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Full-fat insect meals as feed additive – the effect on broiler chicken growth performance and gastrointestinal tract microbiota

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ABSTRACT. The aim of this study was to evaluate the effect of full-fat insect meals fed 'on top' to broiler chickens on their performance and the microbiota composition in the gastrointestinal tract. A total of 1850 day-old Ross 308 females were used in a set of four independent experiments. The insects *Gryllodes sigillatus*, *Shelfordella lateralis*, *Gryllus assimilis*, *Tenebrio molitor* and *Hermetia illucens* were applied in amounts that varied from 0.05 to 0.2%. In general, the application of insect meals to the diets of broilers did not affect their growth performance over the experimental period. However, the 0.2% additions of *T. molitor* and *H. illucens* increased feed intake at days 15–35 (P = 0.011) and the entire period of feeding (days 1–35; P = 0.018) (Experiment 3). Moreover, in Experiment 4 the supplementation of 0.2% of *S. lateralis* improved body weight gain (days 11–21 and 1–21), feed intake (days 1–10 and 1–21) and feed conversion ratio (days 1–21). The addition of insect meals reduced the pH value of digesta in the crop (Experiments 1 and 2) and in the caeca (Experiment 2). Supplementation with *H. illucens* caused the most significant effect on the microbiota populations in the crop, ileum and caeca (Experiment 3). However, at the higher levels of *S. lateralis* addition to the diets of broilers, the counts of selected microbiota in the crop and ileum increased (Experiment 4). These results indicate that the application of the insect full-fat meals in relatively small amounts can affect the microbiota composition in the gastrointestinal tract of broiler chickens.

Introduction

Insects are a natural source of protein, fat and other nutrients for omnivorous species of poultry in the wild. Furthermore, invertebrates play a crucial role in the survival of young poultry, which is reduced in habitats with low insect availability. It is well-documented that wild birds, including turkeys, need approximately 60% of their diet to come from insects during the first period of life (Rumble and Anderson, 1996), and the chicks of ring-neck pheasants are fully dependent on insects and animal matter intake during the first 3 weeks of life (Loughrey, 1951).

The nutritive value of insects is species- and stage-dependent. The crude protein content varies from 38 to 76%, whereas crude fat ranges between 14 and 43% (Józefiak et al., 2016). To date, in most research, the administration of invertebrates as a replacement for soyabean meal or fish meal in diets has produced satisfactory results (Wang et al., 2005; Hwangbo et al., 2009; Bovera et al., 2015; Maurer et al., 2015). The inclusion of 25% housefly larvae meal in the diet of broiler chickens resulted in better growth performance in comparison with the same amount of fish meal (Pretorius, 2011). Similar results were found by Awoniyi et al. (2003), and the substitution of 25% fish meal with insect meal showed the most efficient results in the case of average weekly gain (WG) and protein efficiency ratio (PER).

Currently, to the best of our knowledge, the effects of full-fat insect meals supplemented to diets in relatively small amounts, i.e. 0.05–0.2%, on broiler chicken growth performance and on the populations of microbiota in the gastrointestinal tract (GIT) have not been investigated.

Insects are also known as a potential source of antimicrobial peptides (AMP) with activities against both Gram-positive and Gram-negative bacteria, which may be exploited in livestock production (Józefiak and Engberg, 2017). AMPs are considered growth and health promoters with modulating effects on the intestinal microbiome and do not result in the development of natural bacterial resistance (Wen and He, 2012; Choi, 2013a,b; Xiao, 2015; Józefiak and Engberg, 2017). Therefore, the aim of the present study was to examine the effect of the application of full-fat insect meals as a feed additive (0.05–0.2%) 'on top' of complete diets on the growth performance and GIT microbiota composition of broiler chickens.

Material and methods

Birds and diets

According to Polish law and EU directive (No 2010/63/EU), the experiments conducted within the study do not require the approval of the Local Ethical Committee for Experiments on Animals in Poznań (Poland).

Four independent *in vivo* experiments using different full-fat insect meals as a supplement for feeding broiler chickens were conducted. In the first experiment, 480 day-old, female ROSS 308 chickens obtained from a commercial hatchery were randomly distributed into 6 treatments using 8 replicate pens per group and 10 birds per pen. The following insect species were used: *Gryllodes sigillatus* (Walker, 1869), *Shelfordella lateralis* (Walker, 1868), the imago or nymphal stage of *Gryllus assimilis* (Fabricius, 1775), *Tenebrio molitor* (Linnaeus, 1758) and *Hermetia illucens* (Linnaeus, 1758). The following treatments were applied:

