Italian Journal of Animal Science

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/tjas20

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To cite this article: Matteo Daghio, F. Scicutella, A. Buccioni, S. Minieri, I. Galigani, S. Passeri, R. Danesi, E. Toni, M. Mele & F. Mannelli (2024) Short- and medium-chain fatty acids and chestnut tannin extract blend as supplement in poultry feeding: effect on animal performances and gut microbiota communities, Italian Journal of Animal Science, 23:1, 288-298, DOI: 10.1080/1828051X.2024.2306856

To link to this article: https://doi.org/10.1080/1828051X.2024.2306856

Published online: 10 Feb 2024.

ITALIAN JOURNAL OF ANIMAL SCIENCE
2024, VOL. 23, NO. 1, 288–298
https://doi.org/10.1080/1828051X.2024.2306856

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RESEARCH ARTICLE

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Short- and medium-chain fatty acids and chestnut tannin extract blend as supplement in poultry feeding: effect on animal performances and gut microbiota communities

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ABSTRACT

Intensive poultry rearing needs preventive strategies against diseases, several of which are little or not containable by vaccinations. Short- and medium-chain fatty acids (SMCFAs) and chestnut tannin (CHT), both by-products of two different industrial production chains, are very effective antimicrobials. The inclusion level of these additives in the diet is fundamental because a detrimental effect on the gut microbiome can occur if the dose is too high, compromising the animal welfare and performance. Hence, the aim of this trial was to test the effect of a blend (BL; 1:1, w/w) of SMCFAs (fatty acids from C4:0 to C9:0) and CHT extract (CTE) as feed additive in broiler feeding. One hundred one-day-old Ross 308 male chicks were randomly assigned to 5 dietary treatments (4 replicates; 20 pens; 5 animals per pen): control diet (CON), FAS diet (with 1.5 g/100g on dry matter (DM) of SMCFAs), CTE diet (with 1.5 g/100g on DM of CHT extract), BL1 diet (with 1.5 g/100g on DM of BL), and BL2 diet (with 3.0 g/100g on DM of BL). Among the parameters evaluated (weight gain; feed intake; feed efficiency; dressing out), the only differences were observed for the feed intake. In particular, the feed intake of CON (2.876 kg), FAS (2.773 kg), CTE (2.882 kg), and BL1 (2.858 kg) were comparable while the feed intake of BL2 (2.717 kg) was lower than the other diets except for FAS which was similar ($p = 0.0010$). The microbial community in the caecum was described by high-throughput sequencing of 16S rRNA gene amplicons and was similar between the treatments both in terms of alpha-diversity and beta-diversity. The results of this trial evidenced that no detrimental effect on growth performances or on gut microbiome occurred when birds were fed the blend of SMCFAs and CHT extracts at the inclusion levels tested in this study.

HIGHLIGHTS

- Short- and medium-chain fatty acids and chestnut tannins are alternatives to conventional antimicrobials
- Natural antimicrobials improve the sustainability of poultry production
- Short- and medium-chain fatty acids and chestnut tannins did not alter the gut microbiota of broiler

ARTICLE HISTORY
Received 5 June 2023
Revised 29 October 2023
Accepted 13 January 2024

KEYWORDS
Broiler; gut health word; polyphenols; fatty acid; antibiotic alternative

Introduction

Intensive poultry rearing needs preventive strategies against infectious diseases, several of which are not preventable or attenuable by vaccinations. The most important poultry disease is necrotic enteritis (NE), due to an overgrowth of *Clostridium perfringens* type A and *Eimeria* spp. (*Eimeria acevulina*, *Eimeria tenella*, *Eimeria maxima*) that are responsible for acute enterotoxaemia with lesions in the host small intestine (Timbermont et al. 2010). This disease is a widespread problem, causing illness in birds and possibly degenerating into animal death and leading to farmers' economic loss (Williams 1999). In intensive management, the presence of a large number of birds in the same pen and on the same litter creates optimal conditions for the proliferation of pathogens and the propagation

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of the NE. When birds suffer from NE, the chronic damage of the intestinal mucosa leads to poor absorption of nutrients, resulting in low growth performances and a decrease in feed efficiency (FE). The most common strategy to preserve or control NE is the prophylactic use of antibiotics. The prophylactic use of antibiotics conflicts with the European Parliament and European Council regulations which banned the growth-promoting and preventive use of antibiotics in animal feeding based on the scientific opinions of the European Commission on food safety (Murphy et al. 2017). Thus, the European Committee recommended phasing out all pharmaceuticals not administered for clinical purposes because antibiotics are responsible for antimicrobial resistance (AMR) by bacteria and environmental pollution (Mehdi et al. 2018). In this context, animal disease prevention needs the employment of more sustainable alternatives, guaranteeing healthy end-products from safe poultry rearing. This is a cornerstone for cutting-edge farms and the 'newest' consumers because new farmers need to properly respond to modern consumers who demand healthier food produced with low impact on the environment.

