benefits of nutraceuticals as potential dietary adjuncts for intensive, antibiotic-free meat-production system.

Materials And Methods

Birds and Housing

A total of 1080, one-day-old Ross 308 mixed-sex broilers were used from a commercial hatchery in Hungary. The experiment was carried out on the experimental farm of University of Debrecen. All broilers were placed in the same barn. Chickens were kept in floor pens covered with wood shavings in a thermostatically controlled house at a stocking density of 650 cm^2/bird. Temperature was 32 °C at placement and gradually decreased by 1.5 °C/week. The birds were exposed to light according as follows: 23L:1D during the first 7 days, 20L:4D between 8 – 28 days and 23L:1D between 29 – 42 days (L = light, D = dark).

Experimental Design And Dietary Treatments

The one-day-old Ross 308 hybrid chickens were randomly placed into 6 experimental groups (3 pens/treatment, 60 birds/pen). The experiment was started at 1 day of age and lasted until 42 days of age. Each group was fed one of the following 6 diets: Control Set1 (CS1), basal diet without any added supplements; Control Set2 (CS2), basal diet including 0.5% β-glucan; Treatment Set3 (TS3), basal diet including 0.5% carotenoids; Treatment Set4 (TS4), basal diet including 0.5% FOS; Treatment Set5 (TS5) basal diet including 0.5% synbiotics; Treatment Set 6 (TS6), basal diet including 0.5% anthocyanins. Broilers were fed with a commercial maize-soybean based basal diet (BD) free of antibiotics according to four phase feeding period: pre-starter 1–9 days, starter 10–21 days, grower 22–31 days, and finisher 32–42 days. All diets were fed in mash form. The components and nutritional composition of BD are given in Table 1. The composition of nutrients of each basal diet was planned to satisfy nutritional requirements of chicken according to National research council. Feed and water were available *ad libitum* during the entire experiment. Broilers were weighed at 1, 10, 21, 32, and 42 days of age. As growth performance parameters, average body weight (BW), average daily gain (ADG) and average daily feed intake (ADFI) were calculated. Mortality was monitored, and it was low (0.56%) so no veterinary interventions were required.

Table 1
Ingredients and chemical composition of the basal diet.

Ingredients	Diets			
	Pre-Starter (Day 1–9)	Starter (Day 10–21)	Grower (Day 22–31)	Finisher (Day 32–42)
Corn, %	33	34	33	32
Wheat, %	27	29	31	32
Soybean meal, solvent extracted (46.0% CP), %	29	24	20	16
Soybean meal, extruded (46.0% CP), %	4	6	4	4
Sunflower meal, extracted, %	-	1	3	4
Feed yeast, %	1	-	-	-
Distillers dried grains with solubles, %	-	1	3	5
Plant fats, %	2	1	3	4
Premix, %	4	4	3	3
Total, %	100	100	100	100
Energy and nutrient contents of the diets				
Dry matter, %	89.06	89.03	89.15	89.15
AME_n poultry, MJ/kg	12.23	12.47	12.81	13.01
Crude protein, %	21.58	20.28	19.05	18.28
Crude fat, %	4.61	4.83	6.22	6.83
Crude fiber, %	3.37	3.51	3.7	3.88
Lysine, %	1.37	1.27	1.17	1.09
Methionine, %	0.57	0.54	0.53	0.49
Methionine + Cysteine, %	0.94	0.9	0.87	0.83
Calcium, %	0.85	0.73	0.71	0.67
Phosphorus, %	0.63	0.55	0.52	0.49
Phosphorus utilization, %	0.45	0.42	0.40	0.35
Sodium, %	0.17	0.16	0.16	0.16
Sodium-chloride, %	0.282	0.252	0.242	0.244

Ingredients	Diets			
	Pre-Starter (Day 1–9)	Starter (Day 10–21)	Grower (Day 22–31)	Finisher (Day 32–42)
Vitamin A, mg/kg	12500.250	12500.250	12500.250	8750.175
Vitamin D3, mg/kg	3000.05	3000.05	3000.05	2100.035
Vitamin E, mg/kg	50.001	50.001	50.001	35
Lasalocid-Sodium, mg/kg	82.500	82.500	82.500	

Determination Of Natural Feed Additives

Carotenoids (TS3) supplement was determined as Remenyik *et al*.[162] and Csernus *et al*.[52] Carotenoids were extracted from Hungarian red sweet pepper powder (in 1–5 g) using dichloroethane:acetone:methanol as solvent mixture in 2:2:1ratio. The mixture was stirred in an ultrasonic water bath for 30 minutes and purified through a Munktell-292 filter paper (VWR International, Debrecen, Hungary). For further purification 0.22 µm PTFE syringe filter (TPP Techno Plastic Products AG, Switzerland) was applied. Afterwards, filtered sample was vaporized at 40 ºC at 0.2 bar and then it was solved in a high-performance liquid chromatographic (HPLC) pigment reagent (isopropanol:acetonitrile:methanol in 55:35:10 proportion) (Merck, Darmstadt, Germany). The HPLC separation was conducted on Phenomenex Kinetex® column (2.6 µm, XB-C18, 100 A, 100 × 4.6 mm) (Phenomenex, Torrance, CA, USA) with the following two gradients elution: A: 11% methanol, B: isopropanol:acetonitrile:methanol (55:35:10 V/V/V%) mixture. Gradient elution was performed with the following settings: 0–3 minutes solvent A 100%; 15–20 minutes solvent A 20%; 25–45 minutes solvent B 100%; 48–50 minutes solvent A 100%. For detection Diode Array Detector (DAD) was applied with 0.6 ml/minutes flow rate. Sample was injected in 10 µL volume and DAD detection was applied at 460 and 350 nm. The HPLC profile and carotenoids compounds with the greatest identified areas are involved in additional file [see Additional file 1].

Fructooligosaccharides supplement (TS4) was determined as Csernus *et. al*.[52] Hungarian red sweet pepper was also applied to extract fructooligosaccharides (FOSs) with high arabino-galactose content. To assess the composition of oligosaccharides HP 5890 Gas chromatograph was applied with SP-2380 capillary column (30 m x 0.25 mm, 0.2 µm). Samples were lyophilized and extracted with trifluoracetic acid:acetic acid:water in 5:75:20 proportion as solvent. Oligosaccharides were turned into alditol-acetate. After reduction step, sugars were shifted to sugar alcohols (alditols), which remove interfering isomers and anomers. Reduction was performed with NaBH4 at alkine pH. Acetylation was also performed with acetic anhydride in pyridine. The feed gas was nitrogen at 1.2 mL/min flow rate. The injector temperature was set to 300 ºC and split ratio was 1:20. Flame Ionization Detector (FID) was used for identification of

oligosaccharides. The GC profile and the identified monomer units of oligosaccharides are involved in additional file [see Additional file 2]

The synbiotics supplement (TS5) contained probiotics (*Bifidobakterium bifidum, B.infantis, B.lactis, B.longum, Lactobacillus acidophylus, L.buchneri L.casei, L.paracasei, L.plantarum, L.salivarius, L.lactis*), prebiotics (Fructo-xylo-, mannooligosaccharide and arabinogalactan), vitamins (B group vitamins, vitamin C, D2, D3, E and K2), unsaturated fatty acids (ω-3,ω-6, ω-9) mineral and trace element contents (Sodium, Potassium, Calcium, Iodine and Phosphorous) and lactose. The GC profile and the identified monomer units of oligosaccharides are involved in additional file [see Additional file 3].

Anthocyanins supplement (TS6) was determined as Nemes *et al*.[163] Anthocyanins were extracted from Hungarian sour cherry. Cherries were deseeded and homogenised, then methanol:water:acetic acid solution in 25:24:1 ratio was utilized to extract anthocyanins. The sample was mixed with Magnetic stirrer MSH 300 (BioSan, Riga, Latvia) through 1 hour. Filtering and centrifugation was performed at 10.000 RPM for 5 min, then a simple fraction was carried out in pre-conditioned tubes (Superclean ENVI-18 SPE tubes). For pre-conditioning 5 mL MeOH, 5 mL H2O, then 1 mL of fruit sample was used. The elution was conducted with methanol containing 20% H2O and vaporized at 40 ºC. Sample was dried in vacuum to reach a powder formula. VWR-Hitachi ChromasterUltraRs UHPLC (Hitachi, Tokyo, Japan) was used to anthocyanin profile determination using a Phenomenex Kinetex ® column (2.6 µm, XB-C18, 100 A, 100 × 4.6 mm) (Phenomex, Torrance, CA, USA). Two solvents were applied for gradient elution A: MeOH and B:3% formic acid with the following parameters: 0 min solvent A 15%; 0–25 min solvent A 30%; 25–30 min solvent A 40%; 30–40 min solvent A 50%. UV-VIS detection was applied at 534 nm and flow rate was kept at 0.7 mL/min on 25ºC and the injection volume was 10 µL. UHPLC profile and the main anthocyanins compounds are involved in additional file [see Additional file 4].

Sample Collection

Stool samples were collected on day 7, 19, 31, 40 of age. In every treatment group 6 broilers (3 pullets and 3 cockerels) were marked and faecal samples were collected from them during the whole experimental period. Pooled faecal samples were also collected in the case of all our experimental groups. Stool samples were collected freshly into specific, sterile, DNase free stool transportation bowls and were immediately placed on ice for maximum 3 hours. Not processed samples were kept at -80 °C until further use.

Sample Preparation And Mechanical Cell Lyses

Bacterial cell suspensions (BS) were prepared from 7–7 g broiler stool samples. 7–7 ml of sterile PBS buffers (Thermo Fisher Scientific, Maryland, USA) were added to the samples and homogenized for 4 min (by vortexing at 350 RPM) [164]. The samples were centrifuged for 5 min at 500 x g. Supernatants were collected and the washing step was repeated 2 times. Supernatants were centrifuged for 20 min at

13.000 x g. Finally, the supernatants were discarded, and the bacterial pellets were dissolved in 3 ml of sterile PBS buffer. 1 ml aliquots of BS were added to PowerBead Tubes (Qiagen, Hilden, Germeny) for mechanical cell lyses. Bacterial cell disruption was performed with MagNa Lyser Instrument (Roche Applied Sxciences; Penzberg, Germeny) set to 5000 x rpm for 30 seconds.

Dna Extraction

Total bacterial genomic DNA was extracted with conventional isolation method. 800 µl sample lysate was mixed with 800 µl of phenol:chloroform:isoamyl alcohol (25:24:1) (Thermo Fisher Scientific, Maryland, USA), and vortexed thoroughly for approximately 20 seconds. After homogenization samples were incubated at room temperature for 3 minutes and centrifuged for 10 minutes at 16.000 x g. After phase separation the upper aqueous layer was carefully collected into a new sterile DNase and RNase free Eppendorf tube. For DNA precipitation a mixture of 1 µL glycogen (20 µg), 7.5 M NH_4OAc (ammonium acetate in 0.5 x volume of the sample) and 100% EtOH (ethanol in 2.5 x volume of the sample) was added to the supernatant. The samples were incubated at -20 °C for overnight, then centrifuged for 30 minutes at 16.000 x g at 4° C to pellet the DNA. The supernatant was carefully discarded without disturbing the pellet and 70% EtOH was added to the sample and shaked by hand for 20 seconds. After that samples were centrifuged at 4 °C for 5 minutes at 16.000 x g and the supernatant was carefully removed. This washing step was repeated 2 times. The DNA pellet was dried at room temperature, and then resuspended in 40 µl of nuclease free water. DNA concentrations were determined using Qubit® Fluorometric Quantitation dsDNA assay kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States) on Clariostar microplate reader (BMG Labtech, Ortenberg, Germany). DNA quantity and quality were ascertained using Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific). DNA integrity (shearing/fragmentation) was measured on a 4200 TapeStation System, (G2991AA, Agilent Technologies; Santa Clara, California, United States). The DNA elutes were stored at −20 °C.