Experiment 1: NC – negative control (without additives); PC – positive control (salinomycin, 60 mg · kg^{-1} diet); GS10 – NC + 0.1% of *G. sigillatus*; SL10 – NC + 0.1% of . *S. lateralis*; GA_I10 – NC + 0.1% of *G. assimilis*, imago stage; and GA_S10 – NC + 0.1% of *G. assimilis*, nymphal stage;

Experiment 2: NC – negative control (without additives); PC – positive control (salinomycin, 60 mg · kg^{-1} diet); GS20 – NC + 0.2% of *G. sigillatus*; SL20 – NC + 0.2% of *S. lateralis*; GA_I20 – NC + 0.2% of *G. assimilis*, imago stage; and GA_S20 – NC + 0.2% of *G. assimilis*, nymphal stage;

Experiment 3: NC – negative control (without additives); SL20 – NC + 0.2% of *S. lateralis*; TM20 – NC + 0.2% of *T. molitor*; and HI20 – NC + 0.2% of *H. illucens*;

Experiment 4: NC – negative control (without additives); PC – positive control (salinomycin, 60 mg · kg^{-1} additives diet); SL05 – NC + 0.05% of *S. lateralis*; SL10 – NC + 0.1% of *S. lateralis*; and SL20 – NC + 0.2% of *S. lateralis*.

All insect species used in the current studies were obtained from commercial source (HiProMine S.A., Robakowo, Poland) in the form of full-fat meals, finely ground and air-dried at 55 °C.

The first trial was conducted to investigate the growth performance, i.e. body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR). Additionally, pH values in the crop, ileal and caecal digesta were measured. Birds were maintained in floor pens (1 × 1 m) for 35 days. The stock density was based on 10 birds per m^2. The housing conditions were the same in all experiments.

The second experiment was conducted with the same experimental design as that in the first, but this trial differed in the amount of insect added, which was doubled (0.2%) compared to the first trial. The number of birds, experimental conditions and the measurements were the same as in the first experiment.

In the third trial, 400 day-old female ROSS 308 chickens were used. Birds were placed into 4 treatments containing 10 replicates per group and 10 birds per pen. *S. lateralis*, *T. molitor* and *H. illucens* were used at 0.2%. The growth performance parameters and the pH values in the digesta were measured as in the first trial. Additionally, populations of microbiota were determined in the crop, ileal and caecal digesta by fluorescent *in situ* hybridization (FISH).

In the fourth experiment, 500 day-old female ROSS 308 chickens were distributed into 5 groups with 10 replicate pens of 10 birds each. The experiment was run for 41 days, and 0.05, 0.1 and 0.2% of *S. lateralis* was added based on the results obtained in the third experiment. The growth performance parameters and the microbiota populations in the same three GIT segments were evaluated.

The compositions of the experimental diets used in all experiments are shown in Table 1. Diets for each period were formulated to be isonitrogenous and isocaloric. The diets were prepared in a mash form; all raw materials were ground by disc mill (Skiold A/S, Sæby, Denmark) at a 2.5-mm disc distance, mixed without any heat treatment and fed *ad libitum* to the birds. Starter diets were offered to all birds from days 1 to 8 (Experiments 1 and 2), 1 to 10 (Experiment 4), or 1 to 14 of age (Experiment 3); grower diets were fed between days 9 to 21 (Experiments 1 and 2), 11 to 21 (Experiment 4), or 15 to 21 of age (Experiment. 3); finisher diets were used from days 22 to 35 (Experiment 1, 2 and 3) or to 41 of age (Experiment 4). The experimental diets were designed to provoke GIT colonization by *Clostridium perfringens* due to the use of viscous cereals (wheat/rye), animal fat (pig lard) and fish meal. Furthermore, no exogenous enzymes were used in the studies. Insect meals were added 'on top' of the basal diets per tonne. The nutritive value of the selected insect species is shown in Table 2.

Table 1. Basal diet composition and nutritive value

Indices	Basal diet[1]		
	starter	grower	finisher
Ingredients, g · kg^{-1}			
wheat	226.1	330.6	150.0
maize grain	201.7	111.8	310.7
rye	150.0	150.0	150.0
soyabean meal	224.7	150.2	183.2
rapeseed meal	100.0	150.0	100.0
fish meal	15.0	19.2	20.0
pig lard	49.5	65.0	65.0
vitamin-mineral premix[2]	3.0	3.0	3.0
phosphate 2-Ca	17.1	10.5	7.8
limestone	3.3	2.3	3.4
NaCl	1.3	1.4	1.1
sodium carbonate	1.7	1.2	1.5
L-lysine-HCl	3.2	2.9	2.2
methionine (88% liquid)	3.0	2.0	2.2
L-threonine	0.7	0.1	0.1
Calculated nutritive value, g · kg^{-1}			
crude protein	208	198	190
crude fat	68.9	83.0	87.1
crude fibre	33.4	37.0	32.5
Na	1.5	1.4	1.4
Ca	8.5	7.0	6.5
P	7.8	6.9	6.1
lysine	12.9	11.8	11.2
methionine	5.9	5.0	5.0
methionine + cystine	9.5	8.7	8.4
threonine	8.4	7.4	7.2
AMEn[3], MJ· kg^{-1}	12.55	12.97	13.28

