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Original article

Effects of *Hermetia illucens* larvae full-fat meal and astaxanthin on the microbiome and histomorphology of the large intestine in piglets

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Abstract

This study evaluated the effects of *Hermetia illucens* (HI) larvae full-fat meal and astaxanthin (AST) on large intestine histomorphometry, microbiota activity, and composition in pigs. Forty-eight pigs (8.7 kg) were divided into six groups: control (0HI), 2.5% HI (2.5HI), 5% HI (5HI), 2.5% HI + AST (2.5HI+AST), 5% HI + AST (5HI+AST), and AST alone (AST). The experiment lasted from 35 to 70 days of age. HI meal increased mucosal thickness ($p<0.01$), crypt depth ($p<0.05$), and width ($p<0.05$). Goblet cell counts increased in the 2.5HI ($p<0.05$), while enterocyte numbers decrease in the AST group ($p<0.01$). Dietary HI meal reduced concentrations of total short-chain fatty acids (SCFA), including butyrate ($p<0.05$), whereas AST increased acetic acid levels in multiple intestinal regions ($p<0.05$). Both additives modified microbial populations: AST increased total bacterial counts ($p<0.001$), while 2.5% HI meal reduced the abundance of the *Bacteroides–Prevotella* cluster ($p<0.001$). Significant interactions were detected for *Lactobacillus/Enterococcus* spp. and Enterobacteriaceae ($p<0.001$). HI meal decreased p-cresol concentrations in the middle colon ($p<0.05$), whereas AST reduced phenol in the distal colon ($p<0.05$) and indole in the middle colon ($p<0.05$). AST increased ammonia levels in the proximal colon ($p=0.001$). These findings suggest that HI meal and AST modulate intestinal fermentation, exhibit anti-inflammatory effects, and regulate microbial populations, potentially reducing harmful metabolites and odor emissions. Their dietary combination may have positive implications for intestinal health.

Keywords: astaxanthin, colon fermentation, colon histomorphometry, *Hermetia illucens*, intestinal microbiota

Introduction

Given the continual increase in the global human population and escalating demand for animal products, livestock farmers face a formidable challenge in ensuring sustainable and profitable practices. In addition to ensuring that the animals maintain good health and appropriate zoohygienic conditions, providing proper nutrition is vital for stimulating their growth, development and health (Yu et al. 2019). Due to the increasing demand for protein as one of the main nutrients, insect protein has received much attention in recent years. *Hermetia illucens* (HI) larvae meals provide feed with a high nutritional value, as they are rich in protein (40-44%) and have a favorable amino acid composition (Kierończyk et al. 2022). In addition, HI larvae meal is rich in antimicrobial peptides (AMP), a promising alternative to antibiotics in livestock farming, which is important given the global problem of increasing antibiotic resistance of bacterial strains (Szczepanik and Świątkiewicz, 2024). Additionally, HI larvae contain the polysaccharide chitin, which is a component of the arthropod exoskeleton, and is one of the most widely occurring biopolymers in nature. It is resistant to digestion and absorption in the small intestine of monogastric animals and travels into the large intestine, where the microbiota utilizes it as a fermentable substrate (Triunfo et al. 2022), in case of piglets it reduces the proliferation of pathogenic bacteria causing postweaning diarrhea (Xu et al. 2018). HI meal used as a feed mixture supplement is already well known in various farm animals feeding. For example, in weaned piglets fed with HI meal was observed better growth performance, less diarrhea incidences (Tang et al. 2022a), improved intestinal barrier function, as well as the intestinal microflora composition and immune homeostasis (Yu et al. 2019). There are currently various types of HI meal available on the feedstuff market, in which the main difference is the fat content, however in the present experiment a full-fat meal was used. The justification for this choice was the presence of specific fatty acid – lauric acid, with immunostimulatory, antimicrobial, and anti-inflammatory properties (Józefiak et al. 2017, Gasco et al. 2020), which are also promising in terms of improving the health status of piglets.

The highly intensive growth of piglets of fast-growing breeds is associated with an accelerated metabolism, which in combination with weaning stress can result in the formation of significant amounts of free radicals (Nussey et al. 2009). The young organism is exposed to damage to cellular structures (cell membrane, lipids, proteins, and even DNA) due to the action of reactive oxygen species or excessive stimulation of NAD(P)H causing oxidative stress (Valko et al. 2007). In order to improves the durability of feed mixture during storage, especially unsaturated fatty acids, but also to help counteract the effects of oxidative stress on the animal body, it is good practice to use antioxidants as nutritional additives. Astaxanthin (AST), a naturally occurring carotenoid, is a powerful antioxidant that also has anti-inflammatory properties and protects against apoptosis by regulating mitochondrial proteins (Uchiyama 2008, Macedo et al. 2010, Choi et al. 2011, Ciaraldi et al. 2023). Research indicates that the systemic stress linked to weaning piglets is a significant contributor to the impairment of intestinal barrier function in early weaned pigs, leading to increased production of free radicals and decreased antioxidant activity (Zhu et al. 2012, 2022). Consequently, antioxidants are advised to safeguard the intestinal epithelium from the harmful effects of free radicals. Studies have reported a modulatory effect of AST on the intestinal microbiota (Uchiyama 2008, Yang et al. 2011). However, no studies have analyzed the effects of AST on the microbiota and metabolic status of the large intestine in pigs.

The authors in earlier publications demonstrated both the effect of AST alone and HI larvae meal and the synergistic effect of both additives (Szczepanik et al. 2023). It was shown that the inclusion of HI larvae meal did not have a negative effect on growth performance, organ weights, and lengths of digestive tract sections, as well as on piglet feed intake itself. Moreover, in the experiment conducted, no significant effect of AST supplementation was observed on the production rates of weaned piglets. This suggests that these supplements do not impair feed palatability. These results are in line with other work on the supplementation of feed rations with these additives at similar rations (Yang et al. 2006, Bergstrom et al. 2009, Biasato et al. 2019). In turn, hematological analyses of the blood showed validity in using both additives together (Szczepanik et al. 2023). The addition of 5% HI larvae meal lowered the piglets' red blood cell parameters, which is physiologically unfavorable. When AST was included in the feed ration, these parameters improved significantly, suggesting a strong antioxidant effect of this additive and protection against oxidative stress probably caused by increased lauric acid concentration (Alfhili and Aljuraiban 2021, Lang et al. 2014). Other analyses by the authors (Szczepanik et al. 2024) showed that HI larvae meal increased the length and width of villi in the duodenum and in combination with AST improved the ratio of their length to the depth of the crypts. In addition, administration of HI meal at 2.5% together with AST increased the width of the muscularis, which potentially improves intestinal peristalsis. To the authors' knowledge, there are no studies that have analyzed the effects of AST, as a potent antioxidant,

administered together with HI meal on the microbiota and metabolic status of the large intestine in pigs.