A chicken with optimal gut health may be less susceptible to NE because nutrients are better absorbed, and the immune system is more responsive, and the gut environment is less suitable for the proliferation of pathogens (Van Immerseel et al. 2005; Choct 2009). Several *in vitro* and *in vivo* experiments demonstrated the effectiveness of natural bioactive compounds, as preventive strategies and effective alternatives to antibiotics, against *Clostridium perfringens*, *Eimeria* spp. and *Salmonella* spp. (Tosi et al. 2013; Hofacre et al. 2020). The great advantage is that these substances do not induce or slightly induce AMR (Scicutella et al. 2021; Redondo et al. 2015). Their chemical and physical properties are numerous and strongly linked to the chemical structure. Among them, short and medium-chain fatty acids (SMCFAs) (respectively, short-chain fatty acids - SCFAs $<$ C5:0 and medium-chain fatty acids - MCFAs from C6:0 to C9:0) and polyphenols (PPs) are promising (Sunkara et al. 2012; Tosi et al. 2013; Mannelli et al. 2019a).

Short- and medium-chain fatty acids stimulate the development of intestinal villi and they are an energy source for enterocytes (Antongiovanni et al. 2010; Hofacre et al. 2020). These molecules have a small and linear structure, are highly soluble in water and can be absorbed by bacteria cells. Moreover, the endogenous synthesis of the host-defense proteins is in an inverse correlation with the aliphatic hydrocarbon chain length of free fatty acids (FFAs), with a decreasing efficiency from SCFAs to long-chain fatty acids (LCFAs) (Sunkara et al. 2012). Moreover, their antimicrobial effect is species-specific (Van Immerseel et al. 2005). After the dissociation in the cell cytoplasm (PKa $\approx$ 5), SMCFAs impede DNA replication causing a short circuit of the cell or down-regulate the expression of multiple genes required for pathogen invasion (Sunkara et al. 2012; Yadav and Jha 2019). Short- and medium-chain fatty acids are naturally present in milk or in certain vegetable oils or produced by anaerobic fermentation of bacteria cultured on biowaste (de Oliveira et al. 2020; Reddy et al. 2020).

Polyphenols are a widespread family of bioactive compounds, synthesised by plants as a response to pathogen attacks or environmental stress. Their mechanisms of action are various and the most known one is the ability to form complexes with proteins (Tsao 2010). Among PPs, tannins are potent antimicrobials but can show anti-nutritional effects (Hassan et al. 2020). Monogastrics are more sensitive than ruminants to tannins that may be detrimental to intestinal villi development, compromising animal growth performances. Several hydrolysable tannins (HTs) seem to be more detrimental than condensed ones (CTs) because of the toxic effect of the resorcinol produced from Gallotannins in the gut during digestion (Vadivel and Pugalenthi, 2010). Among HTs, several authors found that chestnut tannin (CHT, from *Castanea sativa* Mill.) is well tolerated by poultry (Mannelli et al. 2019a), making it a promising candidate as a preventive tool against microbial infections in broiler production.

Considering the protective effects of SMCFAs on intestinal villi and the antimicrobial properties of SMCFAs and CHT, a blend composed of SMCFAs and CHT extract could be a suitable alternative to antibiotics. The effect of a SMCFAs and CHT extract mixture on gut microbiota, which has a prominent role in nutrient digestion, absorption, metabolism, and the overall health and growth performances of poultry, is not well known (Yadav and Jha 2019). Hence, this study aimed to investigate the effect of two blends (BLs) of SMCFAs and pure extract of CHT (on growth performances and on the intestinal microbiota of broiler Ross 308.

Material and methods

Diet compositions: CHT, SMCFAs and BL

The SCFAs and MCFAs mixture (SMCFA; from C4:0 to C9:0) is a bio-refinery by-product of vegetable oil produced by Matrica S.p.A. (La Marinella – 07046, Porto Torres, Sassari, Italy). The CHT is obtained by the steam

distillation process of the woodchips resulting from chestnut wood processing and was provided by Gruppo Mauro Saviola (Viadana – 46019 Mantova, Italy).

The BL product of free SMCFA and CHT extract (commercial name TANBIOTIC) was produced and provided by CFS Europe S.p.A (CFS Europe S.p.A, *via* Agostino De Pretis 6, 48123 Ravenna – Italy).

The SMCFAs profile was determined and quantified as follows: firstly, the samples were solubilised in H_2O and, after acidification (pH = 3), were extracted using hexane, and then were trans-esterified according to Molkentin and Precht (2000). Secondly, the identification and quantification of each FA was carried out by gas-chromatography according to Secchiari et al. (2003) using C2:0 and C12:0 as internal standards (Merck KGaA, Darmstadt, Germany). The CHT extract fractions were characterised according to Bargiacchi et al. (2017) and resulted rich in gallic acid, vescalagin and castalagin. The tannic-acid equivalents of CHT were titrated according to Burns (1963) and resulted in 750 g/kg on DM (75% of tannins in the extract). The free SMCFAs, CHT extract and BL profiles were not reported in any table being patented.