Negative and positive controls. To minimize laboratory contamination sterile surgical gloves and face masks were used and all DNA extraction steps were performed with sterile or sterilized equipment under a class II laminar air-flow cabinet. Negative isolation control (NIC) experiments were simultaneously conducted by substituting samples with PCR grade water. Elutes of the NIC samples were conveyed for V3-V4 amplicon - PCR and indexing was performed under DNA free UV sterilized AirClean® PCR workstations/cabinets. At each PCR clean-up steps of the library preparation NIC amplicons were also validated on 4200 Tape Station System (G2991AA, Agilent Technologies; Santa Clara, California, United States) using Agilent D1000 ScreenTapes (5067–5365) and Agilent Genomic DNA reagents. Host background nucleic acid contaminations were also monitored with real-time PCR using GAPDH assay on eluted gDNAs.

Library Construction And Sequencing

Standard library preparation was performed according to Illumina (San Diego, California, United States) 16S Metagenomic Sequencing Library Preparation protocol (15044223 Rev. B). The V3 and V4 hypervariable regions of bacterial 16S rRNA gene were sequenced with Illumina MiSeq benchtop sequencer generating amplicons of ~ 460 by using the universal primer set: 341F-5' CCTACGGGNGGCWGCAG 3'and 785R-5' GACTACHVGGGTATCTAATCC 3' primers flanked by Illumina overhang adapter sequences (forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG 3', reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG 3') (Sigma Aldrich, Missouri, US). After completion of the amplicon PCR with 2 x KAPA HiFi HotStart ReadyMix dual indexing of the samples with adaptor sequences (i7-N7xx-12 items, i5-S5xx-8 items) was performed using the Illumina Nextera XT Index Kit (FC-131-1001/2). PCR cleanups and amplicon size selections were carried out with KAPA Pure Beads (KAPA Biosystems) based on the technical data sheet (KR1245 – v3.16) of the manufacturer resulting in final ~ 580–630 bp libraries. Every time, verifications were done with PCR Agilent D1000 screen tapes (5067–5582) and D1000 Reagents (5067–5583). The 16S amplicon libraries for each sample were quantified with qPCR, normalized with respect to amplicon sizes and pooled into a single library in equal molar quantities. Finally, 5 µl of pooled 4 nM DNA library pool was prepared for sequencing on Illumina MiSeq platform. The library pool was denatured with 0.2 M NaOH and diluted to 8 pM final concentration. Sequencing was carried out with MiSeq Reagent Kit v3–618 cycle (MS-102-3003) following manufacturer's protocols (Illumina, Inc., San Diego, CA, USA). Paired-end sequencing (2 × 301 nt) was performed on Illumina MiSeq platform with 5% PhiX spike-in quality control (PhiX Control Kit v3 - FC-110-3001).

Sequence Processing And Analysis

The Illumina BaseSpace software was used to demultiplex the paired end reads and construct FASTQ files. The sequencing data were analysed using the Quantitative Insight Into Microbial Ecology (Qiime 2, ver 2019.7) [165]. The presence of adapter sequences (CTGTCTCTTATACACATCT) were checked and trimmed from the 3' end of the reads with Cutadapt Software integrated in the Qiime 2 pipeline. DADA2 software was used for quality trimming, filtering and for chimera removal. Sequences were clustered into ASVs, with 97% similarities in sequences [166]. The trimming parameters were set as follows: for the forward reads 1 bases were cropped from the start and the length was set to 300 bases; for the reverse reads 9 bases were cropped from the start of the reads and the length was set to 223 bases.

Bioinformatic Analyses

Multiple sequence alignment was performed with the Mafft software [167], and reads were taxonomically classified using Naïve Bayesian classifier trained on the Silva (ver132) [168] reference database by selecting mapping points according to the forward-reverse primer set that was used for amplifying the 16S rRNA gene's V3-V4 regions of the bacterial community (341F, 806R). Phylogenetic trees were constructed with FastTree plugin [169]. The QIIME2 pipeline was applied to perform alpha and beta

diversity tests. For sample normalization a 11 500 read depth was set. In the case of alpha diversity Shannon's index [170], Faith's phylogenetic diversity index [171], Simpson evenness [172], and Chao-1 index [173] were calculated in the QIIME2 pipeline. For beta diversity analysis weighted/unweighted UNIFRAC distances [174] and Bray-Curtis dissimilarities [175] were measured. Alpha diversity differences were compared using the Kruskal-Wallis test. Beta diversity group significances were calculated with Permutational multivariate analysis of variance (PERMANOVA) pseudo F statistical test. QIIME2 artifact files were exported from the pipeline and converted to TSV files which were used with different visualization packages. Heatmaps were generated in Python (ver3.6.5) with Seaborn package; area, donout plots were constructed with pandas and matplotlib packages. Boxplots, violin plots and line plots were constructed using GraphPad Prism statistical software. R (ver 3.6.2) was used to visualize bubble plots and polar plots. Differential heat tree was created with the metacoder R package [176]. In the case of differential heat trees differences were determined using a Wilcoxon rank-sum test. LEfSe analysis was performed with bioBakery tools developed by Huttenhower lab [177]. Spearman correlation matrices were calculated and visualised with R statistical software using the corrplot package (https://github.com/taiyun/corrplot).

Statistical Analysis Of Growth Performance

The main effects of the bioactive compounds on growth performance was analyzed using one-way analysis of variance (one-way ANOVA), Tukey's multiple comparison test was conducted at significance level of $P < 0.05$.

Abbreviations

ADFI
Average daily feed intake
ADG
Average daily gain
ASV
amplicon sequence variant
BD
Basal diet
BS
Bacterial cell suspension
BSH
Bile salt hydrolase
BW
Average body weight
CS1
Control Set 1

CS2
Control Set 2
DAD
Diode array detector
F/B ratio
Firmicutes to Bacteroides ratio
Faith’s PD
Faith’s phylogenetic diversity
FOS
Fructooligosacharide
GC
Gas chromatography
GIT
Gastrointestinal tract
HPLC
High performance liquid chromatography
LDA
Linear discriminant analysis
LEfSe
Effect size
NIC
negative isolation control
SCFA
Short-chain fatty acid
TS3
Basal diet with carotenoid supplements
TS4
Basal diet with fructooligosacharid supplements
TS5
Basal diet with synbiotic supplements
TS6
Basal diet with anthocyanin supplements
WHO
World Health Organization

Declarations

Availability of data and materials

All sequence data used in the analyses were deposited in the Sequence read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra) under SUB7398678 Sample IDs, meta data and corresponding accession numbers are summarized in Supplementary figure S1.

Ethics approval

Sampling procedures were carried out in accordance with the ethics committee's approved guidelines (DEMAB/12 – 7/2015).

Consent for publication

Not applicable.

Competing interests

The authors declared that they have no competing interests.

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References

1. Clavijo V, Flórez MJV. The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: A review. Poult Sci. 2018;97:1006–21.
2. Lan Y, Verstegen MWA, Tamminga S, Williams BA. The role of the commensal gut microbial community in broiler chickens. Worlds Poult Sci J. 2005;61:95–104.
3. Borda-Molina D, Seifert J, Camarinha-Silva A. Current Perspectives of the Chicken Gastrointestinal Tract and Its Microbiome. Comput Struct Biotechnol J. 2018;16:131–9.
4. Tickle PG, Hutchinson JR, Codd JR. Energy allocation and behaviour in the growing broiler chicken. Sci Rep. 2018;8:1–13.
5. Bornelöv S, Seroussi E, Yosefı S, Benjamini S, Miyara S, Ruzal M, et al. Comparative omics and feeding manipulations in chicken indicate a shift of the endocrine role of visceral fat towards reproduction. BMC Genom. 2018;19:295.

6. Wilson JB, Julian RJ, Barker IK. Lesions of right heart failure and ascites in broiler chickens. Avian Dis. 1988;32:246–61.
7. Paxton H, Anthony NB, Corr SA, Hutchinson JR. The effects of selective breeding on the architectural properties of the pelvic limb in broiler chickens: a comparative study across modern and ancestral populations. J Anat. 2010;217:153–66.
8. Wideman RF, Chapman ME, Hamal KR, Bowen OT, Lorenzoni AG, Erf GF, et al. An inadequate pulmonary vascular capacity and susceptibility to pulmonary arterial hypertension in broilers. Poult Sci. 2007;86:984–98.
9. Julian RJ. Ascites in poultry. Avian Pathol J WVPA. 1993;22:419–54.
10. Shiraishi J-I, Yanagita K, Fukumori R, Sugino T, Fujita M, Kawakami S-I, et al. Comparisons of insulin related parameters in commercial-type chicks: Evidence for insulin resistance in broiler chicks. Physiol Behav. 2011;103:233–9.
11. Cheema MA, Qureshi MA, Havenstein GB. A comparison of the immune response of a 2001 commercial broiler with a 1957 randombred broiler strain when fed representative 1957 and 2001 broiler diets. Poult Sci. 2003;82:1519–29.
12. Allen HK, Stanton TB. Altered egos: antibiotic effects on food animal microbiomes. Annu Rev Microbiol. 2014;68:297–315.
13. Ben Lagha A, Haas B, Gottschalk M, Grenier D. Antimicrobial potential of bacteriocins in poultry and swine production. Vet Res. 2017;48:22.
14. Castanon JIR. History of the use of antibiotic as growth promoters in European poultry feeds. Poult Sci. 2007;86:2466–71.
15. Shea KM. Antibiotic resistance: what is the impact of agricultural uses of antibiotics on children's health? Pediatrics. 2003;112:253–8.
16. van der Waaij D, Nord CE. Development and persistence of multi-resistance to antibiotics in bacteria; an analysis and a new approach to this urgent problem. Int J Antimicrob Agents. 2000;16:191–7.
17. Jin LZ, Ho YW, Abdullah N, Jalaludin S. Growth performance, intestinal microbial populations, and serum cholesterol of broilers fed diets containing Lactobacillus cultures. Poult Sci. 1998;77:1259–65.
18. Grindstaff JL, Brodie ED, Ketterson ED. Immune function across generations: integrating mechanism and evolutionary process in maternal antibody transmission. Proc Biol Sci. 2003;270:2309–19.
19. Hamal KR, Burgess SC, Pevzner IY, Erf GF. Maternal antibody transfer from dams to their egg yolks, egg whites, and chicks in meat lines of chickens. Poult Sci. 2006;85:1364–72.
20. Oakley BB, Lillehoj HS, Kogut MH, Kim WK, Maurer JJ, Pedroso A, et al. The chicken gastrointestinal microbiome. FEMS Microbiol Lett. 2014;360:100–12.
21. Torok VA, Ophel-Keller K, Loo M, Hughes RJ. Application of Methods for Identifying Broiler Chicken Gut Bacterial Species Linked with Increased Energy Metabolism. Appl Environ Microbiol. 2008;74:783–91.