Very interesting aspect of the present study is the focus on the status of the large intestine, where the main microbiological fermentation processes take place and which is rarely taken into account in the assessment of the effectiveness of feed additives. It is assumed that the inclusion of insect meal in concentrations of 2.5% and 5% in the diet of pigs, administered alone or in combination with AST, will result in beneficial modifications of selected components of the intestinal microflora and changes in the histomorphometric and metabolic parameters of the digestive tract. As a result, the combination of these dietary factors is expected to improve the overall health and functioning of pigs. This study aimed to evaluate the effects of *Hermetia illucens* larvae full-fat meal and astaxanthin on the histomorphometric characteristics of the large intestine, as well as on the activity and composition of the intestinal microbiota.

Materials and Methods

Ethical statement

All procedures included in this study relating to the use of live animals agreed with the First Local Ethics Committee for Experiments with Animals in Cracow, Poland (Resolution No. 420/2020, date 22.07.2020). Throughout the experimental period, a veterinarian regularly monitored the health status of postweaning pigs.

Animals – feeding and housing, and experimental design

The experiment was conducted on forty-eight 35-day-old postweaning pigs (barrows) (Polish Landrace) weighing approximately 8.7 kg ± 0.2 kg. The pigs were divided into six groups, with eight pigs in each: group I – control; group II – addition of 2.5% *Hermetia illucens* larvae meal (2.5HI); group III – addition of 5% HI (5HI); group IV – addition of 2.5% HI and astaxanthin (2.5HI+AST); group V – addition of 5% HI and AST (5HI+AST); and group VI – addition of AST (AST). HI larvae meal was a full-fat product from commercial sources (HiProMine S.A, Robakowo, Poland). The meal used contained 426 g of protein, 91 g of chitin defined as fibre, and 264 g of fat. Lauric acid accounted for 36% of the total. The selected inclusion levels of the insect meal were determined based on the optimisation of animal production costs and are consistent with the dose ranges reported in contemporary international studies. AST originated from *Haematococcus pluvialis* (Podkowa AD 1905 Lublin, Poland) and was added at 0.025 g per 1 kg of feed mixture. Piglets were fed an isoprotein, isoenergetic diet meeting Polish standards (Grela & Skomiał, 2020). Diet composition was previously detailed by Szczepanik et al. (2023), and basic chemical analyses followed AOAC methods (2009). The experimental fattening lasted for 35 days. Pigs had ad libitum access to feed and water and were housed in a mechanically ventilated room at 18-20°C, ~55% humidity, 15 air exchanges/hour, and a natural day/night light cycle. All pigs were provided with environmental enrichment throughout the experimental period. Enrichment consisted of manipulable materials, including hanging chains and toys, which were accessible ad libitum. At the end of the experiment, all pigs were slaughtered by approved stunning using a Blitz penetrating pin device (Turbocut Jopp GmbH, Germany).

Sample collections

For histological analysis, approximately 4 cm sections were taken from the middle of the proximal part of the colon (1/4 of the large intestine length) and placed in buffered formaldehyde solution (4%). The digestive contents for microbial community analysis by fluorescence *in situ* hybridization (FISH) were taken from the proximal part of the colon, placed in cryogenic tubes, frozen initially in liquid nitrogen, and then in a low-temperature freezer (-80°C). Additionally, digesta samples were taken from the proximal, middle, and distal colon (1/4, 1/2, and 3/4 of the length of the colon, respectively) for pH measurements and analyses of short-chain fatty acids (SCFA), ammonia, and phenolic compound concentrations.

Analysis of histomorphometry

After 24 hours of storage in formalin solution, the proximal part of the large intestine tissue fragments were cut into sections approximately 0.5 cm in length and placed in histology cassettes. These samples were then dehydrated in graded ethanol solutions (up to 70Tissue cassettes were processed in an automatic tissue processor (CITADEL 2000, Thermo Scientific, Germany) using a graded ethanol series (50-100%), xylene and paraffin (all reagents from Idalia, Poland). To ensure result reliability, two paraffin blocks were prepared per large intestine sample. Each block was sectioned into 4 μm slices using a microtome (Microm HM 340 E, Thermo Scientific, Germany), and two slides were prepared from each block. The intestinal tissue sections were stained using the Alcian blue-PAS method to facilitate goblet cell counting. Stained slides were examined using a light microscope (Axio Lab.A1, Zeiss) with an Olympus EP50 camera with software

Table 1. Oligonucleotide probes used in the study.

Target	Probe	Sequence (5′ to 3′)
Enterobacteriaceae	*Enter1432*	CTT TTG CAA CCC ACT
Bacteroides-*Prevotella* cluster	*Bac303*	CCAATGTGGGGGACCTT
Clostridium leptum subgroup	*Clept1240*	GTTTTRTCAACGGCAGTC
Clostridium coccoides-Eubacterium rectale cluster	*Erec482*	GCTTCTTAGTCARGTACCG
Clostridium perfringens	*Cperf191*	GTAGTAAGTTGGTTTCCTCG
Lactobacillus spp./Enterococcus spp.	*Lab158*	GGTATTAGCAYCTGTTTCCA

EPview (both Olympus, Tokyo, Japan) and ImageJ version 1.53 (US National Institute of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/index.html). In the large intestine, the following parameters were measured: mucosal width, crypt depth, and width (measured at the midpoint of the crypt depth), number of goblet cell count, and number of enterocytes count per 100 µm crypt section. The width of the muscularis was also assessed with the distinction between the longitudinal and circular muscle layers. For each pig, 15 measurements were collected for every parameter. These measurements were then averaged to obtain a single representative value for each animal.