Animals, dietary treatments and experimental design

Animal handling was under Italian Government guidelines (D.lgs 26/2014, art. 2 comma d and f). One hundred one-day-old Ross 308 male chicks, comparable for body weight, were provided by a local hatchery (RI.PRO.COOP Società Cooperativa Agricola, San Vittore di Cesena – 47020 Forlì, Italy), and they were vaccinated against Marek's disease, infectious bronchitis and Newcastle disease. The number of animals per pen, the number of replication units, and the power analysis were computed by G*Power 3.1. The pen was considered as the experimental unit according to Bello et al. (2016). Birds were allotted in 20 pens (5 animals per pen, singularly identified by leg ring from 1 day of life) and reared on coconut fibre litter. Animals were randomly assigned to 5 dietary treatments (4 pens for each diet and 5 animals per pen). The control diet (CON, negative control) was a commercial diet not containing antibiotics, tannins or SMCFAs as additives. Soybean meal was the main protein source, and maize meal and soybean oil were the energy sources. The formulation is reported in Table 1. Then, two other diets were formulated as positive controls adding to CON respectively free SMCFAs or CHT extract: diet FAS containing 1.5 g/100g on dry matter (DM) of free SMCFAs, and diet CTE containing 1.5 g/100g on DM of CHT extract. Finally, the treated diets were formulated by adding to CON the BL at two different inclusion levels: diet BL1 and BL2 containing 1.5 g/100g on DM and 3 g/100g on DM of BL, respectively. The inclusion level was determined on the basis of previous experiments (Leeson et al. 2005; Antongiovanni et al. 2010; Mannelli et al. 2019a). The diets were formulated to meet animal requirements at each different growing age according to Subcommittee on Poultry Nutrition, Committee on Animal Nutrition, Board on Agriculture, and National Research Council (1994): starter (0–7 d); grower (8–21 d) and finisher (22–35 d). The metabolisable energy (ME; kcal) was estimated according to Sauvant et al. (2002). Moreover, the experimental diets, for each feeding period, were formulated to be isoproteic and isoenergetic. In particular, in FAS, BL1 and BL2 an amount of sunflower oil was replaced with the corresponding lipid amount of SMCFAs to make diets comparable for energy content with respect to CON.

Table 1. Ingredient composition and major nutritional traits of the control diet (starter, grower and finisher periods).

Ingredients (g/100 g)	Starter (0–7 d)	Grower (8–21 d)	Finisher (22–35 d)
Maize	52.05	57.00	58.00
Soy bean meal	35.50	33.10	31.05
Maize glutein	3.00	–	–
Sunflower oil	4.50	5.25	6.30
Dicalcium phosphate	1.90	1.90	1.90
Calcium carbonate	1.50	1.50	1.20
Sodium bicarbonate	0.25	0.25	0.25
Sodium clhoride	0.25	0.25	0.25
DL Metionine	0.25	0.25	0.25
Lisinae HCl	0.15	0.15	0.15
Choline chloride	0.15	0.15	0.15
[a]Vitamin mineral premix	0.50	1.90	0.50
Composition			
DM[b], g/kg of fresh feed	895	863	879
g/kg on DM			
CP[c]	245	213	206
EE[d]	72	91	95
CF[e]	51	53	55
NDF[f]	94.7	98.5	99.1
ADF[g]	45.3	45.6	45.72
ADL[h]	12.3	13.3	15.7
Ash,	73	72	69
Calcium	12	11	11
Phosphorus	7	7	7
Lysine	12	11	10
Methionine	4	3	3
ME[i] kcal/kg	2950	3010	3090

[a]Vitamin premix (per kg of diet): vitamin A, 12,000 IU; vitamin D3, 1,000 IU; vitamin E, 30 IU; vitamin K3, 3 mg; vitamin B1, 2 mg; vitamin B2, 3 mg; vitamin B6, 4 mg; vitamin B12, 0.015 mg; nicotinic acid, 30 mg; folic acid, 5 mg; choline chloride, 550 mg. 2; CuSO4, 10 mg; FeSO4, 70 mg; ZnSO4, 50 mg; manganese (MnSO4), 60 mg; I [Ca(IO3)2], 0.5 mg; Na2SeO3, 0.2 mg.
[b]DM: dry matter.
[c]CP: crude protein.
[d]EE: ether extract.
[e]CF: crude fibre.
[f]NDF: neutral detergent fibre.
[g]ADF: acid detergent fibre.
[h]ADL: acid detergent lignin.
[i]ME: Metabolisable Energy estimated according to Sauvant et al. (2002).

From the 1st to the 10th day, chicks were maintained under artificial mothers (poultry heat red lamp mod 1345567, TINGHAO 200w). The light regimen was 18 h of light followed by 6 h of dark and the environmental temperature was maintained at 28 °C. The feeds were provided *ad libitum* and animals had free access to water. On days 0, 8, 22 and 35 body weights were recorded individually, whereas feed intake (FI) was recorded per pen. The individual FI was calculated by dividing the total amount of feed consumed in the pen by the number of animals. Feed efficiency was calculated as the estimated ratio of the individual FI and weight gain (WG), for each group. Animal welfare was monitored during the whole period of the trial (Committee on Animal Health and Animal Welfare, 2000) and faeces were randomly sampled in all pens from litters and analysed for pathogens detections in particular for Coccidia and Nematoda, according to the McMaster method (Mes et al. 2007). To evaluate intestinal activity each enclosure was virtually divided into 4 equally sized areas, observing how much and how many areas were used for the faeces deposition, using a scoring from 1 to 3 (1 = little used, 2 = average used, 3 = very used), and the compactness (Dou et al. 2009). The same operator was employed in the observational process, in order to avoid variations.

At the end of the trial, on day 35, all the birds were slaughtered, plucked, and eviscerated. Then, the carcases were weighed for dressing out evaluation.

The gut of 2 birds per pen (8 replicates for treatment), chosen at random, was sampled for microbiota analyses as described below. All samples were stored at −80 °C until the analyses. The same operator was employed in the sampling procedure, in order to avoid variations.

Feeds analyses

Samples of the five diets were analysed for DM, crude protein (CP), ether extract (EE), and ash according to the procedures 976.06, 920.39, and 942.05 of AOAC methods, respectively (AOAC 1995). Neutral detergent fibre (NDF), acid detergent fibre (ADF), and lignin (ADL) were determined according to Van Soest et al. (1991) using heat-stable amylase and sodium sulphite. Results were inclusive of residual ash.