22. Mohd Shaufi MA, Sieo CC, Chong CW, Gan HM, Ho YW. Deciphering chicken gut microbial dynamics based on high-throughput 16S rRNA metagenomics analyses. Gut Pathog. 2015;7:4.
23. Kers JG, Velkers FC, Fischer EAJ, Hermes GDA, Stegeman JA, Smidt H. Host and Environmental Factors Affecting the Intestinal Microbiota in Chickens. Front Microbiol. 2018;9:235.
24. Forder REA, Howarth GS, Tivey DR, Hughes RJ. Bacterial modulation of small intestinal goblet cells and mucin composition during early posthatch development of poultry. Poult Sci. 2007;86:2396–403.
25. Brisbin JT, Gong J, Sharif S. Interactions between commensal bacteria and the gut-associated immune system of the chicken. Anim Health Res Rev. 2008;9:101–10.
26. Mwangi WN, Beal RK, Powers C, Wu X, Humphrey T, Watson M, et al. Regional and global changes in TCRalphabeta T cell repertoires in the gut are dependent upon the complexity of the enteric microflora. Dev Comp Immunol. 2010;34:406–17.
27. Sommer F, Bäckhed F. The gut microbiota–masters of host development and physiology. Nat Rev Microbiol. 2013;11:227–38.
28. Stanley D, Hughes RJ, Moore RJ. Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. Appl Microbiol Biotechnol. 2014;98:4301–10.
29. Stanley D, Denman SE, Hughes RJ, Geier MS, Crowley TM, Chen H, et al. Intestinal microbiota associated with differential feed conversion efficiency in chickens. Appl Microbiol Biotechnol. 2012;96:1361–9.
30. Gordon HA, Pesti L. The gnotobiotic animal as a tool in the study of host microbial relationships. Microbiol Mol Biol Rev. 1971;35:390–429.
31. Apajalahti J, Kettunen A, Graham H. Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. Worlds Poult Sci J. 2004;60:223–32.
32. Nava GM, Bielke LR, Callaway TR, Castañeda MP. Probiotic alternatives to reduce gastrointestinal infections: the poultry experience. Anim Health Res Rev. 2005;6:105–18.
33. Diaz Carrasco JM, Casanova NA, Fernández Miyakawa ME. Microbiota. Gut Health and Chicken Productivity: What Is the Connection? Microorganisms. 2019;7.
34. Li X, Cao Z, Yang Y, Chen L, Liu J, Lin Q, et al. Correlation between Jejunal Microbial Diversity and Muscle Fatty Acids Deposition in Broilers Reared at Different Ambient Temperatures. Sci Rep. 2019;9:1–12.
35. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 2006;444:1027–31.
36. Hou Q, Kwok L-Y, Zheng Y, Wang L, Guo Z, Zhang J, et al. Differential fecal microbiota are retained in broiler chicken lines divergently selected for fatness traits. Sci Rep. 2016;6:37376.
37. Samuel BS, Hansen EE, Manchester JK, Coutinho PM, Henrissat B, Fulton R, et al. Genomic and metabolic adaptations of Methanobrevibacter smithii to the human gut. Proc Natl Acad Sci U S A. 2007;104:10643–8.

38. Larsen N, Vogensen FK, Berg FWJ, van den, Nielsen DS, Andreasen AS, Pedersen BK, et al. Gut Microbiota in Human Adults with Type 2 Diabetes Differs from Non-Diabetic Adults. PLOS ONE. 2010;5:e9085.
39. Zhang C, Zhang M, Wang S, Han R, Cao Y, Hua W, et al. Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. ISME J. 2010;4:232–41.
40. Palau-Rodriguez M, Tulipani S, Isabel Queipo-Ortuño M, Urpi-Sarda M, Tinahones FJ, Andres-Lacueva C. Metabolomic insights into the intricate gut microbial–host interaction in the development of obesity and type 2 diabetes. Front Microbiol. 2015;6:1151.
41. Toivanen P. Normal intestinal microbiota in the aetiopathogenesis of rheumatoid arthritis. Ann Rheum Dis. 2003;62:807–11.
42. Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proc Natl Acad Sci. 2005;102:11070–5.
43. Zhang C, Zhang M, Pang X, Zhao Y, Wang L, Zhao L. Structural resilience of the gut microbiota in adult mice under high-fat dietary perturbations. ISME J. 2012;6:1848–57.
44. Bäckhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, et al. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A. 2004;101:15718–23.
45. Bai SP, Wu AM, Ding XM, Lei Y, Bai J, Zhang KY, et al. Effects of probiotic-supplemented diets on growth performance and intestinal immune characteristics of broiler chickens. Poult Sci. 2013;92:663–70.
46. Neijat M, Habtewold J, Shirley RB, Welsher A, Barton J, Thiery P, et al. Bacillus subtilis Strain DSM 29784 Modulates the Cecal Microbiome, Concentration of Short-Chain Fatty Acids, and Apparent Retention of Dietary Components in Shaver White Chickens during Grower, Developer, and Laying Phases. Appl Environ Microbiol [Internet]. American Society for Microbiology; 2019;85:e00402-19.
47. Barbara AJ, Trinh HT, Glock RD, Glenn Songer J. Necrotic enteritis-producing strains of Clostridium perfringens displace non-necrotic enteritis strains from the gut of chicks. Vet Microbiol. 2008;126:377–82.
48. Moore RJ. Necrotic enteritis predisposing factors in broiler chickens. Avian Pathol J WVPA. 2016;45:275–81.
49. Antonissen G, Eeckhaut V, Van Driessche K, Onrust L, Haesebrouck F, Ducatelle R, et al. Microbial shifts associated with necrotic enteritis. Avian Pathol J WVPA. 2016;45:308–12.
50. 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.
51. Barreto MSR, Menten JFM, Racanicci AMC, Pereira PWZ, Rizzo PV. Plant extracts used as growth promoters in broilers. Braz J Poult Sci Fundação APINCO de Ciência e Tecnologia Avícolas. 2008;10:109–15.
52. Csernus B, Biró S, Babinszky L, Komlósi I, Jávor A, Stündl L, et al. Effect of Carotenoids, Oligosaccharides and Anthocyanins on Growth Performance, Immunological Parameters and

Intestinal Morphology in Broiler Chickens Challenged with Escherichia coli Lipopolysaccharide. Animals (Basel). 2020;10:347.

53. Shang Y, Kumar S, Thippareddi H, Kim WK. Effect of Dietary Fructooligosaccharide (FOS) Supplementation on Ileal Microbiota in Broiler Chickens. Poult Sci. 2018;97:3622–34.
54. Cengiz Ö, Köksal BH, Tatlı O, Sevim Ö, Ahsan U, Üner AG, et al. Effect of dietary probiotic and high stocking density on the performance, carcass yield, gut microflora, and stress indicators of broilers. Poult Sci. 2015;94:2395–403.
55. Sarangi NR, Babu LK, Kumar A, Pradhan CR, Pati PK, Mishra JP. Effect of dietary supplementation of prebiotic, probiotic, and synbiotic on growth performance and carcass characteristics of broiler chickens. Vet World. 2016;9:313–9.
56. Delzenne N, Aertssens J, Verplaetse H, Roccaro M, Roberfroid M. Effect of fermentable fructo-oligosaccharides on mineral, nitrogen and energy digestive balance in the rat. Life Sci. 1995;57:1579–87.
57. Porcheron G, Chanteloup NK, Trotereau A, Brée A, Schouler C. Effect of fructooligosaccharide metabolism on chicken colonization by an extra-intestinal pathogenic Escherichia coli strain. PloS One. 2012;7:e35475.
58. Drywień M, Frąckiewicz J, Górnicka M, Gadek J, Jałosińska M. Effect of probiotic and storage time of thiamine and riboflavin content in the milk drinks fermented by Lactobacillus casei KNE-1. Rocz Panstw Zakl Hig. 2015;66:373–7.
59. Waititu SM, Yitbarek A, Matini E, Echeverry H, Kiarie E, Rodriguez-Lecompte JC, et al. Effect of supplementing direct-fed microbials on broiler performance, nutrient digestibilities, and immune responses. Poult Sci. 2014;93:625–35.
60. Knarreborg A, Simon MA, Engberg RM, Jensen BB, Tannock GW. Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages. Appl Environ Microbiol. 2002;68:5918–24.
61. Park YH, Hamidon F, Rajangan C, Soh KP, Gan CY, Lim TS, et al. Application of Probiotics for the Production of Safe and High-quality Poultry Meat. Korean J Food Sci Anim Resour. 2016;36:567–76.
62. Macfarlane GT, Macfarlane S. Bacteria, colonic fermentation, and gastrointestinal health. J AOAC Int. 2012;95:50–60.
63. Wang H-B, Wang P-Y, Wang X, Wan Y-L, Liu Y-C. Butyrate enhances intestinal epithelial barrier function via up-regulation of tight junction protein Claudin-1 transcription. Dig Dis Sci. 2012;57:3126–35.
64. Eeckhaut V, Van Immerseel F, Croubels S, De Baere S, Haesebrouck F, Ducatelle R, et al. Butyrate production in phylogenetically diverse Firmicutes isolated from the chicken caecum. Microb Biotechnol. 2011;4:503–12.
65. Eeckhaut V, Van Immerseel F, Teirlynck E, Pasmans F, Fievez V, Snauwaert C, et al. Butyricicoccus pullicaecorum gen. nov., sp. nov., an anaerobic, butyrate-producing bacterium isolated from the caecal content of a broiler chicken. Int J Syst Evol Microbiol. 2008;58:2799–802.

66. Liu Y, Balkwill DL, Aldrich HC, Drake GR, Boone DR. Characterization of the anaerobic propionate-degrading syntrophs Smithella propionica gen. nov., sp. nov. and Syntrophobacter wolinii. Int J Syst Bacteriol. 1999;49 Pt 2:545–56.
67. Blanchfield B, Gardiner MA, Pivnick H. Nurmi Concept for Preventing Infection of Chicks by Salmonella: Comparison of Fecal Suspensions and Fecal Cultures Administered into the Crop and in Drinking Water. J Food Prot. 1982;45:345–7.
68. Pandey KR, Naik SR, Vakil BV. Probiotics, prebiotics and synbiotics- a review. J Food Sci Technol. 2015;52:7577–87.
69. Bonnet M, Mailhe M, Ricaboni D, Labas N, Richez M, Vitton V, et al. Genome sequence and description of Traorella massiliensis gen. nov., sp. nov., a new bacterial genus isolated from human left colon. New Microbes New Infect. 2019;29:100520.
70. Irisawa T, Saputra S, Kitahara M, Sakamoto M, Sulistiani null, Yulineri T, et al. Bacteroides caecicola sp. nov. and Bacteroides gallinaceum sp. nov., isolated from the caecum of an Indonesian chicken. Int J Syst Evol Microbiol. 2016;66:1431–7.
71. Kim H-J, Eom S-J, Park S-J, Cha C-J, Kim G-B. Lactobacillus alvi sp. nov., isolated from the intestinal tract of chicken. FEMS Microbiol Lett. 2011;323:83–7.
72. Spanier AM, Shahidi F, Parliment TH, Mussinan C, Ho C-T, Contis ET, et al. Production of short chain fatty acid esters by pseudomonas fragi CRDA 037 grown on a synthetic medium. Food Flavors Chem. 2001; 318–27.
73. Park SH, Lee SI, Ricke SC. Microbial Populations in Naked Neck Chicken Ceca Raised on Pasture Flock Fed with Commercial Yeast Cell Wall Prebiotics via an Illumina MiSeq Platform. PLOS ONE. 2016;11:e0151944.
74. Hill TCJ, Walsh KA, Harris JA, Moffett BF. Using ecological diversity measures with bacterial communities. FEMS Microbiol Ecol. 2003;43:1–11.
75. Komaroff AL. The Microbiome and Risk for Obesity and Diabetes. JAMA. 2017;317:355–6.
76. O'Hara AM, Shanahan F. Gut microbiota: mining for therapeutic potential. Clin Gastroenterol Hepatol Off Clin Pract J Am Gastroenterol Assoc. 2007;5:274–84.
77. Bogusławska-Tryk M, Piotrowska A, Burlikowska K. Dietary fructans and their potential beneficial influence on health and performance parameters in broiler chickens. J Cent Eur Agric. 2012;13:272–91.
78. Zeng Z, Zhang S, Wang H, Piao X. Essential oil and aromatic plants as feed additives in non-ruminant nutrition: a review. J Anim Sci Biotechnol. 2015;6:7.
79. Feng W, Ao H, Peng C. Gut Microbiota, Short-Chain Fatty Acids, and Herbal Medicines. Front Pharmacol. 2018;9:1354.
80. Saulnier DMA, Molenaar D, de Vos WM, Gibson GR, Kolida S. Identification of Prebiotic Fructooligosaccharide Metabolism in Lactobacillus plantarum WCFS1 through Microarrays. Appl Environ Microbiol. 2007;73:1753–65.