Analysis of digesta pH and SCFAs

The pH of the digestive contents was measured in three sections of the large intestine using a SevenMulti pH-meter (Mettler-Toledo, Warsaw, Poland). SCFA analysis was performed according to the procedure described by Barszcz et al. (2011) on an HP5890 Series II gas chromatograph (Hewlett Packard, Waldbronn, Germany) with a flame-ionization detector and Supelco Nukol fused silica capillary column (30 m × 0.25 mm internal diameter, 0.25 mm film). Helium served as the carrier gas. The concentrations of each acid were determined relative to an internal standard (isocaproic acid) using a mixture of SCFA standard solutions.

Analysis of ammonia and aromatic compounds

Phenol, p-cresol, and indole concentrations in the large intestine digestive contents were analyzed according to the method described by Taciak et al. (2015) using a Shimadzu GC-2010 gas chromatograph (Japan) equipped with an AOC-20i autosampler and a split/splitless injector. 5-methylindole was used as an internal standard to calculate the concentration of aromatic compounds. The ammonia concentration was determined spectrophotometrically using the method described by Taciak et al. (2015). The method is based on the reaction of ammonium ions with Nessler's reagent and the analysis was performed on a MAXMAT PL biochemical analyzer (Erba Diagnostics France SARL, Montpellier, France). A standard curve was prepared using ammonium chloride solution and the absorbance was measured at 425 nm.

Microbiota analysis

FISH analysis of the proximal part of the large intestine (1/4 of the length) was performed according to a previously described protocol (Józefiak et al. 2013). In brief, the samples were thawed and then retorted. Two individuals per sample were combined at a ratio of 1:1 within the same group (n=4). For FISH analysis, 100 µl of each digestive mixture was collected and diluted in PBS (1:1000). A vacuum pump (Vaccum Pump KNF, Vagobletort-Neuberg, Trenton, NJ, USA) was subsequently used to filter the sample through 0.22 µm polycarbonate filters (Frisenette K02BP02500, Knebel, Denmark). After vacuuming, the filters were transferred to cellulose disks for dehydration in a series of ethanol (99.8% purity, POCH, Avantor Performance Materials Poland S.A., Gliwice, Poland) solutions (50, 80, and 96%, each for 3 minutes). A series of identical filters was prepared for each sample to determine optimal hybridization. The oligonucleotide probes used in this study are presented in Table 1. Hybridization was performed in 50 µl of hybridization buffer (0.9 M NaCl; 20 mM Tris/HCl, pH 7.2; 0.01% SDS) containing the oligonucleotide probes. After 24 h of incubation at 37°C in a water bath, the filters were washed twice with wash buffer (20 mM Tris/HCl, pH 7.2; 0.01% SDS; 5 mM EDTA) and then with distilled water. The filter was subsequently dried and mounted on subject slides via VectaShield (Vector laboratories No. H-1000, Burlingame, CA, USA) containing 4',6-diamidino-2-phenylindole (DAPI) and coverslips. The prepared slides were incubated overnight at 4°C and then viewed under an Axio Imager M2 microscope (Carl Zeiss, Obenkochen, Germany). To count selected microbial populations, ZEN lite software (Carl Zeiss, Obenkochen, Germany) and ImageJ (version 1.53; US National Institutes of Health, Bethesda, MD, USA) were used. For each sample, 5 microscope images (n=20) were used for further calculations, and the final results were expressed in log CFU per g of digesta.

Statistical analysis

Histomorphometric data and microbiota activity indices are presented as means ± standard error of the mean (SEM). Data were analyzed using two-way analysis of variance (two-way ANOVA), and multiple comparisons were performed with Bonferroni correction using Statistica® v.13.3 (StatSoft Inc., Tulsa, OK, USA). Differences were considered statistically significant at $p<0.05$, and trends were considered when $p<0.10$. The experiment was conducted in a 3 × 2 factorial design, with the statistical model including two main factors – the inclusion level of Hermetia illucens larvae meal in the feed (0%, 2.5%, and 5%) and the presence of AST (present vs. absent) – as well as their interaction. Individual piglets served as experimental units (n=8 per group). Normality of residuals in the two-way ANOVA model was assessed using the Shapiro–Wilk test, and homogeneity of variances was verified using Levene's test.

Furthermore, to determine changes in selected microbial populations, the large intestine digesta were pooled by two individuals, and five photographs of each sample were taken per treatment (n=20). The normality of the data was assessed using the Shapiro–Wilk test, and the homogeneity of variance was evaluated using Bartlett's test. Differences among treatments were determined via Dunn's test with Benjamini–Hochberg adjustments for multiple comparisons, followed by the application of a significant Kruskal–Wallis or Scheirer–Ray–Hare test. The analyses were conducted via RStudio (v. 2024.04.0 + 735; 2024 RStudio, Inc., Boston, USA). The model employed in the analysis was as follows:

$$Y_{ijk} = \mu + \alpha_i + \beta_j (\alpha\beta)_{ij} + \delta_{ijk}$$

where: Y_{ijk} represents the observed dependent variable, μ represents the overall mean, α_i represents the effect of HI larvae full-fat meal, β_j represents the effect of AST, $(\alpha\beta)_{ij}$ represents the interaction between HI larvae meal and AST, and δ_{ijk} represents the random error.

Results

Results of histomorphometric analyses

The results of all histomorphometric measurements of the proximal colon are presented in Fig. 1. The interaction effect was significant for the number of goblet cells and enterocytes in the crypts, as well as the width of the longitudinal muscle layer ($p<0.05$). The HI meal significantly affected the width of the mucosa, as well as the depth and width of the crypts ($p<0.05$). Conversely, the effect of AST was significant only for the width of the crypts ($p<0.05$). In the group receiving the AST supplement alone, the mucosa was significantly wider than that in both HI meal groups (2.5HI: $p<0.05$; 5HI: $p<0.001$). Significantly deeper crypts were observed in the group receiving AST alone than in the group receiving both 5% HI and AST ($p<0.05$). More goblet cells per 100 µm crypt were counted in the 2.5HI treated group compared to the 5HI group ($p<0.05$), and the control group ($p<0.01$). Furthermore, the group whose diet was supplemented with 5% HI and AST meal had more goblet cells than the group with only AST ($p<0.01$). More goblet cells were also counted in the group treated with 2.5HI meal and AST together, than in the group with AST alone ($p<0.05$). A lower number of enterocytes was recorded in the group treated with AST alone than in the groups receiving HI meal at both levels with AST ($p<0.001$). The group with AST alone showed a significantly wider longitudinal muscle layer compared to the groups with the addition of both AST and HI at both levels ($p<0.01$). There were no significant differences in the circular muscle layer width or total muscle layer width.