[1] starter diets were offered to all birds from days 1 to 8 (Experiments 1 and 2), 1 to 10 (Experiment 4), or 1 to 14 of age (Experiment 3); grower diets were fed between days 9 to 21 (Experiments 1 and 2), 11 to 21 (Experiment 4), or 15 to 21 of age (Experiment. 3); finisher diets were used from days 22 to 35 (Experiment 1, 2 and 3) or to 41 of age (Experiment 4); [2] provided per kilogram of diet: IU: vit. A 11 166, cholecalciferol 2500; mg: vit. E 80, menadione 2.50, vit. B_{12} 0.02, folic acid 1.17, choline 379, D-pantothenic acid 12.50, riboflavin 7.0, niacin 41.67, thiamine 2.17, D-biotin 0.18, pyridoxine 4.0, ethoxyquin 0.09, Mn (MnO_2) 73, Zn (ZnO) 55, Fe ($FeSO_4$) 45, Cu ($CuSO_4$) 20, I (CaI_2O_6) 0.62, Se (Na_2SeO_3) 0.3; [3] AMEn – apparent metabolizable energy corrected to zero nitrogen balance

Table 2. Nutritive value of selected insect species, g · kg^{-1} DM

Indices	*Gryllus sigillatus*	*Shelfordella lateralis*	*Gryllus assimilis*		*Tenebrio molitor*	*Hermetia illucens*
	imago	imago	nymph	imago	larvae	larvae
Crude protein	613 ± 49	546 ± 25	638 ± 29	564 ± 25	563 ± 25	404 ± 18
Crude fat	195 ± 18	261 ± 23	168 ± 15	238 ± 21	253 ± 20	335 ± 30
Crude fibre	60 ± 6	89 ± 9	94 ± 9	70 ± 7	85 ± 8	97 ± 10
Crude ash	65.6 ± 5.9	54.6 ± 4.9	53.6 ± 4.8	64.2 ± 5.8	45.3 ± 1.8	71.3 ± 6.4

Data and sample collection

The growth performance parameters in each experiment were measured using a replicate pen serving as the experimental unit. The FI and BWG of the chickens were determined on days 8 and 35 (Experiments 1 and 2) or 14 and 35 (Experiment 3) or 10, 21 and 41 (Experiment 4). In all trials, on day 28 from each experimental group, 10 randomly selected chickens were killed by cervical dislocation. In the first and second trial, one additional randomly-chosen bird was chosen from two separate replications. For analyses of the gastrointestinal contents (microbiota population and pH), the digesta of crop, ileum and caeca from individual birds (10 per treatment) were collected by gentle squeezing. A part (5 g) of each digesta sample was immediately packed and sealed in sterilized plastic bags. Samples were stored at −80 °C for the analysis of the microbiota composition by FISH of single bacterial cells. The digesta pH value was measured using a combined glass and reference electrode (pH 100L; VWR International, Leuven, Belgium). For pH value determining, as well as microbiota counts, the experimental unit was 1 bird randomly chosen from each replication (10 birds per treatment in total).

Microbial community analysis by fluorescent *in situ* hybridization (FISH)

All details of sample preparation and FISH analyses for bacteria enumeration from crop, ileal and caecal digesta are described in Józefiak et al. (2013). Briefly, samples of the gastrointestinal content taken during bird dissection were immediately frozen and stored in –80 °C. For FISH analysis, 100 µl of digesta were diluted in phosphate-buffered saline (PBS) and pipetted onto 0.22 µm polycarbonate filters (K02BP02500, Frisenette ApS, Knebel, Denmark) and vacuumed (LABOPORT Vacuum pomp, KNF, Freiburg, Germany). After vacuuming, the filters were transferred onto cellulose discs for dehydration in an ethanol series (50, 80 and 96%, 3 min each). For each sample, a series of identical filters was prepared to allow the determination of optimal hybridization. The oligonucleotide probes used in these studies are described in detail in Table 3 (Józefiak et al., 2016). Hybridizations were carried out in 50 µl of hybridization buffer (0.9 M NaCl; 20 mM Tris/HCl, pH 7.2; 0.01% SDS) containing the oligonucleotides probes. After hybridization, filters were washed with washing buffer (20 mM Tris/HCl, pH 7.2; 0.01% SDS; 5 mM EDTA) for 20 min at 48 °C. The filters were rinsed gently in distilled water, air-dried, and mounted on object glasses with VectaShield (No. H-1000, Vector Laboratories, Burlingame, CA, USA) anti-fading agent containing DAPI (4',6-diamidino-2-phenylindole). To distinguish the total count (DAPI) of bacteria from other particles in GIT content samples, the filters were left in 4 °C for 1 h in the dark until visualized using a Microscope Axio Imager M2 (Carl Zeiss, Thornwood, NY, USA).