81. Polansky O, Sekelova Z, Faldynova M, Sebkova A, Sisak F, Rychlik I. Important Metabolic Pathways and Biological Processes Expressed by Chicken Cecal Microbiota. Appl Environ Microbiol. 2015;82:1569–76.
82. Meimandipour A, Shuhaimi M, Hair-Bejo M, Azhar K, Kabeir BM, Rasti B, et al. In vitro fermentation of broiler cecal content: the role of lactobacilli and pH value on the composition of microbiota and end products fermentation. Lett Appl Microbiol. 2009;49:415–20.
83. Yoshii K, Hosomi K, Sawane K, Kunisawa J. Metabolism of Dietary and Microbial Vitamin B Family in the Regulation of Host Immunity. Front Nutr. 2019;6:48.
84. Cani PD, Hul MV, Lefort C, Depommier C, Rastelli M, Everard A. Microbial regulation of organismal energy homeostasis. Nat Metab. 2019;1:34–46.
85. Clarke G, Stilling RM, Kennedy PJ, Stanton C, Cryan JF, Dinan TG. Minireview: Gut Microbiota: The Neglected Endocrine Organ. Mol Endocrinol. 2014;28:1221–38.
86. Zhu N, Wang J, Yu L, Zhang Q, Chen K, Liu B. Modulation of Growth Performance and Intestinal Microbiota in Chickens Fed Plant Extracts or Virginiamycin. Front Microbiol. 2019;10:1333.
87. Danzeisen JL, Kim HB, Isaacson RE, Tu ZJ, Johnson TJ. Modulations of the Chicken Cecal Microbiome and Metagenome in Response to Anticoccidial and Growth Promoter Treatment. PLOS ONE. 2011;6:e27949.
88. Flickinger EA, Van Loo J, Fahey GC. Nutritional responses to the presence of inulin and oligofructose in the diets of domesticated animals: a review. Crit Rev Food Sci Nutr. 2003;43:19–60.
89. Bodet C, Grenier D, Chandad F, Ofek I, Steinberg D, Weiss EI. Potential oral health benefits of cranberry. Crit Rev Food Sci Nutr. 2008;48:672–80.
90. Pourabedin M, Zhao X. Prebiotics and gut microbiota in chickens. FEMS Microbiol Lett. 2015;362:fnv122.
91. Liu F, Li P, Chen M, Luo Y, Prabhakar M, Zheng H, et al. Fructooligosaccharide (FOS) and Galactooligosaccharide (GOS) Increase Bifidobacterium but Reduce Butyrate Producing Bacteria with Adverse Glycemic Metabolism in healthy young population. Sci Rep. 2017;7:11789.
92. Li X, Liu L, Li K, Hao K, Xu C. Effect of fructooligosaccharides and antibiotics on laying performance of chickens and cholesterol content of egg yolk. Br Poult Sci. 2007;48:185–9.
93. Ford AC, Quigley EMM, Lacy BE, Lembo AJ, Saito YA, Schiller LR, et al. Efficacy of prebiotics, probiotics, and synbiotics in irritable bowel syndrome and chronic idiopathic constipation: systematic review and meta-analysis. Am J Gastroenterol. 2014;109:1547–61. quiz 1546, 1562.
94. Gong J, Yu H, Liu T, Gill JJ, Chambers JR, Wheatcroft R, et al. Effects of zinc bacitracin, bird age and access to range on bacterial microbiota in the ileum and caeca of broiler chickens. J Appl Microbiol. 2008;104:1372–82.
95. Ruggiero P. Use of probiotics in the fight against Helicobacter pylori. World J Gastrointest Pathophysiol. 2014;5:384–91.

96. Pandit RJ, Hinsu AT, Patel NV, Koringa PG, Jakhesara SJ, Thakkar JR, et al. Microbial diversity and community composition of caecal microbiota in commercial and indigenous Indian chickens determined using 16 s rDNA amplicon sequencing. Microbiome. 2018;6:115.
97. Djeffal S, Mamache B, Elgroud R, Hireche S, Bouaziz O. Prevalence and risk factors for Salmonella spp. contamination in broiler chicken farms and slaughterhouses in the northeast of Algeria. Vet World. 2018;11:1102–8.
98. Ibrahim RA, Cryer TL, Lafi SQ, Basha E-A, Good L, Tarazi YH. Identification of Escherichia coli from broiler chickens in Jordan, their antimicrobial resistance, gene characterization and the associated risk factors. BMC Vet Res. 2019;15:159.
99. Fasina YO, Newman MM, Stough JM, Liles MR. Effect of Clostridium perfringens infection and antibiotic administration on microbiota in the small intestine of broiler chickens. Poult Sci. 2016;95:247–60.
100. Tabo D, Diguimbaye CD, Granier SA, Moury F, Brisabois A, Elgroud R, et al. Prevalence and antimicrobial resistance of non-typhoidal Salmonella serotypes isolated from laying hens and broiler chicken farms in N'Djamena, Chad. Vet Microbiol. 2013;166:293–8.
101. El-Gedaily A, Paesold G, Chen CY, Guiney DG, Krause M. Plasmid virulence gene expression induced by short-chain fatty acids in Salmonella dublin: identification of rpoS-dependent and rpo-S-independent mechanisms. J Bacteriol. 1997;179:1409–12.
102. Ren J, Sun K, Wu Z, Yao J, Guo B. All 4 bile salt hydrolase proteins are responsible for the hydrolysis activity in Lactobacillus plantarum ST-III. J Food Sci. 2011;76:M622–8.
103. Gu X-C, Luo X-G, Wang C-X, Ma D-Y, Wang Y, He Y-Y, et al. Cloning and analysis of bile salt hydrolase genes from Lactobacillus plantarum CGMCC No. 8198. Biotechnol Lett. 2014;36:975–83.
104. Elkins CA, Moser SA, Savage DC. Genes encoding bile salt hydrolases and conjugated bile salt transporters in Lactobacillus johnsonii 100–100 and other Lactobacillus species. Microbiol Read Engl. 2001;147:3403–12.
105. Jayashree S, Pooja S, Pushpanathan M, Rajendhran J, Gunasekaran P. Identification and characterization of bile salt hydrolase genes from the genome of Lactobacillus fermentum MTCC 8711. Appl Biochem Biotechnol. 2014;174:855–66.
106. Chae JP, Valeriano VD, Kim G-B, Kang D-K. Molecular cloning, characterization and comparison of bile salt hydrolases from Lactobacillus johnsonii PF01. J Appl Microbiol. 2013;114:121–33.
107. Franz CM, Specht I, Haberer P, Holzapfel WH. Bile salt hydrolase activity of Enterococci isolated from food: screening and quantitative determination. J Food Prot. 2001;64:725–9.
108. Wijaya A, Hermann A, Abriouel H, Specht I, Yousif NMK, Holzapfel WH, et al. Cloning of the bile salt hydrolase (bsh) gene from Enterococcus faecium FAIR-E 345 and chromosomal location of bsh genes in food enterococci. J Food Prot. 2004;67:2772–8.
109. Kim G-B, Yi S-H, Lee BH. Purification and characterization of three different types of bile salt hydrolases from Bifidobacterium strains. J Dairy Sci. 2004;87:258–66.

110. Grill J, Schneider F, Crociani J, Ballongue J. Purification and Characterization of Conjugated Bile Salt Hydrolase from Bifidobacterium longum BB536. Appl Environ Microbiol. 1995;61:2577–82.
111. Rossocha M, Schultz-Heienbrok R, von Moeller H, Coleman JP, Saenger W. Conjugated bile acid hydrolase is a tetrameric N-terminal thiol hydrolase with specific recognition of its cholyl but not of its tauryl product. Biochemistry. 2005;44:5739–48.
112. Jones BV, Begley M, Hill C, Gahan CGM, Marchesi JR. Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. Proc Natl Acad Sci U S A. 2008;105:13580–5.
113. Ridlon JM, Harris SC, Bhowmik S, Kang D-J, Hylemon PB. Consequences of bile salt biotransformations by intestinal bacteria. Gut Microbes. 2016;7:22–39.
114. Swann JR, Want EJ, Geier FM, Spagou K, Wilson ID, Sidaway JE, et al. Systemic gut microbial modulation of bile acid metabolism in host tissue compartments. Proc Natl Acad Sci U S A. 2011;108(Suppl 1):4523–30.
115. Lin J. Antibiotic growth promoters enhance animal production by targeting intestinal bile salt hydrolase and its producers. Front Microbiol. 2014;5:33.
116. Hill GM, Cromwell GL, Crenshaw TD, Dove CR, Ewan RC, Knabe DA, et al. Growth promotion effects and plasma changes from feeding high dietary concentrations of zinc and copper to weanling pigs (regional study). J Anim Sci. 2000;78:1010–6.
117. Urdaneta V, Casadesús J. Interactions between Bacteria and Bile Salts in the Gastrointestinal and Hepatobiliary Tracts. Front Med. 2017;4:163.
118. Freetly HC, Dickey A, Lindholm-Perry AK, Thallman RM, Keele JW, Foote AP, et al. Digestive tract microbiota of beef cattle that differed in feed efficiency. J Anim Sci. 2020; pii:skaa008.
119. Lobionda S, Sittipo P, Kwon HY, Lee YK. The Role of Gut Microbiota in Intestinal Inflammation with Respect to Diet and Extrinsic Stressors. Microorganisms. 2019;7:271.
120. Dieterich W, Schink M, Zopf Y. Microbiota in the Gastrointestinal Tract. Med Sci. 2018;6:116.
121. Forgie AJ, Fouhse JM, Willing BP. Diet-Microbe-Host Interactions That Affect Gut Mucosal Integrity and Infection Resistance. Front Immunol. 2019;10:1802.
122. Chelakkot C, Ghim J, Ryu SH. Mechanisms regulating intestinal barrier integrity and its pathological implications. Exp Mol Med. 2018;50:103.
123. Hänninen A, Toivonen R, Pöysti S, Belzer C, Plovier H, Ouwerkerk JP, et al. Akkermansia muciniphila induces gut microbiota remodelling and controls islet autoimmunity in NOD mice. Gut. 2018;67:1445–53.
124. Chambers ES, Viardot A, Psichas A, Morrison DJ, Murphy KG, Zac-Varghese SEK, et al. Effects of targeted delivery of propionate to the human colon on appetite regulation, body weight maintenance and adiposity in overweight adults. Gut. 2015;64:1744–54.
125. Sanderson IR. Short chain fatty acid regulation of signaling genes expressed by the intestinal epithelium. J Nutr. 2004;134:2450S–2454S.