Digesta pH and short-chain fatty acid concentration

The results of the pH measurements and SCFA analysis performed in the large intestine digesta are presented in Table 2. There was no effect of interaction between the two nutritional factors. HI full-fat meal and AST on these parameters. The results of pH measurements and SCFA analysis in the large intestine contents are presented in Table 2. No significant interactions between the two nutritional factors, i.e., full-fat HI meal and AST, were observed. In the proximal colon, total SCFA concentration was higher in the group without HI meal compared to the 2.5% HI group and increased in the groups supplemented with AST. Similarly, acetic acid ($p<0.05$) and propionic acid ($p<0.05$) were higher in the control group compared to the 2.5% HI group, and acetic acid was further increased by AST supplementation ($p<0.01$). Butyric acid was significantly higher in the group without HI meal compared to the 5% HI group ($p<0.05$). The pH measured in the digestive content of this section was not affected by any of the tested supplements. In the middle section of the large intestine, AST supplementation increased the acetic acid concentration ($p<0.05$), and 5% HI meal supplementation decreased its concentration compared to the control group ($p<0.05$). No significant differences were noted in the concentrations of propionic, isobutyric, butyric, isovaleric, or valeric acids. Higher pH values were observed in the groups treated with 5% HI meal than in the groups without meal ($p<0.05$). In the

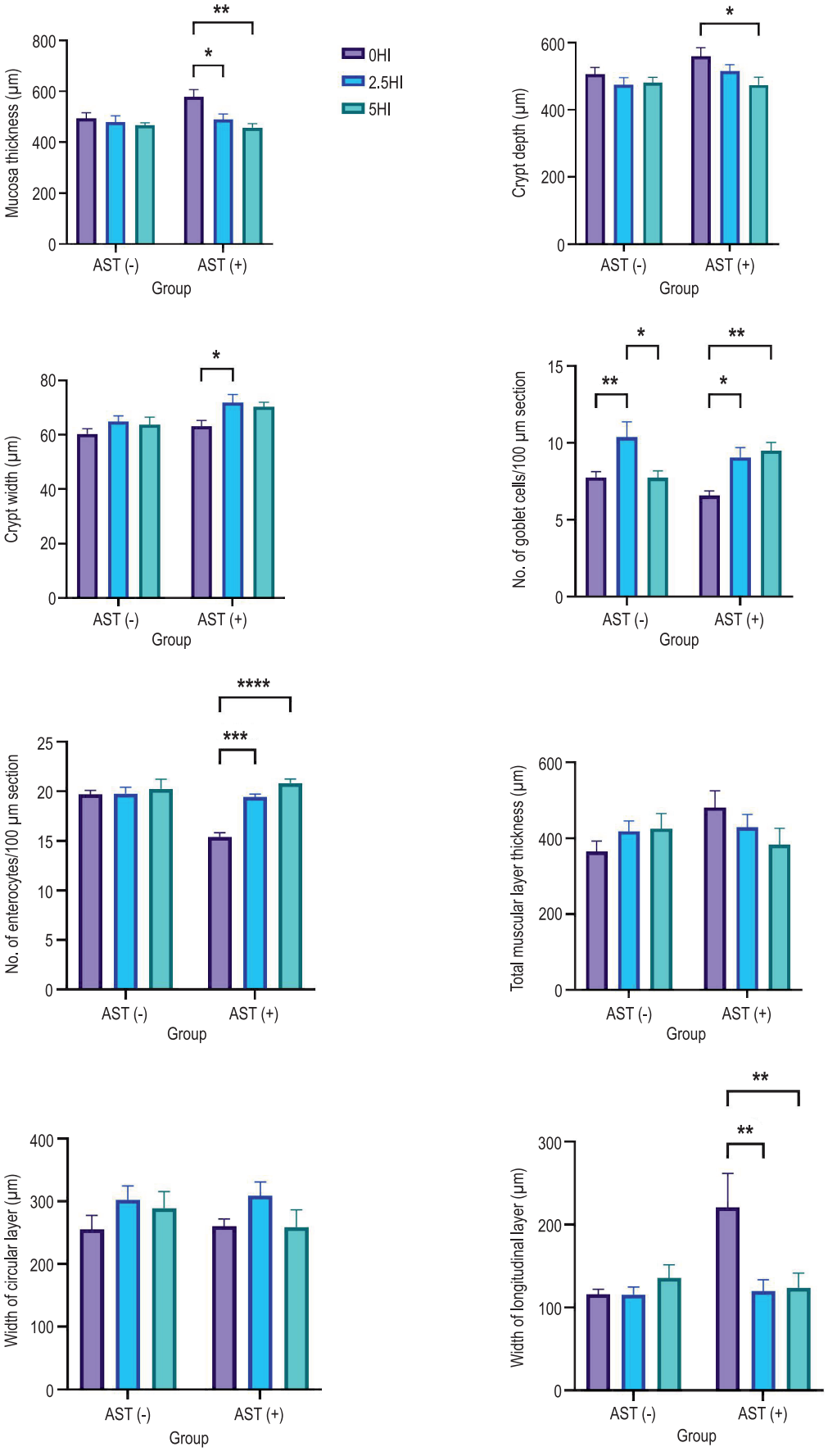

Fig 1. Effects of Hermetia illucens larvae full-fat meal and astaxanthin in the diet on histomorphometry in the large intestine of weaned piglets.

Table 2. Effect of *Hermetia illucens* larvae full-fat meal and astaxanthin on the pH and concentration of short-chain fatty acids (μmol/g of digest) in the large intestine of piglets.

