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RESEARCH ARTICLE

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Beyond soybean meal: investigating the effects of dietary protein alternatives on gut health, liver function and microbiota in traditional slow-growing chicken breeds

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ABSTRACT

The use of soybean meal in animal feed is crucial for nutrition but has a significant environmental impact, especially related to deforestation and biodiversity loss. With the increasing demand for sustainable agricultural practices, it becomes essential to explore alternatives to soybean meal. This study evaluated the effect of a soy-free diet, replaced with fava beans and pea protein, on an Italian slow-growing chicken breed: the Bianca di Saluzzo. The results indicate that the experimental diet did not compromise the growth, final weight or intestinal health of the animals, demonstrating a good tolerance and adaptability of the breed to these alternative ingredients. Furthermore, the analysis of the intestinal microbiota showed a positive impact, with an increase in organic acids such as succinic and citric, which could improve intestinal health and metabolic efficiency. Some changes in liver enzyme levels were observed, such as the increase in glutamate-oxaloacetate transaminase, an enzyme involved in amino acid metabolism. This increase could indicate a higher efficiency in protein metabolism, suggesting that the diet based on alternative ingredients could support an improvement in liver metabolism. Although this aspect deserves further investigation, it could represent a positive effect of the diet on liver function and overall health of the animals. Further studies are needed to fully understand the long-term implications of these dietary modifications, particularly in relation to the gut microbiota and metabolism.

HIGHLIGHTS

- Soy-free diet supports sustainable poultry production.
- No impact on gut health and growth.
- Slow-growing breeds adapt well to alternative proteins.

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Introduction

The use of soybean meal in animal feed is a significant component of modern agricultural practices, with both nutritional and environmental implications. With growing consumer interest in sustainable and environmentally friendly agricultural practices, it is increasingly important to understand these implications. Soybean meal is widely used as a primary source of protein in animal feed due to its high protein content and favourable amino acid profile, which are beneficial for animal growth and productivity (Lambo et al. 2024). However, the environmental impact of soybean cultivation and processing is an important consideration. To address these concerns, there is ongoing work to promote more sustainable soy production practices and explore alternative protein sources for

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animal feed, aiming to reduce the reliance on soybean meal. These alternatives include insect meals, algae and other plant-based proteins, which can provide similar nutritional benefits with a lower environmental impact (Bellezza Oddon et al. 2021; Zampiga et al. 2023). The adoption of such alternatives is driven by consumer demand for more sustainable products and the agricultural sector's commitment to reduce its carbon footprint (Wileman et al. 2024). Nonetheless, challenges persist in balancing livestock nutritional requirements with environmental sustainability, as well as in conducting comprehensive analyses that consider all aspects, including the effects on animal health, such as gut health.

The importance of a holistic approach to improve and maintain intestinal health in chickens is underlined by the multifaceted nature of intestinal health, which is influenced by a variety of factors including diet, microbiome and environmental conditions. These different elements and their interactions are to be considered as a tool to optimise overall health and productivity in poultry. The intestinal microbiome plays a crucial role in maintaining intestinal health, influencing nutrient absorption, immune function and disease resistance. Maintaining a balanced intestinal microbiome is of great importance in preventing pathogenic infections and promoting efficient nutrient utilisation in chickens (Khan et al. 2020). Moreover, the complexity of intestinal health requires a comprehensive strategy that combines these various elements. It is important to use a systems approach, where the interactions between diet, microbiome and host immune system are considered collectively to achieve optimal intestinal health outcomes (Williams 2005). The intricate nature of the gut ecosystem and the necessity for tailored dietary strategies to optimise health outcomes are well understood and widely recognised (Velten et al. 2018).