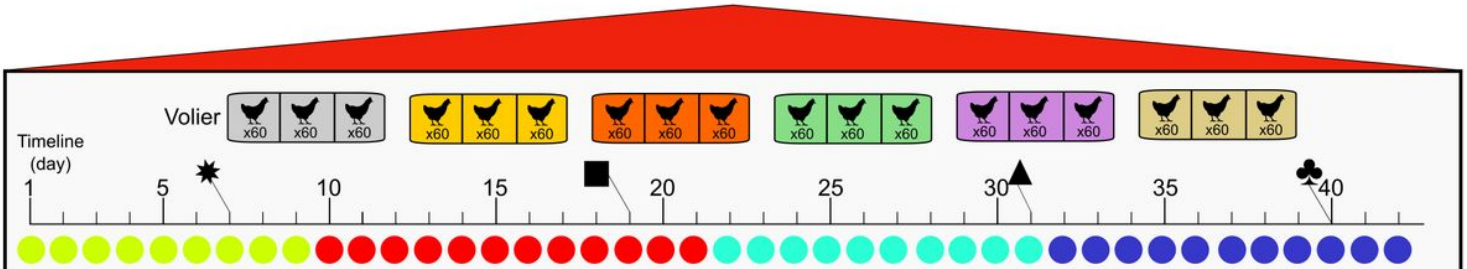
126. Kardinaal AF, Kok FJ, Ringstad J, Gomez-Aracena J, Mazaev VP, Kohlmeier L, et al. Antioxidants in adipose tissue and risk of myocardial infarction: the EURAMIC Study. Lancet Lond Engl. 1993;342:1379–84.
127. Mariat D, Firmesse O, Levenez F, Guimarães V, Sokol H, Doré J, et al. The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. BMC Microbiol. 2009;9:123.
128. Yeoman CJ, Chia N, Jeraldo P, Sipos M, Goldenfeld ND, White BA. The microbiome of the chicken gastrointestinal tract. Anim Health Res Rev. 2012;13:89–99.
129. Vázquez-Baeza Y, Gonzalez A, Smarr L, McDonald D, Morton JT, Navas-Molina JA, et al. Bringing the Dynamic Microbiome to Life with Animations. Cell Host Microbe. 2017;21:7–10.
130. Shimizu J, Kubota T, Takada E, Takai K, Fujiwara N, Arimitsu N, et al. Propionate-producing bacteria in the intestine may associate with skewed responses of IL10-producing regulatory T cells in patients with relapsing polychondritis. PloS One. 2018;13:e0203657.
131. El Hage R, Hernandez-Sanabria E, Calatayud Arroyo M, Props R, Van de Wiele T. Propionate-Producing Consortium Restores Antibiotic-Induced Dysbiosis in a Dynamic in vitro Model of the Human Intestinal Microbial Ecosystem. Front Microbiol. 2019;10:1206.
132. van der Beek CM, Dejong CHC, Troost FJ, Masclee AAM, Lenaerts K. Role of short-chain fatty acids in colonic inflammation, carcinogenesis, and mucosal protection and healing. Nutr Rev. 2017;75:286–305.
133. Pan D, Yu Z. Intestinal microbiome of poultry and its interaction with host and diet. Gut Microbes. 2014;5:108–19.
134. Zhang B, Yang X, Guo Y, Long F. Effects of dietary lipids and Clostridium butyricum on the performance and the digestive tract of broiler chickens. Arch Anim Nutr. 2011;65:329–39.
135. Lyu Y, Wu L, Wang F, Shen X, Lin D. Carotenoid supplementation and retinoic acid in immunoglobulin A regulation of the gut microbiota dysbiosis. Exp Biol Med Maywood NJ. 2018;243:613–20.
136. Crittenden R, Bird AR, Gopal P, Henriksson A, Lee YK, Playne MJ. Probiotic research in Australia, New Zealand and the Asia-Pacific region. Curr Pharm Des. 2005;11:37–53.
137. Baldwin S, Hughes RJ, Van TTH, Moore RJ, Stanley D. At-hatch administration of probiotic to chickens can introduce beneficial changes in gut microbiota. PLOS ONE. 2018;13:e0194825.
138. Ocejo M, Oporto B, Hurtado A. 16S rRNA amplicon sequencing characterization of caecal microbiome composition of broilers and free-range slow-growing chickens throughout their productive lifespan. Sci Rep. 2019;9:1–14.
139. Fernández J, Redondo-Blanco S, Gutiérrez-del-Río I, Miguélez EM, Villar CJ, Lombó F. Colon microbiota fermentation of dietary prebiotics towards short-chain fatty acids and their roles as anti-inflammatory and antitumour agents: A review. J Funct Foods. 2016;25:511–22.
140. Bäckhed F, Fraser CM, Ringel Y, Sanders ME, Sartor RB, Sherman PM, et al. Defining a Healthy Human Gut Microbiome: Current Concepts, Future Directions, and Clinical Applications. Cell Host Microbe. 2012;12:611–22.

141. Wilkins LJ, Monga M, Miller AW. Defining Dysbiosis for a Cluster of Chronic Diseases. Sci Rep. 2019;9:1–10.
142. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature. 2012;489:220–30.
143. Louis P, Flint HJ. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS Microbiol Lett. 2009;294:1–8.
144. Baxter NT, Schmidt AW, Venkataraman A, Kim KS, Waldron C, Schmidt TM. Dynamics of Human Gut Microbiota and Short-Chain Fatty Acids in Response to Dietary Interventions with Three Fermentable Fibers. mBio. 2019;10:e02566-18.
145. Keesing F, Belden LK, Daszak P, Dobson A, Harvell CD, Holt RD, et al. Impacts of biodiversity on the emergence and transmission of infectious diseases. Nature. 2010;468:647–52.
146. Kumar S, Shang Y, Kim WK. Insight Into Dynamics of Gut Microbial Community of Broilers Fed With Fructooligosaccharides Supplemented Low Calcium and Phosphorus Diets. Front Vet Sci. 2019;6:95.
147. Masuda M, Ide M, Utsumi H, Niiro T, Shimamura Y, Murata M. Production potency of folate, vitamin B(12), and thiamine by lactic acid bacteria isolated from Japanese pickles. Biosci Biotechnol Biochem. 2012;76:2061–7.
148. Etxeberria U, Fernández-Quintela A, Milagro FI, Aguirre L, Martínez JA, Portillo MP. Impact of polyphenols and polyphenol-rich dietary sources on gut microbiota composition. J Agric Food Chem. 2013;61:9517–33.
149. Yadav S, Jha R. Strategies to modulate the intestinal microbiota and their effects on nutrient utilization, performance, and health of poultry. J Anim Sci Biotechnol. 2019;10:2.
150. Teng P-Y, Kim WK. Review. Roles of Prebiotics in Intestinal Ecosystem of Broilers. Front Vet Sci. 2018;5:245.
151. van der Wielen PWJJ, Keuzenkamp DA, Lipman LJA, van Knapen F, Biesterveld S. Spatial and temporal variation of the intestinal bacterial community in commercially raised broiler chickens during growth. Microb Ecol. 2002;44:286–93.
152. Wlodarska M, Willing BP, Bravo DM, Finlay BB. Phytonutrient diet supplementation promotes beneficial Clostridia species and intestinal mucus secretion resulting in protection against enteric infection. Sci Rep. 2015;5:9253.
153. Berkhoff HA, McCorkle FM, Brown TT. Pathogenicity of various isolates of Alcaligenes faecalis for broilers. Avian Dis. 1983;27:707–13.
154. Brisbin JT, Gong J, Parvizi P, Sharif S. Effects of lactobacilli on cytokine expression by chicken spleen and cecal tonsil cells. Clin Vaccine Immunol CVI. 2010;17:1337–43.
155. Christensen HR, Frøkiaer H, Pestka JJ. Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. J Immunol Baltim Md 1950. 2002;168:171–8.

156. Kaplan H, Hutkins RW. Metabolism of Fructooligosaccharides by Lactobacillus paracasei 1195. Appl Environ Microbiol. 2003;69:2217–22.
157. Tanaka H, Hashiba H, Kok J, Mierau I. Bile salt hydrolase of Bifidobacterium longum-biochemical and genetic characterization. Appl Environ Microbiol. 2000;66:2502–12.
158. Duncan SH, Louis P, Flint HJ. Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. Appl Environ Microbiol. 2004;70:5810–7.
159. Costa MC, Arroyo LG, Allen-Vercoe E, Stämpfli HR, Kim PT, Sturgeon A, et al. Comparison of the fecal microbiota of healthy horses and horses with colitis by high throughput sequencing of the V3-V5 region of the 16S rRNA gene. PloS One. 2012;7:e41484.
160. Ling Z, Liu X, Jia X, Cheng Y, Luo Y, Yuan L, et al. Impacts of infection with different toxigenic Clostridium difficile strains on faecal microbiota in children. Sci Rep. 2014;4:7485.
161. Mountzouris KC, Tsitrsikos P, Palamidi I, Arvaniti A, Mohnl M, Schatzmayr G, et al. Effects of probiotic inclusion levels in broiler nutrition on growth performance, nutrient digestibility, plasma immunoglobulins, and cecal microflora composition. Poult Sci. 2010;89:58–67.
162. Remenyik J, Ledo H, Dudas L, Veres ZS, Fari M. Antioxidant capacity of some red sweet pepper lines and varieties. Cereal Res Comm. 2008;36:1759–62.
163. Nemes A, Homoki J, Kiss R, Hegedus C, Kovacs D, Peitl B, Gal F, Stundl L, Szilvassy Z, Galne Remenyik J. Effect of anthocyanin rich tart cherry extract on inflammatory mediators and adipokines involved in Type 2 diabetes in a high fat diet induced obesity mouse model. Nutrients. 2019;11:1–17.
164. Fidler G, Tolnai E, Stagel A, Remenyik J, Stundl L, Gal F, Biro S, Paholcsek M. Tendentious effects of automated and manual metagenomic DNA purification protocols on broiler gut microbiome taxonomic profiling. Sci Rep. 2020;10:3419.
165. Evan B, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 2019;8,:852–7.
166. Benjamin JC, et al. Dada2: High-resolution sample inference from Illumina amplicon data. Nature methods 2016; 13:10.1038/nmeth.3869.
167. Kazutaka K, Daron MS. Multiple sequence alignment software version 7: improvements in performance and usability. Molecular biology evolution. 2013;4:772–80.
168. Quast C, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Opens external link in new windowNucl. Acids Res. 2013;41:D590–6.
169. Morgan NP, Paramvir SD, Adam PA. Fasttree 2–approximately maximum-likelihood trees for large alignments. PloS one 2010;3:10.1371/journal.pone.0009490.
170. Shannon CE, Weaver W. A mathematical theory of communication. Bell Syst Tech J 1948;27: 10.1002/j.1538-7305.1948.tb01338.x.
171. Daniel PF, Andrew MB. Phylogenetic diversity (PD) and biodiversity conservation: some bioinformatics challenges. Evol Bioinform Online. 2006;2:121–8.
172. Simpson EH. Measurement of diversity. Nature 1949;163:10.1038/163688a0.

173. Chao A. Non-parametric estimation of the number of classes in a population. Scand J Stat. 1984;11:265–70.
174. Catherine L, Rob K. Unifrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol. 2005;12:8228–35.
175. An ordination of the upland forest community of southern
Bray JR, Curtis JT. An ordination of the upland forest community of southern.
176. Wisconsin. Ecology Monographs. 1957;27:325. https://doi.org/10.2307/1942268.
177. Foster ZS, Sharpton TJ, Grünwald NJ, Metacoder. An R package for visualization and manipulation of community taxonomic diversity data. PLoS Comput Biol 2017; 13,:10.1371/journal.pcbi.1005404.
178. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12:R60.

Figures



Diet	Basal Diet(BD) CS1	BD+β-glucane CS2	BD+Carotenoids TS3	BD+FOSs TS4	BD+Synbiotics TS5	BD+Anthocyanins TS6	RMSE
BW (g/bird)							
Day 1	38.9	37.9	38.6	38.6	39.0	38.5	0.5
Day 10	232	226	221	222	225	227	14
Day 21	759^{ab}	795^{b}	769^{ab}	715^{ab}	742^{ab}	726^{a}	38
Day 32	1713	1767.7	1709.9	1735.3	667	1705.3	66
Day 42	2758	2727	2748	2618	2717	2590	98
ADG (g/day/bird)							
Pre-starter (Day 1-9)	19	19	18	18	19	19	1
Starter (Day 10-21)	47.9^{abc}	51.8^{c}	49.8^{ac}	44.9^{b}	47^{abc}	45.3^{ab}	3
Grower (Day 22-31)	87	88	86	93	85	89	5
Finisher (Day 32-42)	104	96	104	88	104	89	10
Day 1-42	65	64	65	61	64	61	2
ADFI (g/day/bird)							
Pre-starter (Day 1-9)	4	3	3	4	4	5	2
Starter (Day 10-21)	50	58	58	56	55	61	15
Grower (Day 22-31)	130^{a}	144^{ab}	148^{ab}	149^{ab}	145^{ab}	159^{b}	16
Finisher (Day 32-42)	114	132	124	127	117	132	15
Day 1-42	73^{a}	83^{b}	81^{ab}	82^{b}	78^{ab}	88^{b}	6

*RMSE: Root mean-square error
BW: Body weight
ADG: Average daily gain
BW and ADG are based on individual values (n=18)
ADFI: Average daily feed intake
ADFI is calculated for pens (n=3)
^{abc} Mean values within a row with different superscripts are significantly different ($P < 0.05$)

Sampling
day 7
day 19
day 31
day 40

Feeding period (fp)
Pre-starter (day 1-9)
Starter (day 10-21)
Grower (day 22-31)
Finisher (day 32-42)

Figure 1

Sampling and treatment strategies and effects of natural compounds on growth performance of broiler chickens. Baseline statistics represent the pivotal indicators of feed efficiency; average daily feed intake (ADFI), and average daily gain (ADG). Poultry were fed with a commercial maize-soybean based basal diet (BD) free of antibiotics, which was formulated for pre-starter (day 1-9), starter (day 10-21), grower (day 22-31) and finisher (day 32-42) production periods. CS1 negative control (BD with no dietary supplement), dietary treatments were provided as mash feed; CS2 positive control (BD incl. 0.5% of β-glucan), TS3 (BD incl. 0.5% of carotenoids), TS4 (BD incl. 0.5% of FOSs); TS5 (BD incl. 0.5% of probiotics), TS6 (BD incl. 0.5% of anthocyanins).