Analysis of the microbial communities

DNA extraction, amplicons preparation and sequencing

The samples for DNA extraction were thawed and DNA was extracted from 250 mg of caecum content using the Fast DNA Spin kit for soil (MP Biomedicals, Solon, OH), as previously reported (Daghio et al. 2021).

The V3-V4 region of the 16S rRNA gene was amplified with Pro341F and Pro805R primers (Takahashi et al. 2014). Sequencing was performed at BMR Genomics (Padova, Italy) by MiSeq Illumina (Illumina, Inc., San Diego, CA, USA) using a 300 bp x 2 paired-end protocol. The sequencing produced a total of 3,116,251 reads with an average of 82,007 ± 3,176 reads per sample (average ± standard error).

The sequences are available at the National Centre for Biotechnology Information (NCBI), BioProject ID PRJNA955126

Statistical analysis

Data related to the FI, WG, and FE of each period were processed as completely randomised design with repeated measures using the MIXED procedure of SAS Institute (2008):

$$Y_{ijkl} = \mu + T_i + D_j + I_k(D) + (T \times D)_{ij} + e_{ijkl} \quad (1)$$

where y_{ijkl} is the observation; μ is the overall mean; D_j is the fixed effect of treatment ($i = 1$ to 5); T_i is the fixed effect of assaying time ($j = 1$ to 5); I_k is the random effect of the replicate nested within the treatment ($k = 1$ to 5); $(T \times D)_{ij}$ is the interaction between treatment and assaying time and e_{ijkl} is the residual error. The covariance structure and the statistical significance were tested as described above.

The data related to FI, WG, and FE of the whole period and dressing out of slaughtered birds were analysed by one-way ANOVA, keeping the factor diet (D) as the fixed one (SAS Institute, 2008):

$$Y_{ij} = \mu + T_i + D_j + e_{ij} \quad (2)$$

where y_{ij} is the observation; μ is the overall mean; D_i is the diet ($i = 1$ to 5) and e_{ij} is the residual error.

Multiple comparisons among means were performed using the Tukey's test. Probability of significant effect due to experimental factors was fixed for $p < 0.05$.

Bioinformatics and statistic elaborations

Bioinformatic elaborations were performed in R 4.0.3 (R Core Team 2020) with the package DADA2 (Callahan et al. 2016), version 1.16.0. Primer sequences were removed using Cutadapt (Martin 2011). Forward reads were truncated at 280 bases and reverse reads were truncated at 250 bases. The reads with expected errors higher than 1 were discarded. Specific error rates were estimated for the forward reads and for the reverse reads and were used to infer the amplicon sequence variants (ASVs) on the dereplicated reads. The read pairs were merged with default parameters

and the chimeric sequences were removed. Taxonomic assignment for each ASV was performed against SILVA 138.1 database (confidence 80%) (Pruesse et al. 2007). The ASVs with a relative abundance lower than 0.01% in all the samples were removed from the whole dataset. A total of 1,730,417 high-quality sequences were obtained with an average of 45,537 ± 1,974 sequences *per* sample (average ± standard error).

The data were further processed using the vegan package, version 2.5.7 (Oksanen et al. 2018) in R 4.0.3 (R Core Team 2020). A randomly rarefied dataset (sample size = 25,000 sequences) was generated. The Chao1 index, the ACE index, the Simpson index and the Shannon index were calculated to estimate the alpha-diversity and a Kruskal-Wallis test was performed to detect significant differences between the conditions. A non-metric multidimensional scaling (NMDS) and a permutational multivariate analysis of variance (PERMANOVA) based on Hellinger transformed ASVs abundance data were performed using the metaMDS and the adonis2 functions, respectively. Both the NMDS and the PERMANOVA were performed on the Bray-Curtis dissimilarity index. A Kruskal-Wallis test was performed to identify the taxa with a different relative abundance between the conditions.

Results

Animal welfare

During the whole trial period, all subjects were always reactive, no direct trauma, lameness or mutilation lesions were observed, and neither symptom due to respiratory or enteric diseases nor mortality episodes occurred. The cloacal area as well as the hocks have always been cleaned. All assays aimed to discover pathogens and the parasitological examinations showed negative results. All groups used uniformly the entire enclosure for faeces deposition that was on average used (score 2).

Animal performance

Considering the whole period of the trial (0–35 days), the growth of animals was similar. The only differences were registered for the ingestion of feeds. In particular, the FI of CON, FAS, CTE and BL1 were comparable while the FI of BL2 was similar to that of FAS but slightly and significantly lower than the others ($p = 0.0010$; Table 2). The same trend was observed in starter and finisher periods where the FAS diet had the lowest FI ($p = 0.0120$ and $p < 0.0001$, respectively).

Table 2. Animal performances of the single growth periods (starter, grower, finisher) and of the whole trial period.