In this context, slow-growing local poultry breeds represent an invaluable genetic heritage, closely linked to the history, culture and traditions of specific geographical areas. These breeds are often the result of long natural selection and adaptation to the local environment and possess unique characteristics that make them particularly valuable in a sustainable agriculture context. Compared to fast-growing commercial breeds, slow-growing local breeds are more adapted to free-range system and low-nutrient diets (Fiorilla et al. 2023). These breeds are also closely linked to the quality of poultry products, offering meat and eggs with a distinctive flavour that respond to the growing demand for high-quality zero-km food products (Franzoni et al. 2021). Valuing these breeds means not only preserving poultry biodiversity, but also supporting production systems that respect the ecological balance and promote long-term sustainability (Hoffmann 2011; Corlett 2020). These aspects are becoming increasingly crucial, indeed, breeding chicken strains that can easily adapt to feeding a variety of local ingredients could help reduce the negative impact of monocultures and competition between food and feed (Kreuzer et al. 2020). Italy is one of the cradle countries valorising native chickens, being home to 53 recognised local chicken breeds, numerous conservation programs and a National Registry that currently includes 22 local breeds (Castillo et al. 2021). Of these breeds, Bianca di Saluzzo is receiving increasing attention from the scientific community and included in the list of Slow Food Presidia (Soglia et al. 2020; Bongiorno et al. 2022). Bianca di Saluzzo is a dual-purpose slow-growing breeds originating from the Piedmont region (Northwest Italy) that thrive in organic and free-range rearing systems (Franzoni et al. 2021).

The study aimed to evaluate the effects of replacing soybean meal with alternative protein ingredients like fava beans and pea protein in the diets of slow-growing chickens. It focused on the indigenous Bianca di Saluzzo breed, used as a model to assess growth, intestinal health, liver metabolism and microbiota in slow-growing chickens. The goal was to determine whether these alternative ingredients are suitable for free-range poultry.

Materials and methods

Birds, husbandry and diets

The study was performed at the University of Turin's poultry facility (north-west Italy) and received approval from the Bioethical Committee of the University of Turin (No. 814715). The experimental design of the present study is reported in detail by Fiorilla et al. (2024), as the current research is part of the same project and was performed using the same birds. In order to provide a brief summary, a total of 96 males Bianca di Saluzzo birds of 39 days of age were individually marked with wing marks and housed in an experimental poultry facility, with six replicates of eight birds per each of the two treatments. Two dietary treatments were used in the trial: the control group (C) fed a conventional diet with soybean meal as its protein source (maize meal 62.0%, soybean meal 32.0%, soybean oil 2.0%; apparent metabolisable energy 11.8 MJ/kg, crude protein, 18.1%, ether extract 3.6% and crude fibre

3.3%), and the experimental group (EXP) fed an alternative diet with the complete replacement of soybean meal with alternative protein ingredients (maize meal 46.1%, field bean 11.0%, pea protein 10.8%, barley 4.7%, sunflower meal 9.5%, maize gluten 11.6%, soybean oil 1.6%; apparent metabolisable energy 11.8 MJ/kg, crude protein 18.0%, ether extract 3.7% and crude fibre 4.8%), the complete list of ingredients is reported in Table 1. Mortality and health status of birds were observed daily throughout the experiment. Commencing from 39 days of age, individual live weight of the animals and feed intake were recorded every 21 days, and the feed conversion ratio (FCR) was calculated. The trial spanned 135 days, (174 days old). Two experimental slaughters were executed at 147 days and at 174 days of age. The day prior to each slaughter, all chickens were individually weighed, and then two birds per pen (24 birds per slaughter) were chosen based on the mean live weight. Following a 12-hour fasting period, the selected birds underwent electric stunning and exsanguination, in compliance with EU legislation (Council Regulation (EC) No. 1099/2009 of 24 September 2009). Plucked and eviscerated carcases were weighed, and the ready-to-cook carcase (RTCC) weight was recorded. The spleen and liver were weighed after evisceration.

Table 1. Ingredients and analysed chemical composition of the commercial and soybean meal-free experimental diet used in the trial.

Diet composition, g/kg	C	EXP
Maize meal	617	461
Soybean meal 44	320	–
Field bean	–	110
Pea protein	–	108
Barley	–	47
Sunflower meal	–	95
Maize gluten	–	116
Soybean oil	20	16
Dicalcium phosphate	13.5	13.5
Calcium carbonate	19	20
Sodium chloride	1.5	1.5
Sodium bicarbonate	1.4	1.4
DL-Methionine	1.7	0.7
L-Lysine	–	4
Vitamin and mineral premix[a]	5.9	5.9
Total	1000	1000
AME, MJ/kg	11.8	11.9
Analysed values		
Proximate composition, %		
Dry matter	90.81	90.27
Crude protein	18.13	18.10
Ether extract	3.59	3.63
Crude fibre	3.28	4.80
Aminoacid composition, g/100 g		
Alanin	6.53	7.00
Arginin	6.53	5.92
Aspartic acid	9.80	8.07
Glutamic acid	17.42	17.76
Glycine	8.17	8.07
Histidin	2.45	2.64
Isoleucine	4.19	3.82
Leucine	8.17	9.69
Lysine	6.53	7.00
Methionine	2.12	2.05
Phenylalanine	5.12	5.17
Proline	6.53	7.00
Serine	4.79	4.52
Threonine	3.76	3.44
Tyrosine	3.10	3.28
Valine	4.79	4.57