Item	Factor 1: insect meal (HI)			Factor 2: astaxanthin (AST)		I	II	III	IV	V	VI	SEM	*p*-value		
	0HI	2.5HI	5HI	-	+	0HI	2.5HI	5HI	2.5HI+AST	5HI+AST	0HI+AST		HI	AST	HIxAST
Proximal large intestine															
pH	6.407	6.546	6.555	6.553	6.453	6.336	6.614	6.710	6.479	6.400	6.479	0.05	> 0.05	> 0.05	> 0.05
Acetic acid	52.123^{b}	43.902^{a}	46.464^{ab}	44.569^{b}	50.423^{a}	51.21	41.198	41.298	46.605	51.63	53.035	1.24	< 0.05	< 0.01	> 0.05
Propionic acid	25.007^{b}	20.675^{a}	21.130^{ab}	21.249	23.292	24.802	20.132	18.814	21.219	23.446	25.211	0.70	< 0.05	> 0.05	> 0.05
Isobutyric acid	1.460	1.499	1.510	1.466	1.514	1.288	1.494	1.615	1.504	1.405	1.633	0.05	> 0.05	> 0.05	> 0.05
Butyric acid	14.721^{a}	12.398^{ab}	11.759^{a}	12.34	13.579	14.108	11.585	11.326	13.21	12.191	15.335	0.48	< 0.05	> 0.05	> 0.05
Isovaleric acid	1.752	1.665	1.691	1.610	1.796	1.312	1.638	1.879	1.692	1.504	2.192	0.07	> 0.05	> 0.05	> 0.05
Valeric acid	3.956	3.299	3.435	3.624	3.502	4.196	3.324	3.352	3.273	3.517	3.716	0.16	> 0.05	> 0.05	> 0.05
Sum of SCFA	98.459^{a}	83.437^{b}	85.988^{ab}	84.857^{b}	93.732^{a}	96.917	79.372	78.284	87.503	93.693	100.001	2.82	< 0.01	< 0.05	> 0.05
Middle large intestine															
pH	6.704^{a}	6.749^{ab}	6.96^{b}	6.808	6.803	6.640	6.853	6.931	6.645	6.988	6.775	0.04	< 0.05	> 0.05	> 0.05
Acetic acid	41.787^{a}	39.372^{ab}	36.444^{b}	37.380^{b}	41.094^{a}	39.787	36.173	36.179	43.028	36.709	43.788	0.82	< 0.05	< 0.05	> 0.05
Propionic acid	18.599	17.656	16.179	16.977	17.978	17.849	16.747	16.336	18.565	16.021	19.350	0.49	> 0.05	> 0.05	> 0.05
Isobutyric acid	1.983	1.570	1.725	1.685	1.833	1.804	1.508	1.744	1.632	1.707	2.161	0.46	> 0.05	> 0.05	> 0.05
Butyric acid	10.993	10.256	9.629	10.245	10.340	10.314	10.134	10.287	10.378	8.970	11.672	0.37	> 0.05	> 0.05	> 0.05
Isovaleric acid	2.320	1.780	2.100	2.000	2.134	2.107	1.740	2.153	1.821	2.046	2.533	0.09	> 0.05	> 0.05	> 0.05
Valeric acid	3.258	2.978	2.858	3.126	2.937	3.455	2.969	2.953	2.988	2.763	3.062	0.13	> 0.05	> 0.05	> 0.05
Sum of SCFA	78.831	70.503	68.934	71.339	74.172	75.097	69.269	69.652	71.736	68.216	82.565	1.80	> 0.05	> 0.05	> 0.05
Distal large intestine															
pH	6.750	6.668	6.881	6.766	6.767	6.752	6.738	6.808	6.599	6.954	6.748	0.05	> 0.05	> 0.05	> 0.05
Acetic acid	38.649	41.421	37.613	37.444	41.012	36.280	39.241	36.810	43.601	38.417	41.017	1.03	> 0.05	> 0.05	> 0.05
Propionic acid	18.051	19.442	17.646	18.057	18.702	17.481	19.158	17.533	19.725	17.760	18.621	0.79	> 0.05	> 0.05	> 0.05
Isobutyric acid	1.863	1.697	1.654	1.608^{b}	1.867^{a}	1.717	1.621	1.487	1.772	1.821	2.009	0.06	> 0.05	< 0.05	> 0.05
Butyric acid	10.350	11.795	9.886	10.498	10.856	9.508	11.423	10.563	12.167	9.209	11.191	0.53	> 0.05	> 0.05	> 0.05
Isovaleric acid	2.298	1.987	2.007	1.906^{b}	2.289^{a}	2.074	1.939	1.706	2.036	2.308	2.522	0.08	> 0.05	< 0.05	> 0.05
Valeric acid	3.009	3.399	3.297	3.308	3.163	3.010	3.554	3.359	3.245	3.236	3.008	0.19	> 0.05	> 0.05	> 0.05
Sum of SCFA	74.219	79.741	72.104	72.821	77.888	70.070	76.937	71.457	82.546	72.751	78.368	2.36	> 0.05	> 0.05	> 0.05

Abbreviations: I – control group, with no feed additives; II – group supplemented with 2.5% *Hermetia illucens* larvae full-fat meal (2.5HI); III – group supplemented with 5% HI larvae meal (5HI); IV – group supplemented with 2.5% HI larvae meal and astaxanthin (2.5HI+AST); V – group supplemented with 5% HI larvae meal and AST (5HI+AST); VI – group supplemented with AST alone (AST). ^{a,b} Values within a row with different superscripts differ significantly at $p<0.05$.

distal part of the colon, the addition of AST increased the isobutyric and isovaleric acid concentrations ($p<0.05$). No other significant differences were found in this section.

Ammonia and aromatic compound concentrations

The interaction between HI meal and AST did not affect the phenol, p-cresol, indole or ammonia concentrations in any segment of the large intestine. There was no significant effect of the HI meal on the phenol concentrations in the proximal, middle and distal sections of the large intestine of the piglets, but the AST lowered its concentration by 20% in the distal section ($p<0.05$) (Table 3). There were no effects of experimental factors on p-cresol concentrations in the proximal large intestine of piglets (Table 3), however, in the middle part of the large intestine, groups supplemented with 2.5% HI meal presented lower concentrations of p-cresol ($p<0.05$) by 16.8%, respectively. This tendency was also observed in the distal large intestine, however it was not statistically confirmed. There were no significant changes in p-cresol concentration when AST was added in to the feed, in any sections of the large intestine of the piglets. There was no effect of HI meal on indole concentrations in all three sections of the large intestine of the piglets (Table 3), but AST supplementation decreased the indole concentration in the middle colon by 4.2% ($p<0.05$). There were no effects of HI

Table 3. Effect of *Hermetia illucens* larvae full-fat meal and astaxanthin on phenolic and indole compounds (μmol/g), and ammonia concentrations (μmol/g) in the large intestine of piglets.