	CON[a]	FAS[a]	CTE[a]	BL1[a]	BL2[a]	sem[c]	P[d]
starter							
WG[b] (kg/bird)	0.333	0.295	0.286	0.291	0.295	0.014	0.1621
FI[b] (kg/bird)	0.452[a]	0.427[b]	0.426[b]	0.433[ab]	0.418[b]	0.007	0.0120
FE[b]	0.1403	1.495	1.538	1.614	1.472	0.090	0.5453
grower							
WG[b] (kg/bird)	0.538	0.548	0.542	0.535	0.558	0.025	0.9664
FI[b] (kg/bird)	0.908	0.907	0.914	0.892	0.887	0.021	0.8665
FE[b]	1.762	1.692	1.722	1.729	1.626	0.074	0.7290
finisher							
WG[b] (kg/bird)	1.033	0.935	0.979	0.951	0.906	0.043	0.2626
FI[b] (kg/bird)	1.515[a]	1.438[b]	1.541[a]	1.531[a]	1.411[b]	0.020	<0.0001
FE[b]	1.489	1.570	1.603	1.983	1.621	0.193	0.4192
whole period							
WG[b] (kg/bird)	1.905	1.778	1.808	1.778	1.760	0.065	0.5022
FI[b] (kg/bird)	2.876[a]	2.773[ab]	2.882[a]	2.858[a]	2.717[b]	0.031	0.0010
FE[b]	1.531	1.586	1.620	1.678	1.574	0.060	0.5344

[a]Diet treatments: CON, negative control; FAS, positive control diet with 1.5 g/100g on DM of SMCFAs added to CON; CTE, positive control diet obtained with 1.5 g/100g on DM of CHT extract added to CON, BL1 obtained with 1.5 g/100g on DM of BL added to CON, BL2 with 3.0 g/100g on DM of BL added to CON.
[b]WG: weight gain; FI: feed Intake; FE: feed efficiency.
[c]sem: Standard error mean.
[d]*p*-value: Probability of significant effect due to experimental diets, different letters for $p < 0.05$.

Nevertheless, these results did not affect WG or FE and no differences were found among groups for these parameters in the whole trial period and in the *per* periods evaluation (Table 2). However, considering the results *per* week (Table 3), the first and the fifth weeks showed significant differences in WG and FI. In the first week, the consumption of BL2 was higher than the other diets and the WG was comparable to that of animals fed CON and BL1 but FE was similar among groups. In the last week of the trial (5th week) CON, CTE and BL1 showed a higher FI than FAS and BL2. A similar trend was evaluated for the WG. No differences were found for FE (Table 3). In the 2nd week, only FI varied being BL2 comparable to CTE and BL1 diets but lower than CON and FAS diets. Even for the dressing out, no differences were found (CON = 81.54%; FAS = 80.57%; CTE = 81.39%; T3 = 81.18%; T3 = 81.27%; $p = 0.7066$).

Microbial characterisation

The microbial communities in the caecum were characterised by high-throughput sequencing of the 16S rRNA gene. The alpha-diversity of the microbial communities was not different ($p > 0.05$) in the caecum of the chickens fed the tested diets (Table 4). The most abundant classes (Figure 1) were *Bacteroidia* (~38% - ~44%) and *Clostridia* (~42% - ~49%) and the most abundant genus (Table 5) was *Bacteroides* (~26% - ~35%). None of the detected genera had a different relative abundance in the caecum samples collected from the animals fed the different diets. Similarly, to

Table 3. Animal performances (WG, FI and FE) of each week.

WG[b] (kg/bird)	Dietary treatment[a]						
week	CON	FAS	CTE	BL1	BL2	sem[c]	P[d]
1	0.068[a]	0.055[c]	0.056[bc]	0.061[abc]	0.066[a]	0.003	0.0026
2	0.272	0.238	0.223	0.229	0.228	0.014	0.0954
3	0.247	0.244	0.225	0.243	0.263	0.015	0.5011
4	0.290	0.303	0.305	0.291	0.295	0.015	0.9513
5	0.441[a]	0.368[b]	0.423[a]	0.439[a]	0.377[b]	0.020	0.0202
FI[b] (kg/bird)	Dietary treatment[a]						
week	CON	FAS	CTE	BL1	BL2	[c]sem	[d]P
1	0.082[b]	0.068[c]	0.078[b]	0.082[b]	0.094[a]	0.002	<0.0001
2	0.369[a]	0.358[a]	0.345[ab]	0.351[ab]	0.324[b]	0.008	0.0012
3	0.340	0.331	0.344	0.351	0.329	0.009	0.4666
4	0.568	0.576	0.568	0.541	0.558	0.014	0.5274
5	0.822[a]	0.758[b]	0.837[a]	0.830[a]	0.775[b]	0.012	<0.0001
FE[b]	Dietary treatment[a]						
week	CON	FAS	CTE	BL1	BL2	[c]sem	[d]P
1	1.234	1.317	1.481	1.404	1.463	0.078	0.0780
2	1.422	1.566	1.687	1.733	1.492	0.134	0.4265
3	2.012	1.394	1.583	1.532	1.279	0.210	0.4785
4	2.055	1.946	1.908	1.946	1.972	0.095	0.8479
5	1.927	2.253	2.003	1.993	2.122	0.119	0.3248

[a]Diet treatments: CON, negative control; FAS, positive control diet with 1.5 g/100g on DM of SMCFAs added to CON; CTE, positive control diet obtained with 1.5 g/100g on DM of CHT extract added to CON, BL1 obtained with 1.5 g/100g on DM of BL added to CON, BL2 with 3.0 g/100g on DM of BL added to CON.
[b]WG: weight gain; FI: feed Intake; FE: feed efficiency.
[c]sem: Standard error mean.
[d]P-value: Probability of significant effect due to experimental diets, different letters for $p < 0.05$.

Table 4. Diversity indexes calculated for ASV abundance.