[a]Vitamin A, Vitamin D3, Betaine anhydrous 600.48 mg, Biotin 0.04 mg, Choline chloride 333.07 mg, Folic acid 0.81 mg, Niacinamide 25.01 mg, Calcium pantothenate 7.28 mg, Vitamin B1 0.75 mg, Vitamin B12 0.02 mg, Vitamin E 18.50 mg, Vitamin K3 2.50 mg, Copper 10.00 mg, Iodine 1.50 mg, Iron 44.01 mg, Manganese 62.01 mg, Selenium 0.25 mg, Zinc 50.01 mg.

Intestinal and organ health

At the age of 147 and 174 days upon each slaughtering, samples of the gut segments, measuring about 5 cm in length, were collected from the duodenum, jejunum, and ileum of 12 animals per group. These segments underwent a rinsing process with a 0.9% saline solution to eliminate their contents. The specific parts of the intestines that were gathered included the duodenal loop, the section preceding Meckel's diverticulum (*jejunum*), and the section prior to the ileocecum junction (*ileum*). Furthermore, specimens from the spleen and the left lobe of the liver were collected too (0.5–1.5 g/organ). All specimens were preserved in a 10% buffered formalin solution, embedded in paraffin wax blocks, sliced into 5-μm sections, put onto glass slides, and stained with haematoxylin and eosin for morphometric and histological assessments. The slide of jejunum was examined by light microscopy and some pictures/each slide were captured with a Nikon DS-Fi1 digital camera (Nikon Corporation, Minato, Japan) coupled to a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany) using a ×2.5 objective lens. Morphometric analysis was performed utilising the ImageJ software (Bethesda, MD) with Fiji distribution (Schindelin et al. 2012).

The evaluated morphometric indices were the villus height (Vh, from the tip of the villus to the crypt), the crypt depth (Cd, from the base of the villus to the submucosa), the villus width (Vw) and the villus height to crypt depth (Vh/Cd) ratio (Laudadio et al. 2012). The villus surface area (VSA) was calculated according to the following formula: $(2\pi)(Vw/2)(Vh)$. These morphometric analyses were performed on 10 well-oriented and intact villi and 10 crypts chosen from the duodenum and jejunum (Qaisrani et al. 2014). The mucosal thickness (MT) and muscular thickness (MuT) thickness were also measured on three standardised points of the gut mucosal and muscular layers per each captured field.

Histopathological variations were scrutinised such as white pulp hyperplasia and depletion in the spleen, hepatocyte degeneration and lymphoid tissue activation in the liver (Biasato et al. 2016). In relation to the histopathological discoveries in the gut, the mucosa and submucosa of every gut segment were examined for inflammatory infiltrates and activation of gut-associated lymphoid tissue. A semiquantitative scoring system was employed to evaluate all identified histopathological changes, categorising them as absent (score = 0), mild (score = 1), moderate (score = 2) or severe (score = 3). The cumulative score for each gut segment was determined by summing up the scores for the mucosa and submucosa. All slides were assessed independently by three different examiners, with any discrepancies being resolved through collective examination using a multi-head microscope until a unanimous agreement was reached.

Liver metabolism

At the second slaughtering (174 days of age), samples from the liver (n = 12) were immediately frozen at −80 °C after collection. Tissues were homogenised in 0.1 M TRIS/HCl buffer solution with 0.1 mM EDTA and Triton X-100 (0.1%, v/v) and centrifuged at 20,000 × g for 30 min at 4 °C, the supernatant was stored at −80 °C. The liver metabolic enzymes alanine aminotransferase (ALT; EC 2.6.1.2), aspartate aminotransferase (AST; EC 2.6.1.1), glutamate dehydrogenase (GDH; EC 1.4.1.2), pyruvate kinase (PK; EC 2.7.1.40), glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) and fructose 1,6-bisphosphatase (FBPase; EC 3.1.3.11) were measured according to Bisswanger (2011). Enzyme activity was measured by changes in NADH/NADPH absorbance at 340 nm in a PowerWavex spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT). Protein content of homogenates was measured with a Pierce™ BCA Protein Assay Kit (Thermo Scientific™, Rockford, IL). Enzyme activity was described as the amount of enzyme required to oxidise/reduce 1 μmol of NADH/NADP per minute and protein at 40 °C.