Item	Factor 1: insect meal (HI)			Factor 2: astaxanthin (AST)		I	II	III	IV	V	VI	SEM	*p*-value		
	0HI	2.5HI	5HI	-	+	0HI	2.5HI	5HI	2.5HI+AST	5HI+AST	0HI+AST		HI	AST	HIxAST
Proximal large intestine															
Phenol	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.004	0.005	0.005	0.005	0.01	> 0.05	> 0.05	> 0.05
p-cresol	0.118	0.110	0.106	0.110	0.113	0.109	0.109	0.112	0.110	0.100	0.128	0.01	> 0.05	> 0.05	> 0.05
Indole	0.070	0.070	0.073	0.071	0.070	0.071	0.069	0.073	0.071	0.072	0.068	0.01	> 0.05	> 0.05	> 0.05
Ammonia	17.658	17.317	16.325	13.769^{b}	20.324^{a}	14.184	14.339	12.644	20.295	19.545	21.131	0.95	> 0.05	0.001	> 0.05
Middle large intestine															
Phenol	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.01	> 0.05	> 0.05	> 0.05
p-cresol	0.131^{a}	0.109^{b}	0.112^{ab}	0.122	0.113	0.136	0.113	0.114	0.104	0.109	0.126	0.01	< 0.05	> 0.05	> 0.05
Indole	0.071	0.068	0.070	0.071^{a}	0.068^{b}	0.073	0.069	0.070	0.067	0.069	0.068	0.01	> 0.05	< 0.05	> 0.05
Ammonia	14.708	14.864	13.129	13.115	15.352	14.404	12.385	12.557	17.342	13.701	15.013	0.30	> 0.05	> 0.05	> 0.05
Distal large intestine															
Phenol	0.004	0.004	0.004	0.005^{a}	0.004^{b}	0.005	0.005	0.005	0.004	0.004	0.004	0.01	> 0.05	< 0.01	> 0.05
p-cresol	0.130	0.119	0.115	0.117	0.126	0.128	0.121	0.101	0.116	0.129	0.133	0.01	> 0.05	> 0.05	> 0.05
Indole	0.068	0.068	0.068	0.068	0.068	0.070	0.067	0.068	0.069	0.068	0.066	0.01	> 0.05	> 0.05	> 0.05
Ammonia	13.810	14.838	14.196	14.858	13.689	14.440	14.164	16.189	15.513	12.451	13.102	0.75	> 0.05	> 0.05	> 0.05

Abbreviations: I – control group, with no feed additives; II – group supplemented with 2.5% *Hermetia illucens* larvae full-fat meal (2.5HI); III – group supplemented with 5% HI larvae meal (5HI); IV – group supplemented with 2.5% HI larvae meal and astaxanthin (2.5HI+AST); V – group supplemented with 5% HI larvae meal and AST (5HI+AST); VI – group supplemented with AST alone (AST). ^{a,b} Values within a row with different superscripts differ significantly at $p< 0.05$.

Table 4. Effect of *Hermetia illucens* larvae ful-fat meal supplementation and astaxanthin on selected microbiota populations (log CFU/ml digest) in the large intestine digesta, as determined by DAPI staining and fluorescence *in situ* hybridization (FISH) in pigs.

Item	Factor 1: insect meal (HI)			Factor 2: astaxanthin (AST)		I	II	III	IV	V	VI	SEM	*p*-value		
	0HI	2.5HI	5HI	-	+	0HI	2.5HI	5HI	2.5HI+AST	5HI+AST	0HI+AST	0.04	HI	AST	HIxAST
DAPI	10.8 ^{a}	10.5 ^{b}	10.7 ^{a}	10.5 ^{b}	10.9 ^{a}	10.7	10.3	10.4	10.8	10.9	10.9	0.14	< 0.05	< 0.001	> 0.05
Bacteroides-*Prevotella* cluster	9.01 ^{a}	8.95 ^{b}	9.56 ^{a}	8.93	9.42	8.58	8.74	9.45	9.16	9.67	9.44	0.16	< 0.001	> 0.05	> 0.05
Clostridium leptum subgroup	8.8	9.36	8.96	8.99	9.05	8.31	9.32	9.34	9.33	8.51	9.3	0.09	> 0.05	> 0.05	> 0.05
Clostridium perfringens	9.36	9.37	9.05	9.35	9.18	9.32	9.5	9.23	9.25	8.87	9.41	0.17	> 0.05	> 0.05	< 0.1
Enterobacteriaceae	8.77	8.57	9.35	9.08	8.72	8.77 ^{b}	9.33 ^{ab}	9.14 ^{b}	7.81 ^{b}	9.57 ^{a}	8.77 ^{b}	0.09	> 0.05	> 0.05	< 0.001
C. coccoides/ *Eubacterium rectale cluster*	9.52	9.19	9.63	9.28	9.6	9.5	8.86	9.49	9.51	9.77	9.53	0.03	> 0.09	> 0.05	> 0.05
Lactobacillus/ *Enterococcus* spp.	9.38	9.3	9.34	9.34	9.34	9.25 ^{ab}	9.51 ^{a}	9.25 ^{ab}	9.10 ^{b}	9.42 ^{ab}	9.50 ^{a}	0.04	> 0.05	> 0.05	< 0.001

Abbreviations: I – control group, with no feed additives; II – group supplemented with 2.5% *Hermetia illucens* larvae full-fat meal (2.5HI); III – group supplemented with 5% HI larvae meal (5HI); IV – group supplemented with 2.5% HI larvae meal and astaxanthin (2.5HI+AST); V – group supplemented with 5% HI larvae meal and AST (5HI+AST); VI – group supplemented with AST alone (AST). ^{a,b} Values within a row with different superscripts differ significantly at $p< 0.05$.

meal addition on the ammonia concentration in the three sections of the large intestine of piglets (Table 3). Feeding diets supplemented with AST significantly increased the ammonia concentration in the proximal part of the large intestine of piglets (p=0.001), but in other segments the ammonia concentration was unaffected by the AST treatment.