Index	FAS[a]	CTE[a]	BL1[a]	BL2[a]	FAS[a]	sem[b]	P[c]
Chao1 index	268	236	271	229	251	16	0.9342
ACE index	268	0.916	271	229	251	16	0.9245
Simpson index	0.925	3.580	0.887	0.892	0.885	0.009	0.4719
Shannon index	3.756	236	3.580	3.518	3.513	0.094	0.9026

[a]Diet treatments: CON, negative control; FAS, positive control diet with 1.5 g/100g on DM of SMCFAs added to CON; CTE, positive control diet obtained with 1.5 g/100g on DM of CHT extract added to CON, BL1 obtained with 1.5 g/100g on DM of BL added to CON, BL2 with 3.0 g/100g on DM of BL added to CON.
[b]sem: Standard error mean.
[c]*p*-value: Probability of significant effect due to experimental diets, different letters for $p < 0.05$.
ASV: Amplicon Sequence Variance.

alpha-diversity analysis, the NMDS plot showed that the microbial communities were not different among the tested conditions (Figure 2) and this observation was further corroborated by the PERMANOVA, which did not show differences among groups ($p = 0.783$).

Discussion

In this work the effect of BL on the productive performances, welfare and gut microbiome of chickens was tested. Animal performance was monitored during a five week the trial (the whole period and single periods were considered), and the growth of birds was very similar regardless of the dietary treatment. The slaughtering weight was reached at the 35th day of life, in compliance with the standard parameters reported in Ross 308 broiler handbook of Aviagen (2018). No differences were found for the dressing out among groups confirming growths comparable among animals. The animal FE of each group was similar, either evaluating data from the whole or single growth periods and considering data weekly collected.

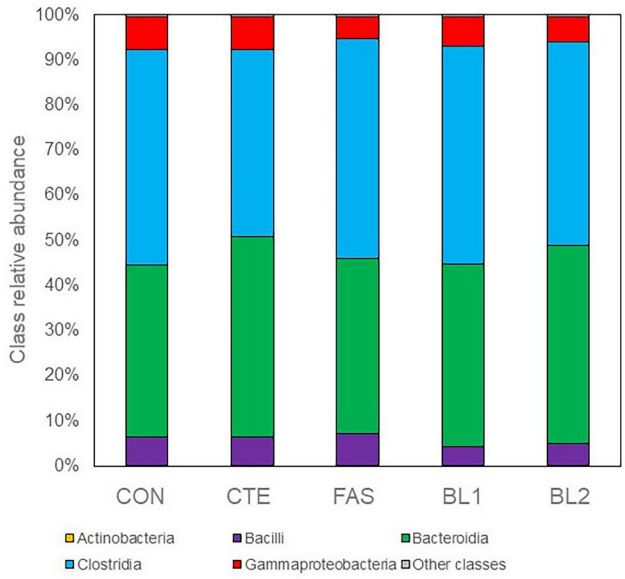

Figure 1. Relative abundance calculated at class level. Diet treatments: CON, negative control; FAS, positive control diet with 1.5g/100g on DM of SMCFAs added to CON; CTE, positive control diet obtained with 1.5 g/100g on DM of CHT extract added to CON, BL1 obtained with 1.5 g/100g on DM of BL added to CON, BL2 with 3.0 g/100g on DM of BL added to CON.

Several studies evidenced the main chemical characteristic of tannins in complexing proteins (Tsao 2010). This property makes difficult the hydrolysis of proteins and peptides to amino acids during digestion and, consequently, nutrient absorption at the gut level, lowering nitrogen retention by animals and increasing protein excretion. Tannins are also selective in complexing amino acids, making them unavailable for absorption. Moreover, most PPs are detrimental to the development of intestinal villi and act as strong antimicrobials, altering the gut microflora (Scicutella et al. 2021). However, tannins such as CHT show different behaviour in terms of target proteins and microorganisms (Mannelli et al. 2019a). Contrasting data on SCFAs salt bio-activities are available and, in particular, on C4:0 and C5:0 salts when these additives are used in poultry diets since the starter period. Several authors reported that sodium butyrate and valerate contribute to good gut health by acting on the microbial community and stimulating the development of intestinal epithelium (Elnesr et al. 2020; Hofacre et al.

Table 5. Genera with a relative abundance of 1% (or higher) in at least one sample in the caecum of chickens fed the tested diets.