Table 3. Oligonucleotide probes used for intestinal microbiota analyses using fluorescent *in situ* hybridization (FISH) (Józefiak et al. (2016))

Target	Probe	Sequence (from 5' to 3')
Bacteroides–Prevotella cluster	Bacto303	CCAATGTGGGGGACCTT
Enterobacteriaceae	Enter1432	CTTTTGCAACCCACT
Lactobacillus spp./ *Enterococcus* spp.	Lab158	GGTATTAGCAYCTGTTTCCA
Clostridium coccoides–Eubacterium rectale cluster	Erec482	GCTTCTTAGTCARGTACCG
Clostridium leptum subgroup	Clept1240	GTTTTRTCAACGGCAGTC
Streptococus spp./ *Lactococcus* spp.	Strc493	GTTAGCCGTCCCTTTCTGG

Statistical analyses

All experiments used a completely randomized design, and data were tested using the GLM procedure of the SAS statistical software package ver. 9.4 (SAS Institute Inc., Cary, NC, USA). In all experiments, means were separated using Duncan's tests following a one-way analysis of variance (ANOVA) based on the following equation:

$$Y_{ij} = \mu + \alpha_i + \delta_{ij}$$

where: Y_{ij} – observed dependent variable; μ – overall mean; α_i – effect of treatment; and δ_{ij} – random error. In cases in which the overall effect was significant ($P \leq 0.05$), means were compared pairwise (pdiff). The results are given as the least squares means with a pooled standard deviation.

Results

Experiments 1 and 2

The performance of the birds (Tables 4 and 5) fed diet with full-fat insect meal supplementation did not differ from NC and PC groups, irrespective of the supplementation amount (0.1 or 0.2%). However, the addition of both 0.1 and 0.2% of *G. assimilis* (nymphal stage) decreased the pH value of the digesta in the crop (Experiment 1, $P = 0.003$; Experiment 2, $P = 0.005$; Table 6), and the addition of 0.1% of *G. sigillatus* caused a similar effect ($P = 0.003$). Moreover, supplementation with 0.2% of *S. lateralis* reduced the pH in the crop and caeca compared with that in NC and PC groups and resulted in the lowest caecal digesta pH ($P = 0.002$) among all treatments.

Table 4. Growth performance of broiler chickens fed diet with 0.1% of insect meals addition (Experiment 1)

Indices[1]	Treatments[2]						SEM[3]	*P*-value
	NC	PC	GS10	SL10	GA_I10	GA_S10		
BWG, g								
days 1–8	153	156	158	153	156	158	1.52	0.885
9–35	1868	1937	1848	1958	1901	1841	22.02	0.575
1–35	2021	2093	2006	2111	2057	1999	22.23	0.614
FI, g								
days 1–8	205	205	208	210	203	203	2.03	0.877
9–35	2980	2975	2954	3071	3052	3001	17.74	0.348
1–35	3184	3180	3162	3281	3255	3203	18.44	0.380
FCR, g · g^{-1}								
days 1–8	1.35	1.33	1.32	1.37	1.31	1.29	0.02	0.745
9–35	1.60	1.55	1.61	1.58	1.61	1.64	0.01	0.556
1–35	1.58	1.53	1.58	1.57	1.58	1.61	0.01	0.586

[1] BWG – body weight gain, FI – feed intake, FCR – feed conversion ratio; [2] treatments: NC – negative control (without additives), PC – salinomycin (60 mg · kg^{-1} diet), GS10 – 0.1% of *Gryllodes sigillatus*, SL10 – 0.1% of *Shelfordella lateralis*, GA_I10 – 0.1% of *Gryllus assimilis* (imago stage), GA_S10 – 0.1% of *Gryllus assimilis* (nymphal stage) (in all groups insect meals were supplemented 'on top'); [3] SEM – standard error of mean

Table 5. Growth performance of broiler chickens fed diets with 0.2% of insect meals addition (Experiment 2)