Microbiota analysis

According to the FISH analysis (Table 4), a significant overall increase in bacterial proliferation in the digesta of the proximal section of the large intestine was observed in the pigs treated with AST (p<0.001). The total bacterial count decreased in the pigs treated with 2.5HI meal (p<0.05), especially the number of *Bacteroides–Prevotella* cluster bacteria (p<0.001). No significant changes were detected in the *Clostridium leptum* subgroup or the *C. coccoides/Eubacterium rectale* cluster. The effect of interaction between both studied dietary factors – HI meal and AST – was observed in the case of the *Lactobacillus/Enterococcus spp*. population, where the simultaneous addition of AST and 2.5% HI meal, reduced their number in con-

trast to the groups where the factors were added individually ($p<0.001$). The interaction also affected the *Enterobacteriaceae* population ($p<0.001$). The highest proliferation was observed in the 5HI+AST group, whereas the control, 5HI, 2.5HI+AST, and AST diets did not exhibit increased growth of this bacterial family. A tendency ($p<0.1$) for interaction was noted regarding the *C. perfringens* population, where the greatest suppressive effect was observed in the 5HI+AST group.

Discussion

The large intestine plays a significant role in reabsorbing water and electrolytes secreted into the intestinal lumen during digestion, excreting toxic substances and metabolic products, and providing an environment for the microbiota to live (Bass and Wershil 2015). There are mechanisms by which imbalances in the homeostasis of the intestinal microbiome contribute to inflammation and metabolic disorders. Colonic epithelial cells maintain a symbiotic relationship with the microbiota, facilitating absorption and contributing to both innate and acquired mucosal immunity. Increased mucosal thickness can indicate inflammation, regenerative proliferation, or neoplastic conditions (Cerilli and Greenson 2012). In this experiment, groups without HI additive exhibited thicker mucosa, though this was not linked to any observable pathological changes. AST supplementation used in this experiment reduced the number of enterocytes which may be due to its anti-inflammatory and antioxidant effects. AST also can control and influence signaling pathways that control cell proliferation and apoptosis which could also affect enterocytes (Kim and Kim 2019). Increased release of lipopolysaccharides from gram-negative intestinal bacteria stimulates inflammation and reduced SCFAs production results in energy deficiency for beneficial intestinal microbes and host intestinal epithelial cells (Di Lorenzo et al. 2019, Wu et al. 2020). SCFAs are formed by microbial fermentation and provide a source of energy for colon epithelial cells. Along the large intestine, the concentrations of major SCFA decrease, while levels of branched-chain fatty acids, ammonia, amines, and phenolic and indole compounds increase due to reduced carbohydrate fermentation and increased proteolysis (Taciak et al. 2017). As a result of the active fermentation of feed substrates, an increase in the concentration of SCFA, which is reflected in a reduced pH in the proximal part of the colon (Macfarlane et al. 1992) is observed. In the present study, it was noted that the addition of HI meal reduced the concentration of SCFAs in the colon contents, which contributed to a slight increase in pH. Despite this, the pH was still in the normal acidic range, and therefore, the pigs were able to produce acidic conditions that facilitated microbial growth and colonization. The effect of AST on SCFA is interesting. For some acids, there is a noticeable increase in concentrations in AST-treated groups. AST is a type of the carotenoid that largely bypass absorption in the small intestine, and makes its way to the large intestine, where it appears to be partially broken down into metabolites that are not fully known (Eroglu et al. 2023). Butyrate has an important effect on cell differentiation and metabolism. It is a source of energy for colonocytes (Barcenilla et al. 2000) and protects against cancer and ulcerative colitis (Walker et al. 2005). SCFAs can also inhibit the proliferation of pathogenic bacteria (Tang et al. 2022a). In this study, in the proximal part of the large intestine, the addition of HI meal reduced the concentration of butyric acid, which appears to be an adverse effect of the diet. The large intestinal microbiota is a major factor involved in metabolism and energy regulation. It has been proven that changes in the composition of intestinal bacteria are linked to pig health and productivity (Maltecca et al. 2020). The intestinal microbiota develops rapidly in the early life of piglets, whereas changes in bacterial taxonomy are particularly evident during the periparturient period. After weaning and cessation of milk consumption, there is a change in the bacterial population. This is particularly true for bacteria of the genus *Lactobacillus*, which consumes mono- and disaccharides of plant origin and simple milk sugars. In contrast, the population of saccharolytic microorganisms, such as *Prevotellaceae*, is increasing, and their significant prevalence contributes to a concomitant decline in Bacteroidaceae (Martínez et al. 2013, Pajarillo et al. 2014). The environmental factors that create a habitat for bacteria are important considerations. These include the supply of substrates, growth factors, and micronutrients, the concentration of antimicrobial compounds, ionic conditions, and the intestinal pH (Walker et al. 2005, Duncan et al. 2009). In the large intestine pH is much greater (above 6.5) than in the initial part of the gastrointestinal tract. Nonetheless, even under such conditions of the large intestine, changes in pH can have a significant impact on microbiota and metabolic activity (Duncan et al. 2009, Merchant et al. 2011). The pH value of the colon lumen is partly determined by host secretions, and partly by the fermentation products of intestinal microorganisms (Duncan et al. 2009). Liu et al. (2023) found that replacing soybean meal with HI meal in pig diets increased SCFA-producing bacteria (*Ruminococcaceae, Faecalibacterium* and *Butyricoccus*) compared to the control group. Piglets fed HI meal had a 25% reduction in potentially pathogenic bacteria, but those receiving