Genus	CON[a] (%)	FAS[a] (%)	CTE[a] (%)	BL1[a] (%)	BL2[a] (%)	sem[b]	P[c]
[*Ruminococcus*] *torques* group	4.08	2.79	3.33	2.60	3.04	0.42	0.9136
Alistipes	1.87	1.82	1.74	1.29	2.20	0.23	0.7737
Anaerofilum	0.17	0.26	0.22	0.18	0.06	0.04	0.1167
Anaerotruncus	0.75	0.66	0.18	0.61	0.16	0.14	0.9516
Bacteroides	26.08	29.59	34.78	31.57	29.76	2.49	0.9118
Blautia	0.78	0.42	0.48	0.30	0.50	0.09	0.8483
Butyricicoccus	0.71	0.35	0.35	0.77	0.75	0.12	0.5064
Christensenellaceae R-7 group	0.39	0.34	0.47	0.31	0.38	0.06	0.8632
Clostridium sensu stricto 1	0.06	0.05	0.03	0.58	0.03	0.11	0.3167
Colidextribacter	0.48	0.31	0.17	0.26	0.35	0.07	0.3392
Dysgonomonas	0.03	0.04	0.12	0.00	0.33	0.07	0.4570
Enterococcus	2.65	2.30	1.24	0.86	0.67	0.42	0.8269
Erysipelatoclostridium	0.78	0.64	0.50	0.52	0.69	0.08	0.9460
Escherichia-Shigella	6.61	4.69	6.65	5.55	4.85	1.02	0.9827
Faecalibacterium	3.96	4.94	6.42	4.79	4.41	0.61	0.8390
Fournierella	0.39	0.56	0.24	0.48	0.15	0.08	0.4023
GCA-900066575	0.05	0.14	0.16	0.20	0.42	0.06	0.7923
Intestinimonas	0.46	0.57	0.52	1.03	0.49	0.12	0.7358
Lachnoclostridium	0.77	0.85	0.61	0.42	0.57	0.08	0.5261
Lactobacillus	0.08	0.04	0.77	0.38	0.64	0.12	0.3417
Ligilactobacillus	0.16	0.64	0.50	0.10	0.52	0.10	0.5270
Macellibacteroides	0.21	0.00	0.00	0.00	0.00	0.04	0.4409
Monoglobus	0.35	0.18	0.25	0.21	0.21	0.04	0.9197
Negativibacillus	0.96	1.77	0.87	1.71	0.91	0.24	0.5675
NK4A214 group	0.59	0.28	0.30	0.47	0.51	0.08	0.8891
Oscillospira	0.20	0.30	0.03	0.29	0.03	0.07	0.4764
Paludicola	0.29	0.02	0.02	0.03	0.07	0.05	0.4379
Parasutterella	0.65	0.20	0.62	0.96	0.72	0.11	0.2861
Rikenella	4.15	3.60	6.19	4.72	4.89	0.63	0.6964
Romboutsia	0.75	0.64	1.60	0.53	0.41	0.17	0.5553
Sellimonas	0.48	0.39	0.60	0.32	0.32	0.05	0.4400
Subdoligranulum	1.23	1.28	1.02	0.61	1.01	0.32	0.5548
Turicibacter	0.41	1.03	0.98	0.41	0.20	0.17	0.4483
Tuzzerella	1.55	3.96	0.71	0.78	1.09	0.60	0.5491
UC5-1-2E3	0.44	0.23	0.34	0.39	0.31	0.06	0.7988
UCG-005	1.93	4.17	2.01	3.54	2.77	0.36	0.1754
Other genera	3.17	2.72	3.60	2.22	3.19		
Unclassified	31.37	27.22	21.40	30.02	32.41		

[a]Diet treatments: CON, negative control; FAS, positive control diet with 1.5 g/100g on DM of SMCFAs sodium salts added to CON; CTE, positive control diet obtained with 1.5 g/100g on DM of CHT extract added to CON, BL1 obtained with 1.5 g/100g on DM of BL added to CON, BL2 with 3.0 g/100g on DM of BL added to CON.
[b]sem: Standard error mean.
[c]*p*-value: Probability of significant effect due to experimental diets, different letters for $p < 0.05$.

2020). Short-chain fatty acids can also increase crypt depth and villus height in the small intestine favouring the digestion and absorption of nutrients and disadvantaging colonisation of pathogens (Adil et al. 2010; Antongiovanni et al. 2010; Kum 2010). Sunkara et al. (2012) found that the oral supplementation of SCFAs (C4:0 and C5.0) can boost host immunity and disease resistance, with the potential for infectious disease control and prevention in animal farming without relying on antibiotics. These authors demonstrated that butyric and valeric acids enhanced antimicrobial host defence peptide gene expression without triggering proinflammatory interleukin-1b production in the chicken cells.

In contrast, other authors in previous studies observed antimicrobial activity of C4:0 on enteric bacteria, promoting the action on gut-associated lymphoid tissue and having a detrimental effect on digestion and absorption of nutrients (Cox et al. 2009). Several studies showed that unprotected C4:0 might also suppress protein-hydrolysing microorganisms by disrupting their energy metabolism and lowering environmental pH. Consequently, the hindgut protein fermentation is reduced limiting the availability and absorption of amino acids (Ricke 2003; Gunal et al. 2006).

In our trial, the uniformity of animal performances during the whole growth period seems to indicate that the presence of CHT and SMCFAs in the diets did not affect protein absorption either they are added alone or blended. Our results are consistent with those reported in Mannelli et al. (2019a) who found that CHT extract and Sn1-monoglycerides of SCFAs are well tolerated by poultry when mixed or alone. In the same study, the effect on the growth of several common pathogens in poultry, such as *Clostridium perfringens*, *Salmonella typhymurium*, *Escherichia coli* and *Campylobacter jejuni,*