Microbiota

Caecum content samples were collected from chickens using sterile equipment to ensure accuracy and prevent contamination. The samples were then immediately frozen at −80 °C for storage until analysis. Total DNA was extracted from each sample using the DNeasy 96 PowerSoil Pro QIAcube HT Kit (Qiagen, Milan, Italy) following the manufacturer's instructions. DNA was quantified by Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE) and standardised at 50 ng/μL.

The analysis of the microbiota of 48 Bianca di Saluzzo chickens was conducted using a metataxonomic approach to identify potential variations in microbiota composition. Utilising the 16S rRNA gene (specifically targeting the V3–V4 regions), PCR products were amplified, purified, tagged and pooled in accordance with the guidelines outlined by Illumina (San Diego, CA) (Klindworth et al. 2013). Subsequently, the raw files (fastq) containing 250-bp paired-end reads produced by the Illumina MiSeq platform (San Diego, CA) with V2 chemistry were processed using QIIME 2 software version 2022.2.0 (Bolyen et al. 2019). Following the methodology described by Callahan et al. (2016), Cutadapt was employed to eliminate primer sequences, and the DADA2 algorithms were applied to denoise the acquired reads through the q2-dada2 plugin within the QIIME 2 environment. Taxonomy classification was executed using the QIIME2 feature-classifier against the Greengenes2 database. To enhance the reliability of sequence reads, amplicon sequence variants (ASV) with a read count of less than five in a minimum of two samples were excluded.

Intestinal volatile fatty acids

The same procedure used to collect caecum content for microbiota analysis was also applied to obtain aliquots for volatile fatty acid (VFA) analysis. Quantification of VFAs was adapted by the method described in Raspa et al. (2022). Caecal samples (about 300 mg) were suspended in about 750 μL 0.1 N H_2SO_4 solution and vortexed. The mixture was then centrifuged at 15,000 × g for 10 min at 4 °C. The supernatant was collected in 2 mL clear glass vials (Sigma-Aldrich, St. Louis, MO). Analyses were performed on a High-Performance Liquid Chromatography by using a Dionex Ultimate 3000 UV/VIS Detector (Thermo Fisher, Wilmington, DE) with a 300 × 7.8 mm Aminex HPX-87H (Bio-Rad, Hercules, CA) and a guard-column. Injected samples (30 μL) were isocratically separated in 0.005 N H_2SO_4 at a flow rate of 0.6 mL/min at 41 °C. VFAs were detected by UV light at 210 nm and identified using an external standard curve (4.95–148.5 mg/100 mL succinic acid; 9–270 mg/100 mL lactic acid; 10.5–314.4 mg/100 mL acetic acid; 9.85–285.5 mg/100 mL propionic acid; 9.4–282.1 mg/100 mL butyric acid; 9.5–285.1 mg/100 mL isobutyric acid; 9.1–273.4 mg/100 mL iso-valeric acid; 9.1–273.2 mg/100 mL

valeric acid; 4.95–148.5 mg/100 mL citric acid) created using standards dissolved in 0.1 N H_2SO_4.