100% HI meal exhibited significantly higher numbers of harmful bacteria. The high level of HI meal corresponds simultaneously to a high level of chitin and this, in turn, can be linked to an increase in pathogenic bacteria. Complete replacement with insect meal had adverse effects on piglet performance, and the possibly because of the extremely high amount of chitin in the diet, as well as the limited amount of chitin-degrading enzyme. The site of interaction between the host and the microorganisms is the mucus produced by goblet cells, which cover the surface of the intestinal epithelium. In the colon, the mucus layer serves as a barrier that impedes direct contact between the epithelium and the microbial population (Biasato et al. 2020). Commensal intestinal microbes depend on diet as a source of nutrients, energy, and mucus as a binding site. The intestinal microbiota and microbial products can modulate mucin synthesis, and secretion, through both direct activation of signaling cascades and indirect production of bioactive factors by the intestinal mucosa (Desantis et al. 2019). Thus, it can be assumed that the increased number of goblet cells will produce more mucus. Such an observation was made by in the present study when adding HI meal at a level of 2.5% increased the number of goblet cells. Some studies report that the effect of HI meal on the intestinal microbiome depends on the amount given in the feed. In a study by Biasato et al. (2019) showed that the inclusion of HI meal in the diet positively affected the cecal microbiota and small intestinal mucin dynamics in piglets, promoting the selection of potentially beneficial bacteria and maintaining a mature mucin secretory architecture without inducing intestinal inflammation. In the present study, the neutral effects of administration of 5% HI meal into piglet diets were observed concerning selected microbiota populations in the large intestine. However, the simultaneous addition of 5% HI meal together with AST resulted in a negative increase in the *Enterobacteriaceae* community. This is difficult to explain, particularly when the separate inclusion of both showed an impact similar to that of the control group. On the other hand, the combination of 5% HI meal and AST tended to positively suppress the proliferation of *C. perfringens*, a common etiological factor of necrotic enteritis (Posthaus et al. 2020). In contrast to the present study, the inclusion of 2% HI larvae meal increased the abundance of *Lactobacillus*, *Ruminococcus*, *Clostridium* cluster IV, and *Prevotella* in the cecum of weanling piglets (Yu et al. 2019). The administration of 4% insect meal also promoted *Lactobacillus*, *Clostridium cluster* XIVa, and *Roseburia* in the colonic digesta (Yu et al. 2019). Finally, Tang et al. (2022b) identified *Ruminoccocus*, *Faecalibacterium prausnitzii*, *Alloprevotella*, and *Lactobacillus reuteri* as biomarkers of HI meal supplementation in pig diet. In the present experiment, the most significant effects were observed for 2.5% HI meal in relation to total bacterial counts and the *Bacteroides-Prevotella* cluster, and for 2.5% HI meal together with AST in terms of *Enterobacteriaceae* and *Lactobacillus*/*Enterococcus* spp. The most positive effect was observed in limiting of lactic acid bacteria population, and Bacteroides, likely due to their ability to deconjugate bile salts (Hou et al. 2020), which can have a detrimental effect on energy availability for the host. Several studies have reported microbiological changes following carotenoid interventions. For example, in an experiment conducted by Nagayama et al. (2014), a diet supplemented with 0.04% yeast-derived AST for eight weeks significantly reduced *Proteobacteria* and Bacteroides in β-carotene oxidase 2 knockout mice. In contrast, this supplementation markedly increased *Actinobacteria* and *Bifidobacterium* in wild-type mice. Wang et al. (2021) found that AST alleviates intestine microflora dysbiosis induced by a high-fat diet by optimizing the ratio of Firmicutes to Bacteroides. Among the bacterial populations analyzed, a small effect of AST on increasing bacterial counts was observed for *Lactobacillus/ Enterococcus* spp. Bacterial metabolites resulting from protein fermentation, such as ammonia, indole and phenolic compounds, are believed to have negative effects on the intestinal mucosa by interfering with its inflammatory responses (Davila et al. 2013, Rist et al. 2013). In the present study, reduced concentrations of p-cresols in the middle part of the large intestine were noted under the influence of the HI meal administered at both levels. Lower concentrations of indole and phenol were also observed in the mid and distal parts of the large intestine, respectively, under the influence of AST supplementation. It can be assumed that the administration of these additives can be beneficial in terms of reducing the concentration of harmful aromatic compounds, and in this way can contribute to reducing odor emissions from livestock production. Furthermore, the decrease in concentrations of phenolic and indole compounds in the present study indicates that both tested supplements may have a beneficial effect on intestinal health, looking at the carcinogenic effects of these listed compounds (Nowak and Libudzisz 2006). Interestingly, the concentration of ammonia in the proximal part of the large intestine increased after adding AST. In weaned piglets, ammonia is increased by high dietary protein amount in the distal part of the colon (Pieper et al. 2014). According to Blachier et al. (2022), higher ammonia levels in the colon caused by a high-protein diet may contribute to the development of diarrhea by disrupting the uptake of butyrate by colonic absorptive cells. This interference hinders butyrate's role in sodium and

water absorption by colonic epithelium. No diarrhea problems were noted in the pigs in the present study, but this should be considered in the future.

Importantly, in addition to health aspects, increasing attention is also being paid to the economic viability of using *Hermetia illucens* meal in pig feed. This component has high nutritional value, but its production and procurement costs are currently higher than those of traditional protein sources such as soybean meal (Shah and Çetingül 2022). However, the growing insect farming sector and increasing availability of raw materials are gradually reducing costs, making insect meal increasingly competitive. In addition, the possibility of using organic waste as a substrate for rearing larvae reduces environmental costs and can improve profitability in sustainable production systems. Therefore, in the future, with increased production scale and technology optimisation, HI meal may become more profitable.

Conclusions

The dietary intervention involving *Hermetia illucens* larvae full-fat meal and astaxanthin supplementation influenced the large intestinal microbiome composition and histomorphometric indices in piglets. The 2.5% of HI full-fat meal in the diet increased the number of goblet cells, suggesting increased mucus production, whereas AST supplementation impacted enterocyte counts, likely through its anti-inflammatory and regulatory effects on cellular pathways. Both dietary factors altered SCFA profiles, with HI full-fat meal reducing butyric acid levels, and AST increasing acetic acid concentrations, indicating their role in modulating intestinal fermentation processes. The combination of HI full-fat meal and AST demonstrated potential benefits in reducing harmful bacterial metabolites, as HI full-fat meal lowered the concentration of p-cresol, whereas AST lowered the phenol and indol concentrations, depending on the section of the large intestine. The study concluded that both tested feed additives, HI full-fat meal and AST, supported the intestinal health of weaned piglets, and potentially mitigated odor emissions from livestock production.

Author Declarations

Ethics approval

All procedures included in this study relating to the use of live animals agreed with the First Local Ethics Committee for Experiments with Animals in Cracow, Poland (Resolution No. 420/2020, date 22.07.2020). Throughout the experimental period, a veterinarian regularly monitored the health status of postweaning pigs.

Use of generative artificial intelligence

The authors confirm that they did not use any generative artificial intelligence methods or AI-assisted methods in the preparation of this manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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