Indices[1]	Treatments[2]						SEM[3]	*P*-value
	NC	PC	GS20	SL20	GA_I20	GA_S20		
BWG, g								
days 1–8	155	155	153	157	157	151	1.46	0.846
9–35	1884	1981	1848	1846	1834	1889	17.72	0.166
1–35	2038	2136	2001	2003	1991	2040	17.84	0.181
FI, g								
days 1–8	203	204	205	212	212	198	1.91	0.276
9–35	3010	3003	2987	2978	2982	2965	15.39	0.973
1–35	3213	3207	3193	3190	3194	3164	15.73	0.970
FCR, g · g^{-1}								
days 1–8	1.32	1.33	1.34	1.35	1.35	1.32	0.02	0.980
9–35	1.60	1.52	1.62	1.62	1.63	1.57	0.01	0.088
1–35	1.58	1.51	1.60	1.60	1.61	1.55	0.01	0.076

[1] see Table 4; [2] treatments: NC – negative control (without additives), PC – salinomycin (60 mg · kg^{-1} diet), GS20 – 0.2% of *Gryllodes sigillatus*, SL20 – 0.2% of *Shelfordella lateralis*, GA_I20 – 0.2% of *Gryllus assimilis* (imago stage), GA_S20 – 0.2% of *Gryllus assimilis* (nymphal stage) (in all groups insect meals were supplemented 'on top'); [3] SEM – standard error of mean

Table 6. Values of pH in the crop, ileum and caeca digesta after additions of 0.1 or 0.2% of insect meals (Experiments 1 and 2)

Digesta	Experiment 1 Treatments[1]						SEM[3]	*P*-value
	NC	PC	GS10	SL10	GA_I10	GA_S10		
Crop	5.49[a]	5.48[a]	4.91[b]	5.15[ab]	5.10[ab]	4.84[b]	0.06	0.003
Ileum	6.25	6.54	5.98	6.53	6.61	6.59	0.08	0.148
Caeca	6.30	6.19	6.13	5.89	6.18	5.94	0.05	0.120
	Experiment 2 Treatments[2]							
	NC	PC	GS20	SL20	GA_I20	GA_S20		
Crop	5.48[a]	5.48[a]	5.33[ab]	4.97[bc]	5.07[abc]	4.88[c]	0.06	0.005
Ileum	6.25	6.54	6.35	6.56	6.62	6.42	0.07	0.691
Caeca	6.30[ab]	6.19[ab]	6.42[a]	5.80[c]	6.16[ab]	5.98[bc]	0.05	0.002

[1] see Table 4; [2] see Table 5; [3] SEM – standard error of mean; [abc] – means with different superscripts within a row are significantly different at $P \leq 0.05$

Table 7. Growth performance of broiler chickens (Experiment 3)

Indices[1]	Tretments[2]				SEM[3]	*P*-value
	NC	SL20	TM20	HI20		
BWG, g						
days 1–14	372	379	383	387	2.44	0.181
15–35	1697	1697	1744	1715	10.93	0.196
1–35	2069	2076	2127	2101	10.52	0.193
FI, g						
days 1–14	542	532	535	545	3.52	0.556
15–35	2553[b]	2592[ab]	2676[a]	2665[a]	15.99	0.011
1–35	3094[b]	3124[ab]	3211[a]	3210[a]	16.90	0.018
FCR, g · g^{-1}						
days 1–14	1.46	1.41	1.40	1.41	0.01	0.122
15–35	1.51[b]	1.53[ab]	1.53[ab]	1.55[a]	0.01	0.014
1–35	1.50	1.51	1.51	1.53	0.01	0.095

[1] see Table 4; [2] treatments: NC – negative control (without additives), SL20 – 0.2% of *Shelfordella lateralis*, TM20 – 0.2% of *Tenebrio molitor*, HI20 – 0.2% of *Hermetia illucens* (in all groups insect meals were supplemented 'on top'); [3] SEM – standard error of mean; [ab] – means with different superscripts within a row are significantly different at $P \leq 0.05$

Experiment 3

Supplementation with *T. molitor* and *H. illucens* increased the feed intake of the birds at days 15–35 ($P = 0.011$) and for the entire trial ($P = 0.018$; Table 7). In the HI20 treatment, the FCR was impaired in comparison with that in the NC group at days 15–35 ($P = 0.014$), but no significant differences were observed for the entire trial (days 1–35). In the crop, statistically significant changes in microbiota were observed (Table 8). In the *T. molitor* treatment, the lowest counts of the *Bacteroides–Prevotella* cluster were observed ($P = 0.001$).