Data analysis

Statistical analyses were performed using R software, Version 4.4.0 (R Foundation for Statistical Computing, Vienna, Austria). Each pen was considered as the experimental unit for the growth performance ($n = 6$ pens per treatment), while the individual bird was considered the experimental unit for the analysis of intestinal and organ health, liver metabolism, microbiota and intestinal VFAs ($n = 12$). The normality of data distribution was assessed using the Shapiro–Wilk test, and the homogeneity of variance was evaluated using Levene's test. Growth performance, intestinal and organ health, liver metabolism and intestinal VFAs were analysed by fitting a general linear model (GLM) with Tukey's post hoc test. The results are expressed as the least square mean and standard error of the mean (SEM). p Values ($p < .05$) were considered statistically significant, while $p < .10$ was considered as tendency. The microbiota α-diversity index (Simpson, Shannon and Chao1) was obtained using vegan package in R (R Foundation for Statistical Computing, Vienna, Austria). Spearman's rank correlation coefficient between microbial ASVs and VFAs was obtained through the function psych and plotted using the corrplot package in R (FDR <0.05). ASV data were first analysed using a *t*-test to assess statistical differences between groups. Additionally, microbiota data were analysed using an unsupervised technique for dimensionality reduction: the *t*-stochastic neighbor embedding (*t*-SNE) technique. *t*-SNE is an unsupervised, non-linear machine learning technique primarily used for data exploration and visualising high-dimensional data (van der Maaten and Hinton 2008). The *t*-SNE technique was performed using Python Software.

Table 2. Slaughter weight (SW), feed conversion ratio (FCR), ready to cook carcase (RTCC) and organs of an indigenous slow-growing chicken breed fed a control or a soybean meal-free experimental diet over the periods 39–147 and 39–174 days of age (means, $n = 6$).

	Diet (*D*)		Age (*A*)			*p* Value		
	C	EXP	147 d	174 d	SEM	*D*	*A*	*D* × *A*
SW, g	2275	2281	2152	2394	45.19	.645	.002	.741
FCR, g/g	4.29	4.07	3.68	4.68	0.092	.068	.012	.959
RTCC, SW%	65.7	65.1	65.4	65.4	0.589	.352	.948	.394
Spleen, SW%	0.17	0.19	0.19	0.17	0.008	.332	.302	.292
Liver, SW%	1.58	1.55	1.51	1.62	0.089	.811	.323	.855

C: control; EXP: soybean meal-free experimental diet; *D* × *A*: interaction diet/age; SEM: standard error of mean.

Results

Growth and slaughtering performance

Table 2 presents the values for live weight and FCR values of the birds. There were no significant differences between the two experimental treatments. As expected, birds reached a higher final live weight at the older slaughter age of 174 days compared to 147 days. Additionally, the FCR was significantly higher at 174 days, showing an increase of +27.2% compared to 147 days ($p < .05$).

Table 2 also includes data on slaughter performance with no significant differences observed in the weights of the RTCC, liver or spleen with respect to diet, age or their interaction.

Table 3. The histomorphometry evaluation (mm) of the jejunum and histopathology evaluation (score 0–3[a]) of the gut, liver and spleen of an indigenous slow-growing chicken breed fed a control or a soybean meal-free experimental diet over the periods 39–147 and 39–174 days of age (means, $n = 12$).

	Diet (*D*)		Age (*A*)			*p* Value		
	C	EXP	147 d	174 d	SEM	*D*	*A*	*D* × *A*
Villus heights	0.94	0.81	0.82	0.93	0.027	.075	.052	.831
Villus width	0.098	0.094	0.100	0.093	0.0032	.622	.279	.438
Crypts depth	0.049	0.053	0.054	0.048	0.0012	.070	.230	.391
Villus heights/crypts depth	16.8	15.5	16.7	18.8	0.76	.103	.083	.505
Mucosa width	1.44	1.32	1.34	1.42	0.0271	.062	.143	.105
Muscularis layers width	0.22	0.22	0.21	0.24	0.0083	.816	.088	.102
Villus area	0.29	0.24	0.26	0.27	0.0118	.062	.672	.529
Duodenum (inflammation)	1.4	1.3	1.3	1.2	0.12	.735	.820	.342
Jejunum (inflammation)	1.0	1.0	1.1	0.9	0.14	.939	.438	.180
Ileal (inflammation)	1.6	1.3	1.7	1.2	0.12	.421	.189	.155
Liver (inflammation)	0.5	0.6	0.5	0.6	0.066	.431	.875	.270
Liver (degeneration)	0.2	0.1	0.2	0.2	0.040	.801	.801	.362
Spleen (hyperplasia)	0.3	0.1	0.2	0.1	0.058	.122	.547	.406
Spleen (depletion)	0.08	0.09	0.06	0.04	0.023	.147	.709	.590

C: control; EXP: soybean meal-free experimental diet; *D* × *A*: interaction diet/age; SEM: standard error of mean.
[a]Score = 0; absent, score = 1; mild, score = 2; moderate, score = 3, severe.