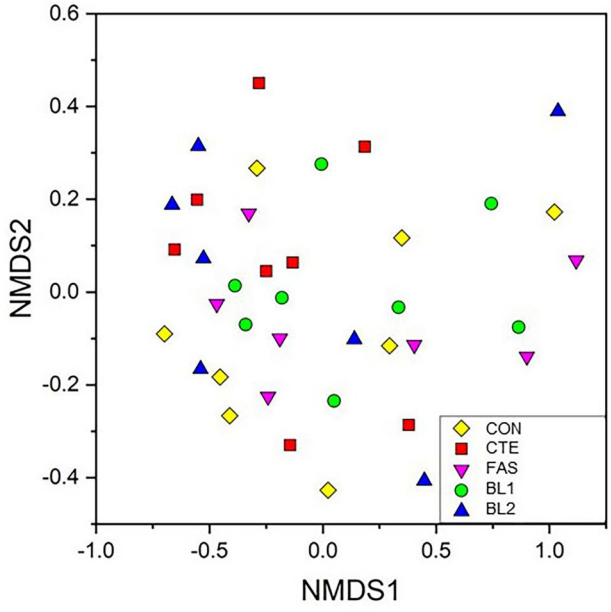

Figure 2. Non-metric multidimensional scaling plot based on Hellinger transformed ASVs abundance data. The microbial community in the caecum of the chickens fed the tested diets are not different. Stress = 0.0993. Diet treatments: CON, negative control; FAS, positive control diet with 1.5g/100g on DM of SMCFAs added to CON; CTE, positive control diet obtained with 1.5 g/100g on DM of CHT extract added to CON, BL1 obtained with 1.5 g/100g on DM of BL added to CON, BL2 with 3.0 g/100g on DM of BL added to CON

was evaluated and the results were promising since the tested compounds were very efficient in lowering the *in vitro* growth of these bacteria.

Observing the animal behaviour during feeding, all diets were voraciously consumed by chickens and even the BL diets seemed to be appreciated by animals in terms of palatability.

It is well known that the first week of life is crucial for animal gut health and its microbial colonisation (Heier et al. 2002; Yassin et al. 2009; Ayaman et al. 2021). In our trial, the data of animal performances were calculated per period and per week to verify if an eventual critical moment occurred during the birds' growth. Considering the first week of the chick life, no differences were registered among animals fed CON, CTE, BL1 and BL2 dietary treatments while chicks fed FAS had the lowest performances in accordance with findings by Gunal et al. (2006) who found that C4:0 could interfere with nutrient absorption in the first week of life. In contrast, no differences among treatments were found for WGs in the starter period, which may be due to a compensative effect that occurred in the second week when animals fed FAS showed an increase in the FI. This trend fits with a good absorption of nutrients related to the correct development of intestinal villi as reported by several authors (Leeson et al. 2005; Antongiovanni et al. 2007). Further investigations are needed to confirm this hypothesis. The faeces excreted by animals fed CTE, BL1 and BL2 were more compact than those of the animals fed CON or FAS, confirming the astringent effect of tannins in animal gut (Bonelli et al. 2018).

At the end of the trial, the bacterial communities in the caecum were characterised by high-throughput sequencing of the 16S rRNA gene. The most abundant classes in the gut of the animal in this trial were *Bacteroidia* and *Clostridia*, and the most abundant genus was *Bacteroides*. This observation is, in accordance with previous studies which reported that the phyla *Firmicutes* and *Bacteroidetes* are the most abundant in the caecum of healthy chickens (Rychlik 2020). The gut microbial communities in the animals fed the tested diets did not show significant differences in terms of alpha- and beta-diversity. This observation suggests that the use of SMCFAs, CHT, or a blend of them, at the inclusion level tested in our study, did not alter the composition of the gut microbiota compared to a control diet. Our observations are in contrast with another study in which the authors tested different tannin-based products on the diet of male Ross 308 chickens (Brugaletta et al. 2020). The authors observed a lower alpha-diversity in the gut of the animals fed a diet supplemented with one of the additives in which the main components were castalagin, gallic acid and vescalagin (main components of CHT extract) provided at a concentration of 50 mg/kg feed, 43 mg/kg feed, 41 mg/kg feed, and 46 mg/kg feed, 40 mg/kg feed, 38 mg/kg feed during the starter-grower I period and the grower II-finisher period, respectively. The dietary treatments led also to a difference in the beta-diversity despite being partially overlapped to the control diet (Brugaletta et al. 2020). Despite the higher concentration of tannins in the diets tested in our study compared to the products tested by Brugaletta et al. (2020), the difference in the results could be linked to the presence of other components in the additives tested by these authors. The absence of alteration in the composition of the gut microbiome of chickens involved in our study is of pivotal importance to apply SMCFAs- or tannin-based antimicrobials in the diet of poultry since several of these additives lead to detrimental effects on animal health (Ricke 2003; Gunal et al. 2006), despite their ability to inhibit the growth of pathogens (Mannelli et al. 2019a).

Conclusion

The replacement of antibiotics with alternative antimicrobial substances is mandatory to make more sustainable poultry meat production. The results of this trial showed that no negative effect on growth performances or on gut microbiota occurred when birds were fed the blend of free SMCFAs and CHT extract at the two inclusion levels tested in the study. Hence, the absence of alteration in the gut microbial community composition seems to show that the use of these antimicrobials in the diet of poultry may be an interesting alternative to antibiotics.

Acknowledgements

The authors thank Mr. Giovanni Bini, Dr. Annunzio Carulli (RI.PRO.COOP Società Cooperativa Agricola, San Vittore di Cesena - 47020 Forlì) and Fabio Nesti srl for technical assistance.

Ethical approval

Animal handling was under Italian Government guidelines (D.lgs 26/2014, art. 2 comma d and f).

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This study was funded by CFS Europe S.p.A *via* Agostino De Pretis 6, 48123 Ravenna Italy. Dr. Sauro Passeri and Dr. Roberta Danesi, belonging to this factory, were included in the author list because of their technical support.

Data availability statement

Data are available under reasonable request. The sequences are available at the National Centre for Biotechnology Information (NCBI), BioProject ID PRJNA955126

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