Table 8. Selected microbiota counts in the crop, ileal and caecal digesta determined by DAPI staining and fluorescent *in situ* hybridization, log cell number · g^{-1} of digesta (Experiment 3)

Indices	Treatments[1]				SEM[2]	*P*-value
	NC	SL20	TM20	HI20		
Crop						
total number of bacteria[3]	8.85	8.61	8.70	8.78	0.09	0.694
Bacteroides–Prevotella cluster	8.48[a]	8.46[a]	8.23[b]	8.41[a]	0.02	0.001
Clostridium leptum subgroup	8.25[bc]	8.42[a]	8.30[ab]	8.16[c]	0.03	0.015
Streptococcus spp./ *Lactococcus* spp.	7.83	8.19	8.04	7.91	0.09	0.741
Clostridium coccoides–Eubacterium rectale cluster	8.28[b]	8.32[b]	8.26[b]	8.45[a]	0.02	0.002
Lactobacillus spp./ *Enterococcus* spp.	7.98[b]	7.89[b]	8.07[ab]	8.23[a]	0.06	<0.0001
Ileum						
total number of bacteria[3]	8.80	8.80	8.63	8.81	0.02	0.193
Bacteroides–Prevotella cluster	8.34	8.47	8.36	8.55	0.01	<0.001
Clostridium leptum subgroup	8.29	8.37	8.36	8.34	0.01	0.081
Streptococcus spp./ *Lactococcus* spp.	8.23	8.24	8.24	8.36	0.03	0.001
Clostridium coccoides–Eubacterium rectale cluster	8.07[c]	8.16[b]	8.20[ab]	8.28[a]	0.01	<0.001
Lactobacillus spp./ *Enterococcus* spp.	8.28[b]	8.25[b]	8.44[a]	8.05[c]	0.04	0.009
Caeca						
total number of bacteria[3]	9.77	9.73	9.56	9.84	0.04	<0.0001
Bacteroides–Prevotella cluster	8.61[c]	8.81[b]	8.60[c]	9.05[a]	0.09	<0.0001
Clostridium leptum subgroup	8.28	8.32	8.43	8.45	0.07	0.061
Streptococcus spp./ *Lactococcus* spp.	8.17[b]	8.33[b]	8.21[b]	8.93[a]	0.20	<0.0001
Clostridium coccoides–Eubacterium rectale cluster	8.22[c]	8.39[bc]	8.48[b]	8.87[a]	0.16	<0.0001
Lactobacillus spp./ *Enterococcus* spp.	8.27[bc]	8.54[ab]	8.17[c]	8.60[a]	0.05	0.007

[1] see Table 7; [2] SEM – standard error of mean; [3] total number of bacteria determined by 4',6-diamidino-2-phenylindole (DAPI) staining; [abc] – means with different superscripts within a row are significantly different at $P \leq 0.05$

In comparisson with the SL20 and TM20 treatments, HI20 lowered the *Clostridium leptum* subgroup counts and increased those of the *Clostridium coccoides–Eubacterium rectale* cluster. Moreover, in the HI20 treatment, the highest numbers of *Lactobacillus* spp./*Enterococcus* spp. were observed compared with those of the NC and SL20 treatments. In comparison with the negative control, the addition of selected insect full-fat meals increased numbers of *Clostridium coccoides–Eubacterium rectale* cluster in the ileal digesta ($P < 0.001$). The lowest *Lactobacillus* spp./*Enterococcus* spp. counts were in the HI20 treatment, whereas an increase was observed in the TM20 treatment. In the caecal digesta, counts of *Bacteroides–Prevotella*, *Clostridium coccoides–Eubacterium rectale* clusters and *Streptococcus* spp./*Lactococcus* spp. were the highest in the HI20 group. *Hermetia illucens* addition also resulted in increased counts of *Lactobacillus* spp./*Enterococcus* spp. compared with those in the control and TM20 groups.

Experiment 4

At days 11–21 and 1–21, supplementation of 0.2% full-fat *S. lateralis* meal improved BWG (Table 9). The final BWG (days 1–41) in the SL20 treatment improved by approximately 3.5%, although the difference was not statistically significant ($P = 0.099$). The feed intake also improved in the SL20 treatment at days 1–10 and 1–21. The FCR in the SL20 treatment at days 1–21 was better than that in the PC ($P = 0.045$) but did not differ from that in the NC.

In the crop, the highest counts of total microbiota were found in the SL20 treatment. The *Clostridium leptum* subgroup and *Clostridium coccoides–Eubacterium rectale* counts increased in all treatments supplemented with *S. lateralis* (Table 10). In the ileal digesta, for most of the microbiota populations, no statistically significant effects of *S. lateralis* meal were observed. The only changes in the microbiota were for *Lactobacillus* spp./*Enterococcus* spp., and the lowest counts were marked in the NC treatment. In the caecal digesta, no significant differences were observed.