Table 4. The hepatic metabolism enzymes (mU/mg protein) at 174 days of age of an indigenous slow-growing chicken breed fed a control or a soybean meal-free experimental diet over the period 39–174 days of age (means, $n = 12$).

	Diet (*D*)			
	C	EXP	SEM	*p* Value
FBPase	25.3	20.3	1.45	.085
PK	32.4	31.7	1.65	.823
G6PDH	2.81	3.57	0.422	.369
ALT	139	171	8.71	.065
AST	472	601	16.9	<.001
GDH	1555	1642	85.2	.611

C: control; EXP: soybean meal-free experimental diet; SEM: standard error of mean; FBPase: Fructose-1, 6-bisphosphatase; PK: Pyruvate Kinase; G6PDH: Glucose-6-phosphate dehydrogenase; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; GDH: Glutamate Dehydrogenase.

Intestinal and organ health and metabolism

Table 3 shows the histopathological alterations recorded in the gut and the main organs. The EXP treatment did not affect the severity of the histopathological scores in any of the organs. The liver showed mild signs of inflammation and degeneration while the spleen presented mild signs of hyperplasia and depletion even though all these results are in the range of physiological status in birds this age and no differences were highlighted between treatments. Moreover, regardless the diet, we observed mild to moderate, multifocal to diffuse, mucosal lymphoplasmacytic infiltrates. The results of the histomorphometry of the jejunum are reported in Table 3. Overall, there were no significant differences in Vh, Vw, Cd, mucosa width or villus area between the control and experimental diet or between the slaughtering at 147 and 174 days of age. Both diet and age showed a tendency in Vh, mucosa width and the ratio of Vh to Cd ($p < .10$). The muscularis layer width showed trends for age. Overall, diet and age did not significantly affect the measured intestinal morphometric parameters.

The effects of the diet on the liver metabolism are reported in Table 4. There were no significant differences between the control and experimental diets for FBPase, PK, G6PDH, ALT and GDH. FBPase and ALT showed trends with *p* values of .085 and .065, respectively. The AST level was significantly higher in the experimental diet compared to the control diet ($p < .001$).

Microbiota

Figure 1 shows the relative concentration of ASVs in caecum in the two dietary treatments C and EXP. The data highlight the variations in the composition of the microbiota between the two treatment groups. Three indicators (Simpson, Shannon and Chao1) were used to assess the microbiota α-diversity (Figure 2). The analysis revealed no significant differences between the experimental treatments. Figure 3(a) illustrates the result of the *t*-SNE analysis of all bacteria. This representation demonstrates that there are no evident differences between the C and EXP treatments, as the results do not form any distinct clusters. Figure 3(b), on the other hand, represents the result of the *t*-SNE analysis considering only the bacterial concentrations that differ between the C and EXP treatments. In this case, a clear clustering of the results based on the two dietary treatments is observed, highlighting significant differences in the composition of the microbiota between the two groups. Figure 4 reports the significantly different bacterial concentrations between the two dietary treatments. *Bacteroides*, *Desulfovibrio*, *Odoribacter* and Prevotellaceae are present in higher amounts in the EXP treatment compared to the C treatment. Conversely, Christensenellaceae, *Enterococcus*, *Lactobacillus* and *Subdoligranulum* are found in higher concentrations in the C treatment compared to EXP. All the related relevant data are reported in Table 1S in the Supplementary Material.

Intestinal volatile fatty acids

Table 5 outlines the results regarding the intestinal VFAs with citric, succinic and valeric acids levels significantly higher in the EXP diet compared to the C diet, while formic and isobutyric levels were significantly lower in the EXP diet than C. Finally, there was a trend for propionic acid, which was lower in the experimental diet. No significant differences were found between diets for acetic, butyric, lactic or the total levels of these parameters. Furthermore, there were no significant results in age effect nor the interaction between diet and age for any of the measured parameters.