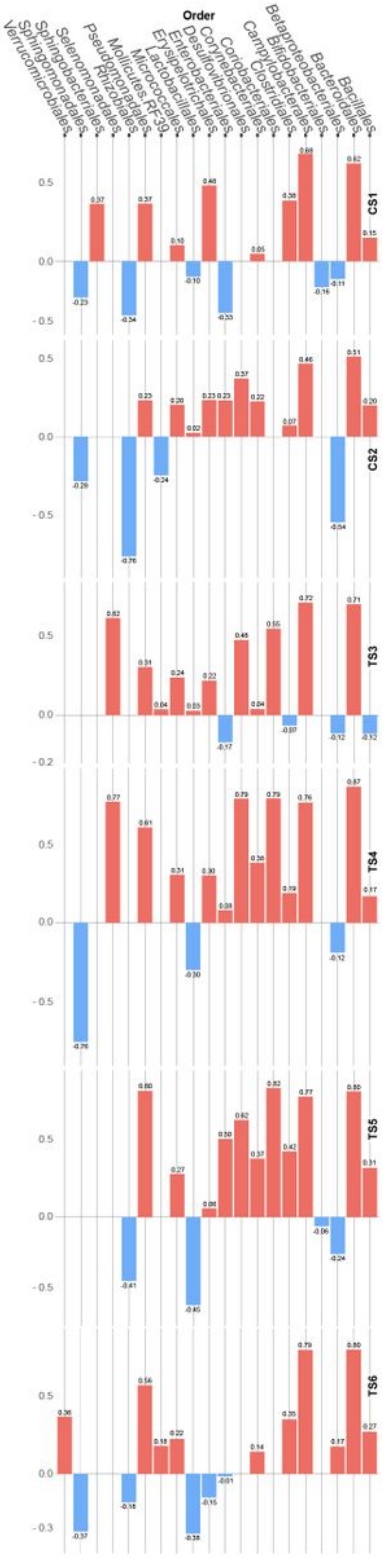


Figure 2

Spearman correlation plots were made to measure associations between BWG and order. The values of correlations vary from −1 to +1 indicating the level of consistency of positive (≥0; red) and negative (<0; blue) correlations. CS1 negative control (BD with no dietary supplement), dietary treatments were provided as mash feed; CS2 positive control (BD incl. β-glucan), TS3 (BD incl. carotenoids), TS4 (BD incl. FOSs); TS5 (BD incl. synbiotics), TS6 (BD incl. anthocyanins).

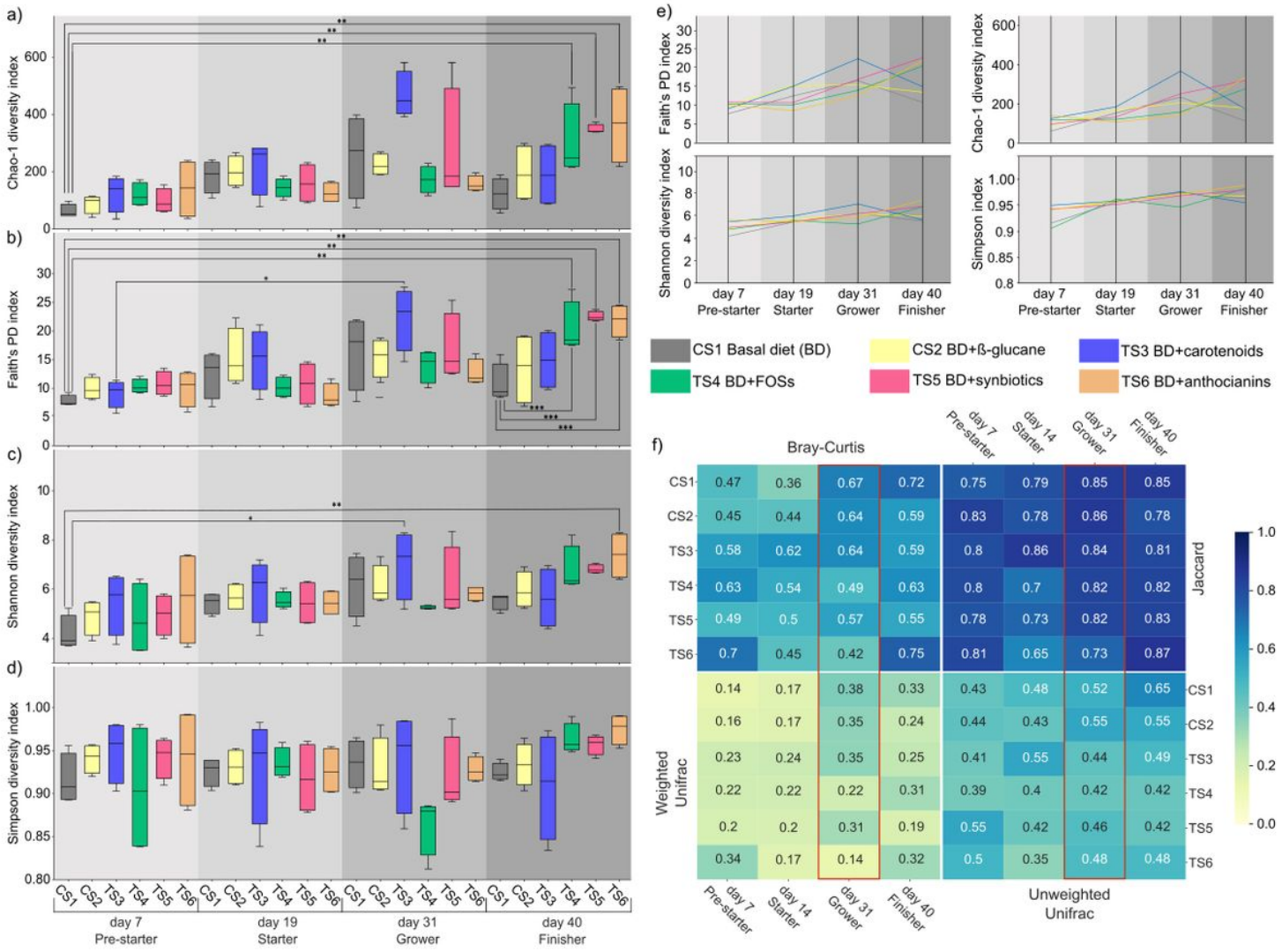


Figure 3

Community diversity distributions represent values of differences within- and between our experimental groups. Statistical comparisons among multiple groups were performed with non-parametric Kruskal-Wallis test, and intergroup differences were tested with Dunn's test. (a-d) Boxplots represent comparisons of alpha diversity metrices of richness; Chao-1 and Faith's PD (a, b) and evenness; Shannon and Simpson (c, d) diversity indices measured in different experimental groups. CS1 negative control (BD with no dietary supplement), dietary treatments were provided as mash feed; CS2 positive control (BD incl. β-glucan), TS3 (BD incl. carotenoids), TS4 (BD incl. FOSs); TS5 (BD incl. synbiotics), TS6 (BD incl. anthocyanins). Asterisks report statistical significance * P≤0,05; **P≤0,01; ***P≤0,001. (e) Line graphs display the age specific tendential changes in alpha diversity metrices observed in six experimental groups coloured accordingly. Data shown are mean values. (f) Sample distances were calculated on the basis of quantitative (Bray-Curtis, weighted UniFrac) and qualitative (Jacquard, unweighted UniFrac) dissimilarity-based statistics.

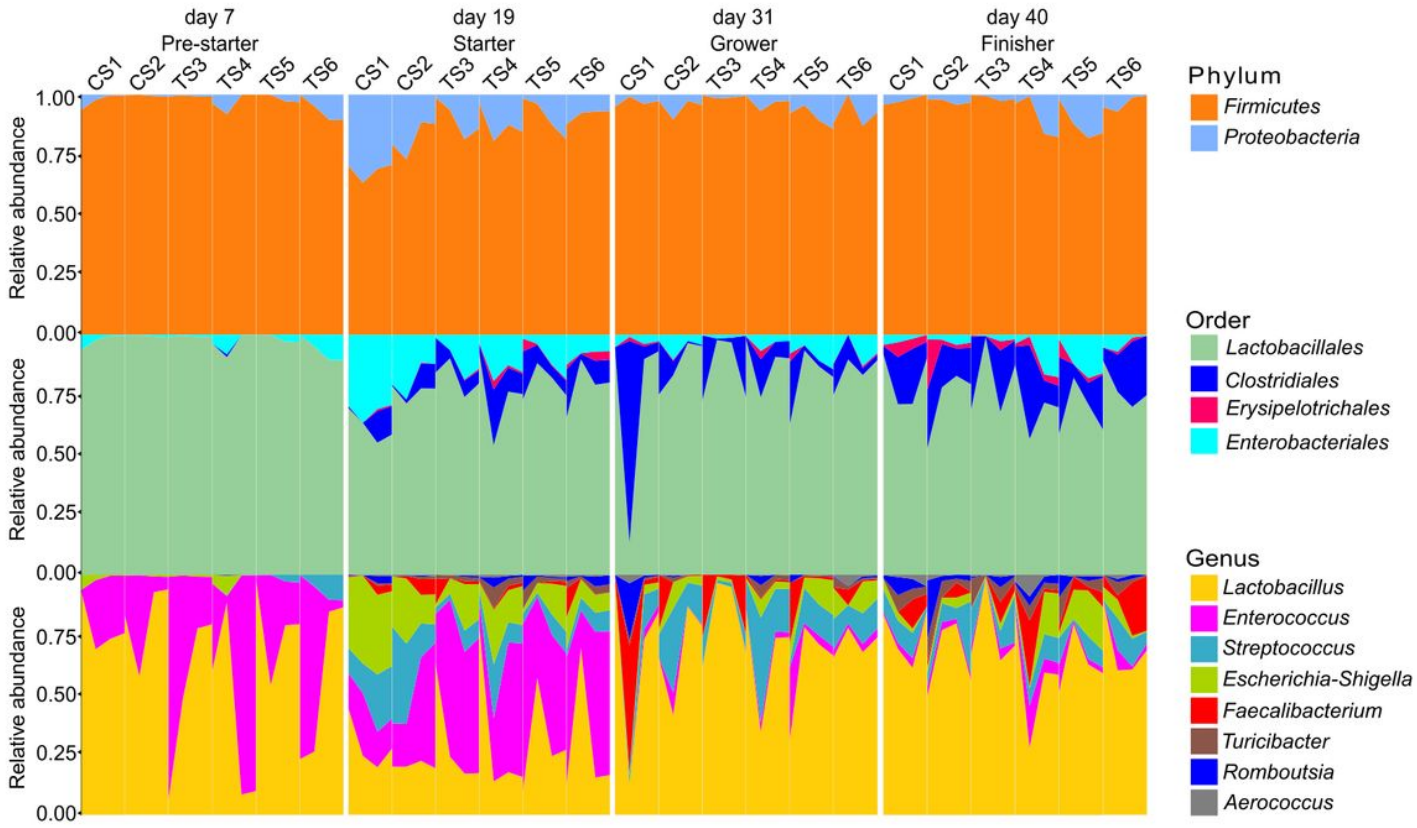


Figure 4

Remarkable temporal variations in healthy core 50% GIT microbiota of broiler. Area plots represent diet related biases in the relative abundances observed in the core phyla, order and genera. CS1 negative control (BD with no dietary supplement), dietary treatments were provided as mash feed; CS2 positive control (BD incl. 0.5% of β-glucan), TS3 (BD incl. 0.5% of carotenoids), TS4 (BD incl. 0.5% of FOSs); TS5 (BD incl. 0.5% of synbiotics), TS6 (BD incl. 0.5% of anthocyanins).