Table 9. Growth performance of broiler chickens (Experiment 4)

Indices[1]	Treatments[2]					SEM[3]	*P*-value
	NC	PC	SL05	SL10	SL20		
BWG, g							
days 1–10	181	177	178	184	189	1.56	0.089
11–21	561^{b}	552^{b}	560^{b}	564^{b}	588^{a}	3.75	0.025
1–21	740^{b}	727^{b}	737^{b}	747^{ab}	776^{a}	5.10	0.028
22–41	2002	2038	2029	2031	2064	8.91	0.295
1–41	2743	2765	2766	2778	2840	11.89	0.099
FI, g							
days 1–10	239^{b}	251^{ab}	244^{ab}	251^{ab}	257^{a}	2.04	0.041
11–10	828	827	831	848	867	5.25	0.067
1–21	1067^{b}	1078^{b}	1075^{b}	1100^{ab}	1124^{a}	6.64	0.041
22–41	3452	3485	3484	3517	3545	14.25	0.299
1–41	4519	4564	4560	4617	4669	19.39	0.123
FCR, g · g^{-1}							
days 1–10	1.32^{b}	1.43^{a}	1.37^{ab}	1.37^{ab}	1.36^{ab}	0.0111	0.048
11–21	1.48	1.50	1.48	1.50	1.47	0.0049	0.218
1–21	1.44^{b}	1.48^{a}	1.46^{ab}	1.47^{ab}	1.45^{b}	0.0051	0.045
22–42	1.72	1.71	1.72	1.73	1.72	0.0051	0.780
1–41	1.65	1.65	1.65	1.66	1.64	0.0039	0.687

[1] see Table 4; [2] treatments: NC – negative control (without additives), PC – salinomycin (60 mg · kg^{-1} diet), SL05 – 0.05% of *Shelfordella lateralis*, SL10 – 0.1% of *Shelfordella lateralis*, SL20 – 0.2% of *Shelfordella lateralis* (in all groups insect meals were supplemented 'on top'); [3] SEM – standard error of mean; [ab] – means with different superscripts within a row are significantly different at $P \leq 0.05$

Table 10. Selected microbiota counts in crop, ileal and caecal digesta determined by DAPI staining and fluorescent *in situ* hybridization (FISH), log cell number · g^{-1} of digesta (Experiment 4)

Indices	Tretments[1]					SEM[2]	*P*-value
	NC	PC	SL05	SL10	SL20		
Crop							
total number of bacteria[3]	9.08^{c}	8.98^{e}	9.15^{b}	9.03^{d}	9.22^{a}	0.01	<0.0001
Clostridium leptum subgroup	8.06^{c}	8.04^{c}	8.19^{b}	8.27^{a}	8.30^{a}	0.01	<0.0001
Clostridium coccoides–Eubacterium rectale cluster	8.01^{c}	8.12^{b}	8.26^{a}	8.32^{a}	8.29^{a}	0.01	<0.0001
Lactobacillus spp./*Enterococcus* spp.	8.53	8.60	8.56	8.34	8.47	0.01	0.01
Ileum							
total number of bacteria[3]	9.10	9.37	9.30	9.35	9.36	0.01	0.21
Clostridium leptum subgroup	8.26	8.56	8.32	8.30	8.21	0.01	0.06
Clostridium coccoides–Eubacterium rectale cluster	8.18	8.42	8.44	8.30	8.26	0.01	0.31
Lactobacillus spp./*Enterococcus* spp.	8.45^{d}	8.60^{b}	8.58^{c}	8.57^{c}	8.63^{a}	0.01	<0.0001
Caeca							
total number of bacteria[3]	10.20	10.10	10.16	10.18	10.07	0.004	0.22
Enterobacteraceae	8.96	8.88	8.83	8.76	8.71	0.01	0.22
Clostridium leptum subgroup	9.32	9.35	9.13	9.09	9.19	0.01	0.52
Clostridium coccoides–Eubacterium rectale cluster	9.41	9.33	9.33	9.43	9.29	0.004	0.12
Lactobacillus spp./*Enterococcus* spp.	9.52	9.57	9.41	9.38	9.42	0.01	0.32

[1] see Table 9; [2] SEM – standard error of mean; [3] total number of bacteria determined by 4',6-diamidino-2-phenylindole (DAPI) staining; [a–e] – means with different superscripts within a row are significantly different at $P \leq 0.05$

Discussion

Insect meals as a novel source of nutrients have been successfully added to the diets of animals, such as broiler chickens, layers, weaned pigs, aquatic animals and rabbits (Makkar et al., 2014). In general, the data suggest that the welfare, growth performance, nutrient digestibility, intestinal morphological features, and even quality of the carcass and meat are not adversely affected by insect additions to the diet. However, the current study was aimed to analyse the potential effects of insect meals used as feed additives, in low amounts ranging from 0.05 to 0.2%, added 'on top' of the diet.

In general, supplementation of the full-fat insect meals did not improve final performance results of the birds. However, the addition of 0.2% *H. illucens* and *T. molitor* increased FI without any adverse effect on the FCR, simultaneously improving BWG by 1.5 and 2.8%, respectively. Whereas the 0.05% addition of *S. lateralis* had a similar effect on BWG, FI and FCR to that of the group supplemented with salinomycin, the 0.2% addition increased BWG up to 3.5% as compared with the NC.

In poultry, all segments of the GIT are colonized by different populations of microbiota (Salanitro et al., 1978; Pan and Yu, 2014; Józefiak and Engberg, 2017). To date, more than 900 bacterial species have been identified, and it is well documented that the disturbance of microbial homeostasis can have significant negative effects on intestinal health and bird performance (Stanley et al., 2014). In the current experiments, the analysed microbiota populations were chosen as gut health indicators on the basis of our earlier research (Józefiak et al., 2011, 2012, 2013, 2016; Ptak et al., 2015; Kierończyk et al., 2016; Dunislawska et al., 2017). Although it cannot be demonstrated that final performance indices of the birds are improved due to supplementation with insect meals, significant changes in microbiota populations were marked. The predominant taxa of the GIT of broiler chickens belong to the phylum *Firmicutes* and consist of mainly cellulolytic and amylolitic *Clostridia* followed by *Bacillus* spp., *Lactobacillus* spp. and *Enterococcus* spp. (Egorova et al., 2016). In the current study, in the ileal digesta, counts of the *Clostridium coccoides–Eubacterium rectale* cluster were the highest in insect supplemented treatments in comparison with NC. Moreover, *Lactobacillus* spp./*Enterococcus* spp. counts increased with supplementation of *T. molitor* meal. *Shelfordella lateralis* supplementation increased the *Clostridium leptum* subgroup and *Clostridium coccoides–Eubacterium rectale* cluster counts in the crop, while *H. illucens* (0.2%) addition increased *Bacteroides–Prevotella*, the *Clostridium coccoides–Eubacterium rectale* cluster and *Streptococcus* spp./*Lactococcus* spp. in the caeca.

The *Clostridium leptum* subgroup includes many species that are members of the genera *Clostridium*, *Eubacterium* and *Ruminococcus*, which are mostly butyrate producing and fibrolytic species that have significant effects on gut health (Lay et al., 2005). Species of this genus produce organic acids, including butyrate, acetate, lactate or formate, but not propionic and succinic acids, as primary products of dietary fibre fermentation. Therefore, similar to the *Clostridium leptum* subgroup, the abundance of *E. rectale* is a good indicator of the butyrate-producing microbiota, which indirectly affect epithelial cell structure and function, particularly in the lower regions of the GIT. Observed changes in the *Clostridium leptum* subgroup, *Clostridium coccoides–Eubacterium rectale,* and *Bacteroides–Prevotella* show that the relatively low inclusion of the insect meals, particularly *S. lateralis* and *H. illucens*, can affect the GIT microbiome. Moreover, supplementation of *S. lateralis* resulted in improvement of BWG of the chickens, and this could be connected to the higher frequency of the *Clostridium leptum* subgroup, as well as *Clostridium coccoides–Eubacterium rectale*. Finally, the addition of insect full-fat meals also reduced the pH value in the crop, particularly with the supplementation of 0.1 or 0.2% of *G. assimilis*, 0.1% of *G. sigillatus* or 0.2% of *S. lateralis*. However, even in lower regions of the GIT (caeca), supplementation with 0.2% of *S. lateralis* decreased the pH value. This acidification effect can also reflect the potential bacteriostatic role of insect meals in the GIT of poultry, while fermentation processes contribute to the formation of short-chain fatty acids and shifts in pH values.

It is well-documented that insects produce AMPs that have broad-spectrum activity against both Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus* (MRSA)*, Klebsiella pneumoniae, Shigella sonnei, Pseudomonas aeruginosa* and *Escherichia coli*, and fungi, viruses and parasites (Park et al., 2014; Yi et al., 2014; Józefiak and Engberg, 2017). Additionally, the chitin content in insect meals could activate the innate immune system because of the fungistatic and immunoadjuvant properties (Dutta et al., 2004). The results of the present study demonstrate that even low supplementation of the full-fat insect to broiler chicken

diets can have a statistically significant impact on selected GIT microbiota populations. Therefore in our opinion, the role of AMPs and/or chitin need more research to clarify their potential effects on broiler chicken gut health.

Conclusions

It can be concluded that supplementation with full-fat insect meals in relatively small amounts, i.e. from 0.05 to 0.2%, in the diet of broiler chickens can modulate microbiota populations in the gastrointestinal tract of broiler chickens.

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