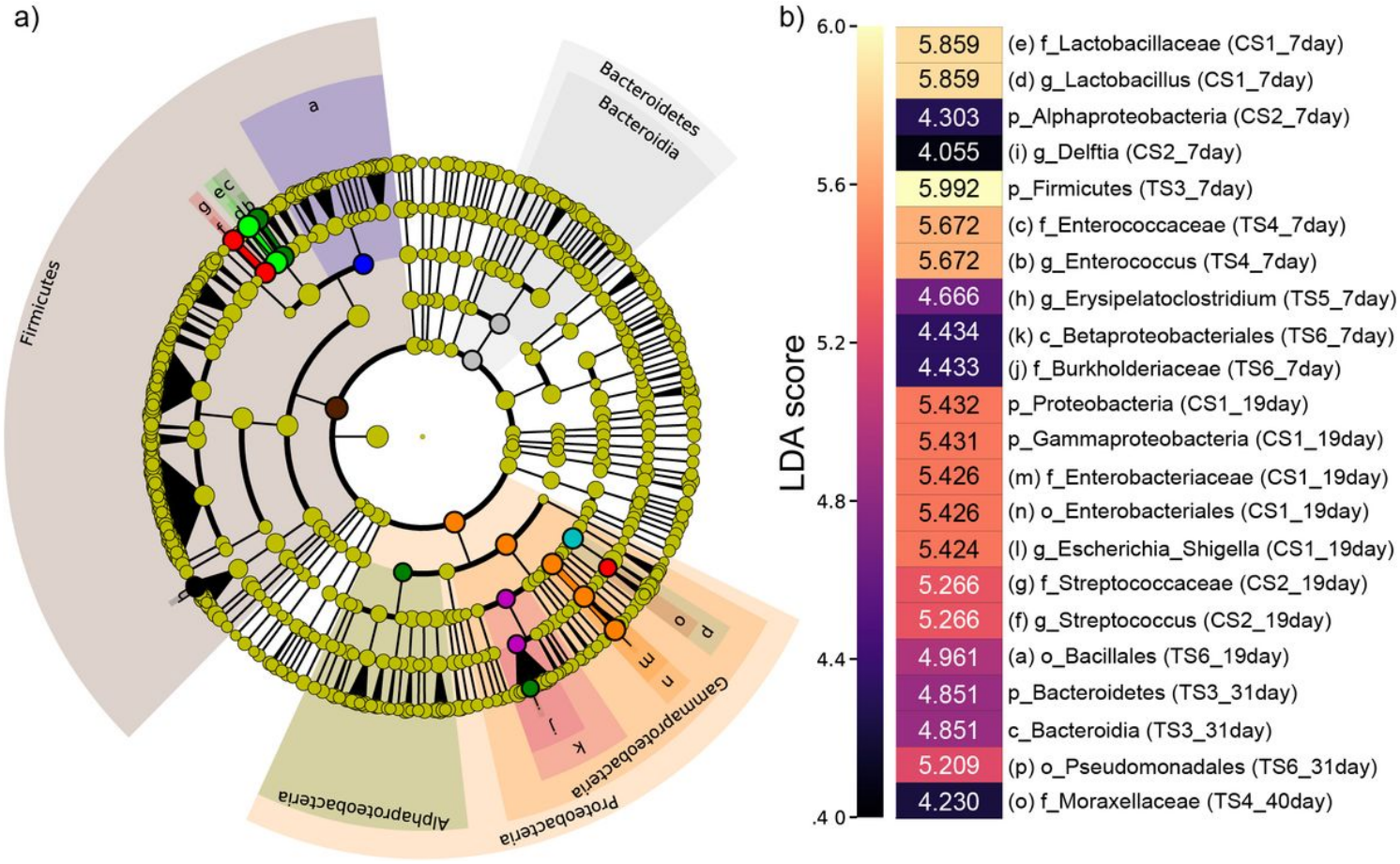


Figure 5

Linear discriminant analysis (LDA) effect size (LEfSe) represents bacterial clades involved in significant taxonomic shifts. (a) The cladogram depicts the phylogenetic distribution of microbial lineages in fecal samples obtained from broiler. (b) A list of 22 significantly enriched bacterial clades ranked with respect to diet and age. Heat-map represents LDA scores (LDA scores > 4.2). CS1 negative control (BD with no dietary supplement), dietary treatments were provided as mash feed; CS2 positive control (BD incl. 0.5% of β-glucan), TS3 (BD incl. 0.5% of carotenoids), TS4 (BD incl. 0.5% of FOSs); TS5 (BD incl. 0.5% of probiotics), TS6 (BD incl. 0.5% of anthocyanins).

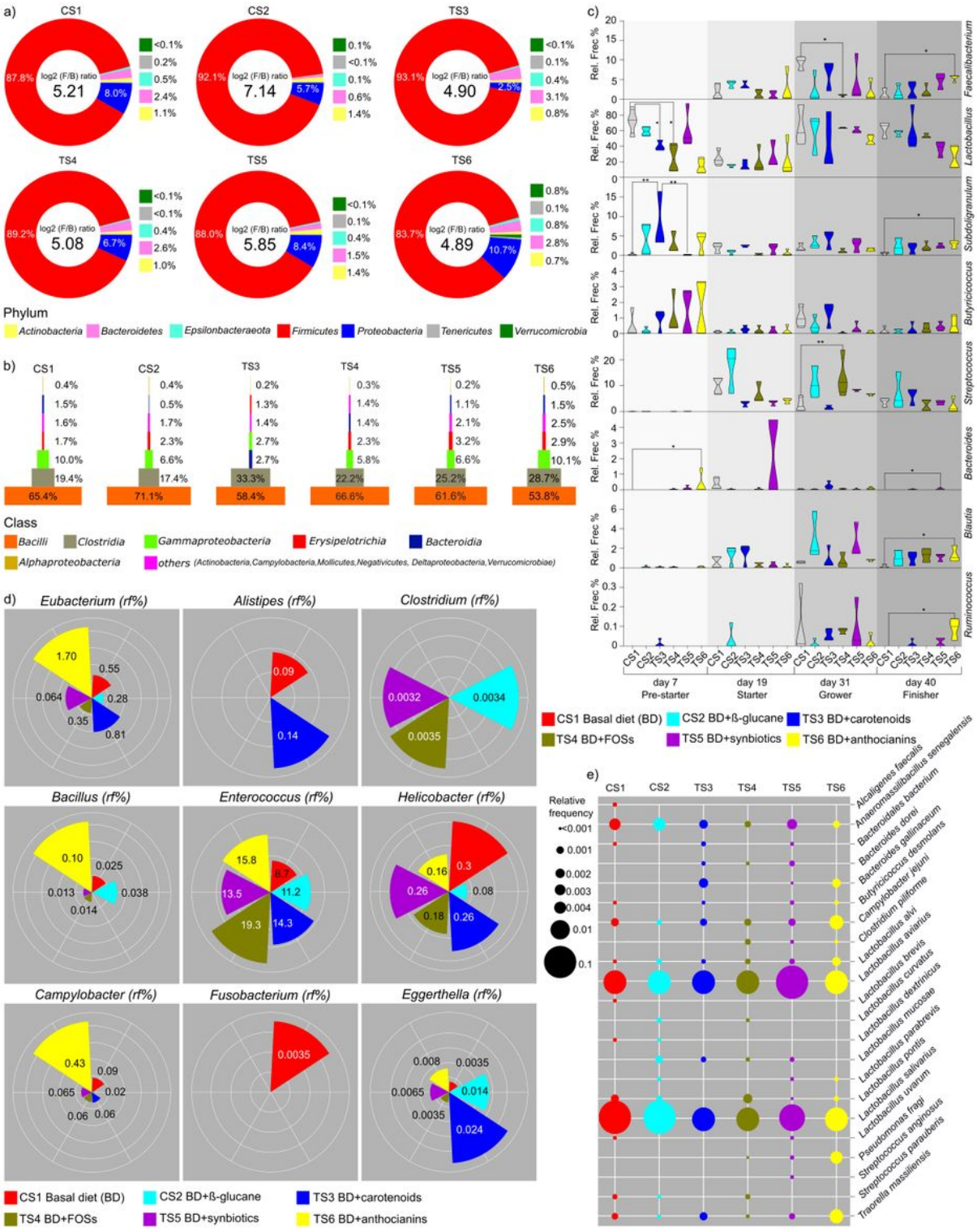


Figure 6

Dietary supplementations induced appreciable shifts in community at every taxonomic level. (a) Donut plots represent the diet induced distortions in the main phyla. Firmicutes to Bacteroides ratios (log2 ratio of F/B relative frequencies %) are also indicated. (b) Pyramid plots show relative abundances of the most relevant classes (c) Violin plots show short chain fatty acid producing genera; Faecalibacterium, Lactobacillus, Subdoligranulum, Butyricicoccus, Streptococcus, Bacteroides, Blautia, Ruminococcus. (d)

Effect of nutraceuticals on the relative frequencies of potential pathogenic and/or zoonotic genera; Eggerthella, and Fusobacterium and Helicobacter and on those associated with enhanced metabolism; Alistipes, Bacillus, Bacteroides, Blautia, Clostridium, and Eubacterium are represented by polar plots. (e) Bubble chart shows 22 dedicated species, where bubble sizes correspond to their relative abundance values.

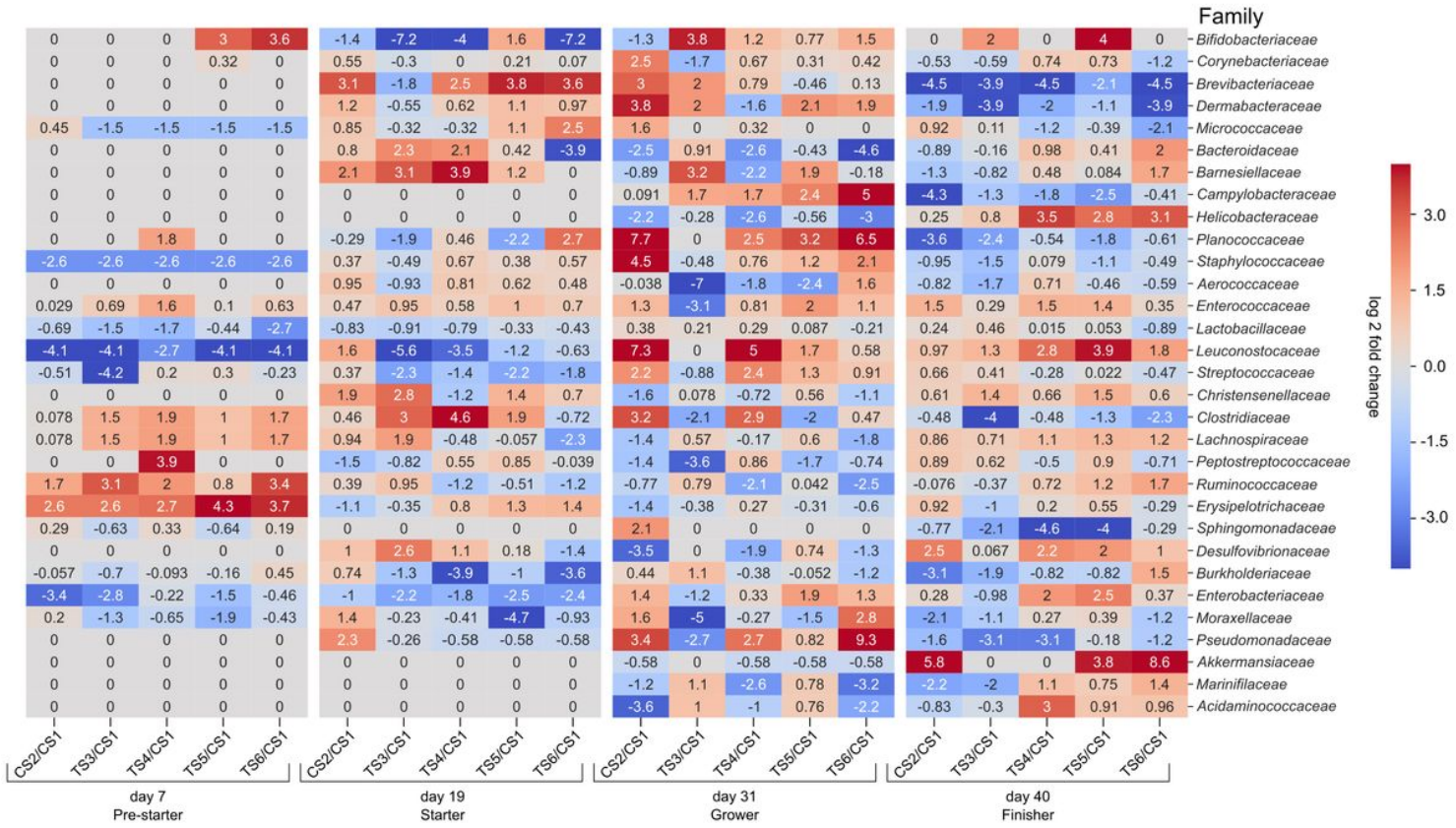


Figure 7

Extents of the estimated differences are illustrated on the annotated heat-map with the normalized log2 fold changes of the specified family abundances. Red scale represents dominance of family owed to special dietary supplementation: log2 (supplemented/non-supplemented diet) > 0, whereas blue scale represents values of increments in favour of negative controls: log2 (supplemented/non-supplemented diet < 0). CS1 negative control (BD with no dietary supplement), dietary treatments were provided as mash feed; CS2 positive control (BD incl. 0.5% of β-glucan), TS3 (BD incl. 0.5% of carotenoids), TS4 (BD incl. 0.5% of FOSs); TS5 (BD incl. 0.5% of synbiotics), TS6 (BD incl. 0.5% of anthocyanins).

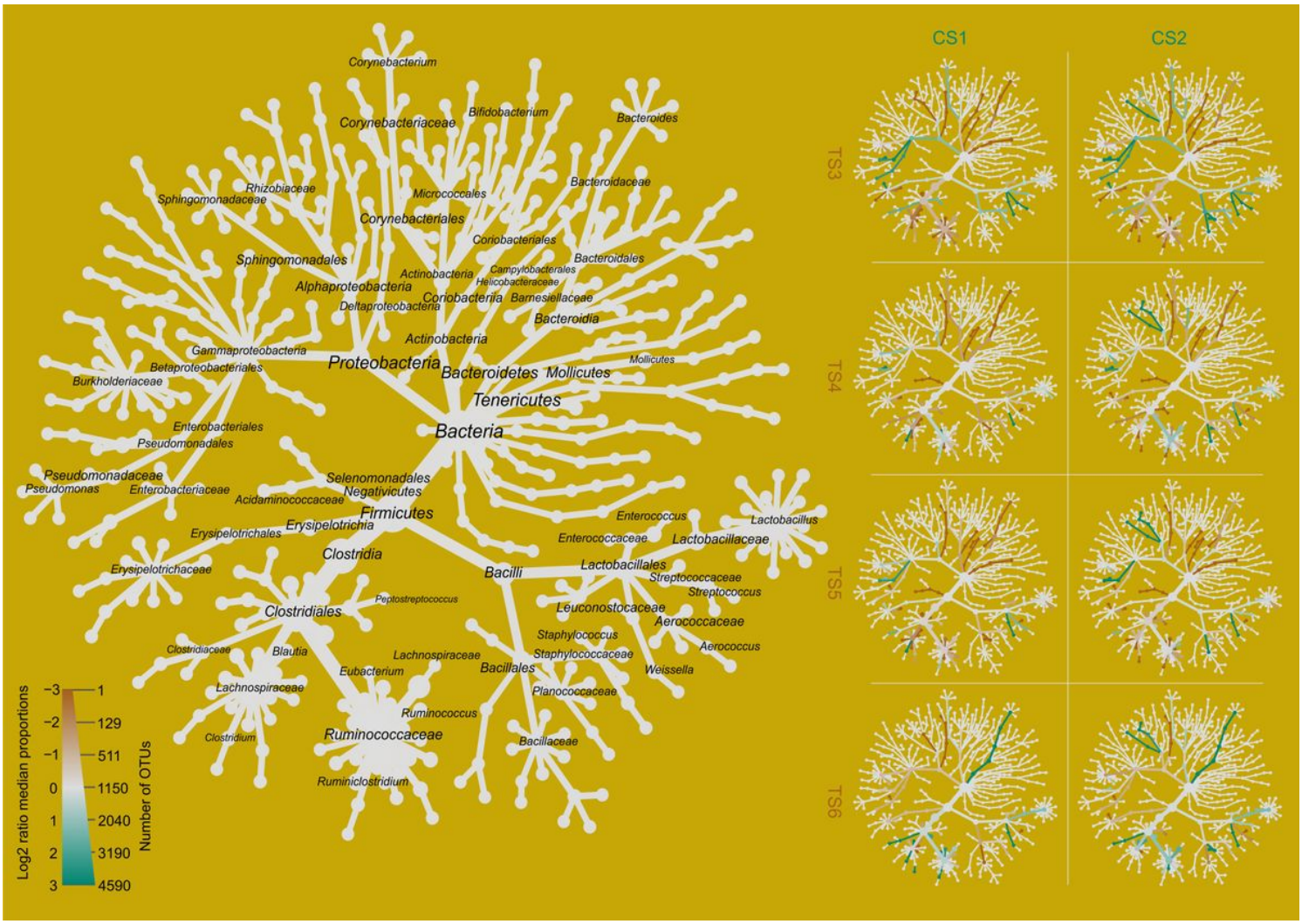


Figure 8

Differential abundance taxonomic heat trees revealed the effects of nutraceuticals. Metacoder differential heat tree illustrates the variation in microbiome phylotypes between experimental groups. Nodes in the heat tree correspond to phylotypes, as indicated by node labels, while edges link phylotypes in accordance to taxonomic hierarchy. Node size corresponds to the number of OTUs observed within a given phylotype. To visualize the effects of dietary supplementation on microbial composition community heat trees were made to represent the effects of the following dietary treatment; CS1 negative control (BD with no dietary supplement), dietary treatments were provided as mash feed; CS2 positive control (BD incl. 0.5% of β-glucan), TS3 (BD incl. 0.5% of carotenoids), TS4 (BD incl. 0.5% of FOSs); TS5 (BD incl. 0.5% of synbiotics), TS6 (BD incl. 0.5% of anthocyanins). The annotated tree on the left functions as a map for the unlabelled trees. Coloured taxons represents the extents of log2 differences in taxa abundances.

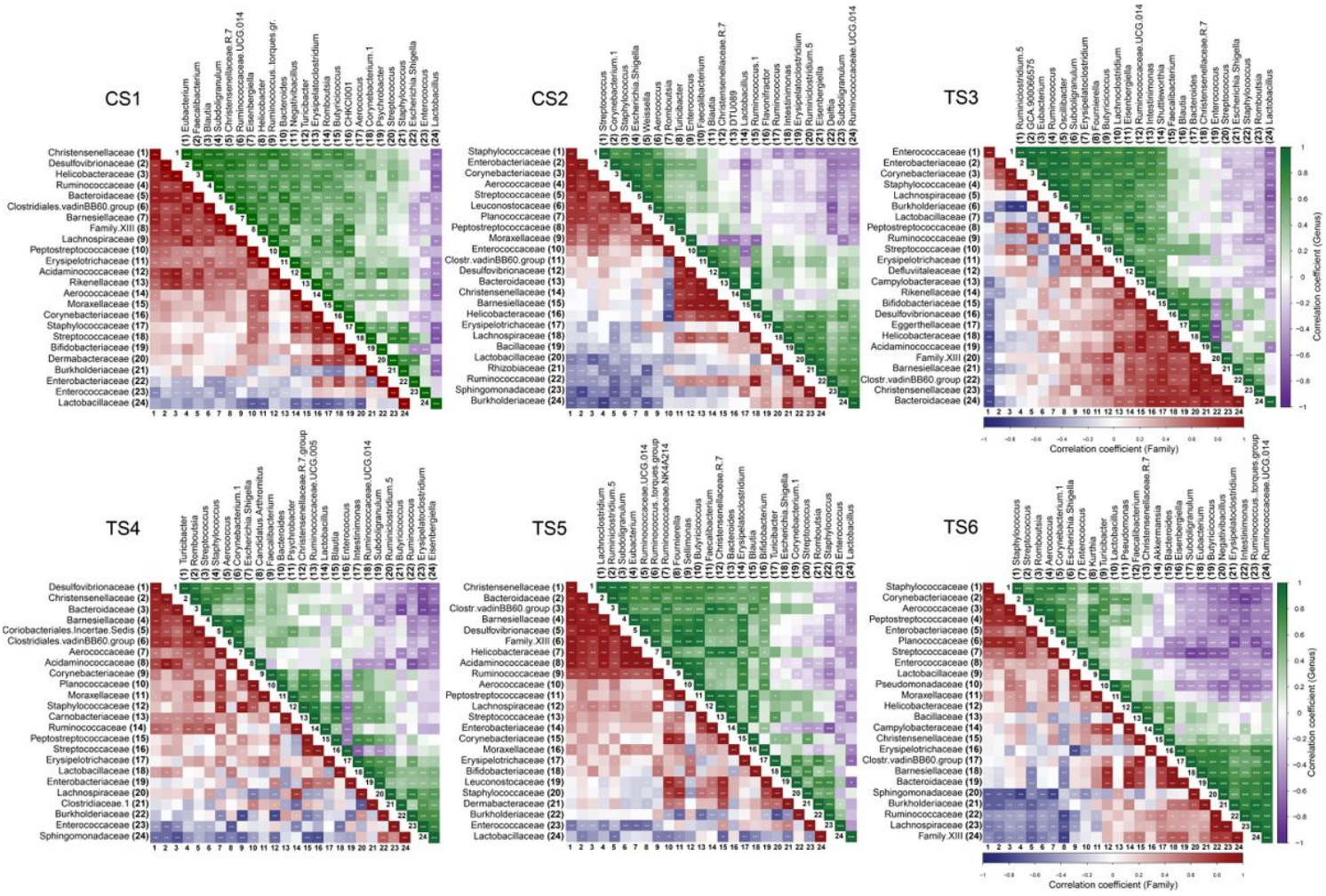


Figure 9

Spearman correlation plots were made on two taxonomic ranks to measure the strength of associations between variables. Extents of colors indicate values of correation coefficients. The values vary from –1 to +1indicating the level of consistency of positive (>0) and negative (<0) correlations. CS1 negative control (BD with no dietary supplement), dietary treatments were provided as mash feed; CS2 positive control (BD incl. 0.5% of β-glucan), TS3 (BD incl. 0.5% of carotenoids), TS4 (BD incl. 0.5% of FOSs); TS5 (BD incl. 0.5% of synbiotics), TS6 (BD incl. 0.5% of anthocyanins).

Supplementary Files

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