Finally, Figure 5 shows a correlation plot representing Spearman's correlation between microbial ASVs and VFAs. Of particular interest is the correlation between the production of *Enterococcus* and isobutyric acid, both of which are higher in the C treatment compared to EXP (Table 5). Conversely, Prevotellaceae, found in greater quantities in the EXP treatment, are correlated with increased production of lactic acid (Table 5). Moreover, the presence of *Lactobacillus*, which was more abundant in the C chickens, negatively correlated with valeric acid production, resulting in lower levels in C chickens compared to EXP

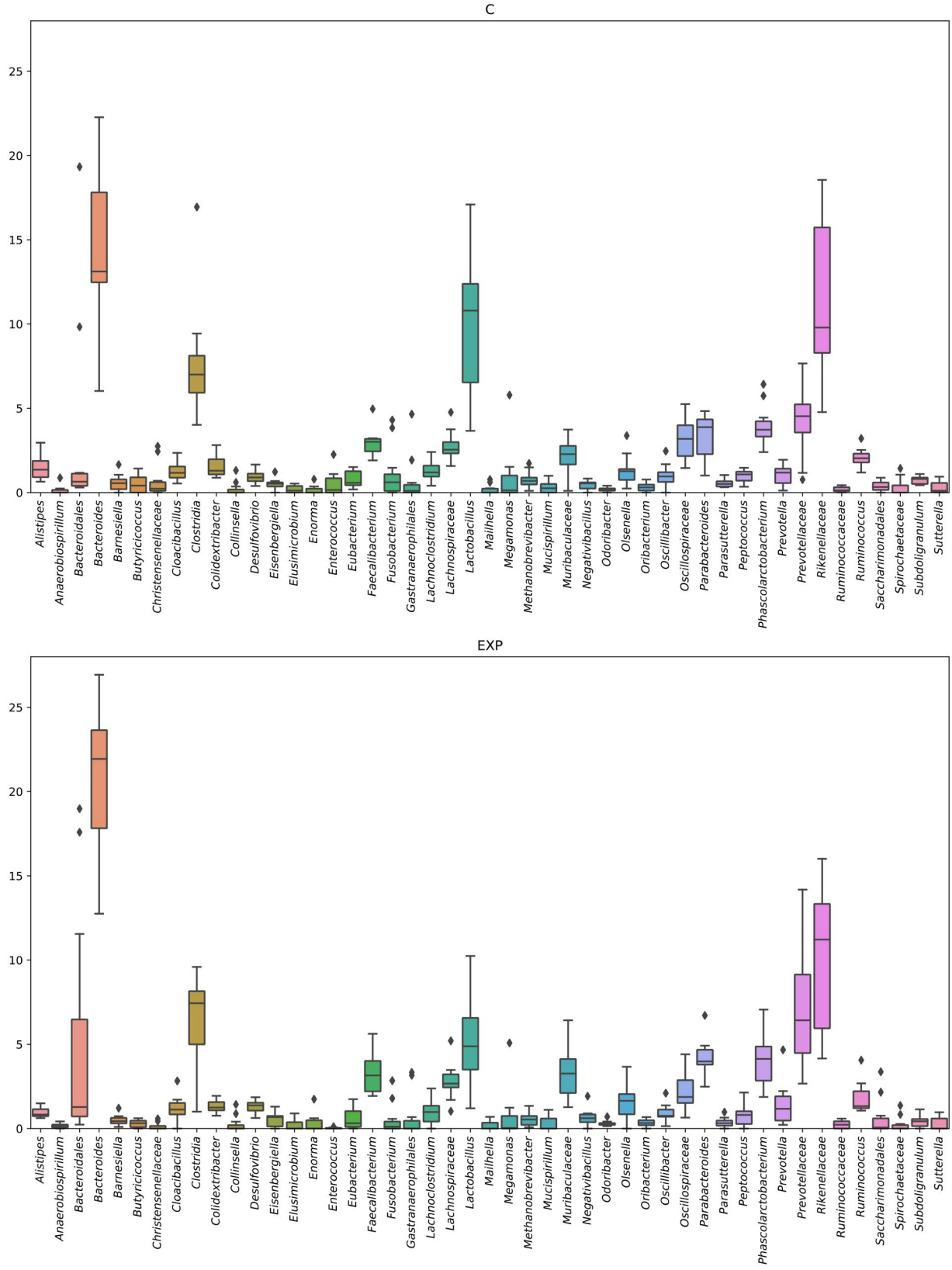

Figure 1. Total amplicon sequence variant of bacteria present in the gut of an indigenous slow-growing chicken breed fed a control or a soybean meal-free experimental diet over the period 39–174 days of age. Mean of 147 and 174 days of age slaughtering.

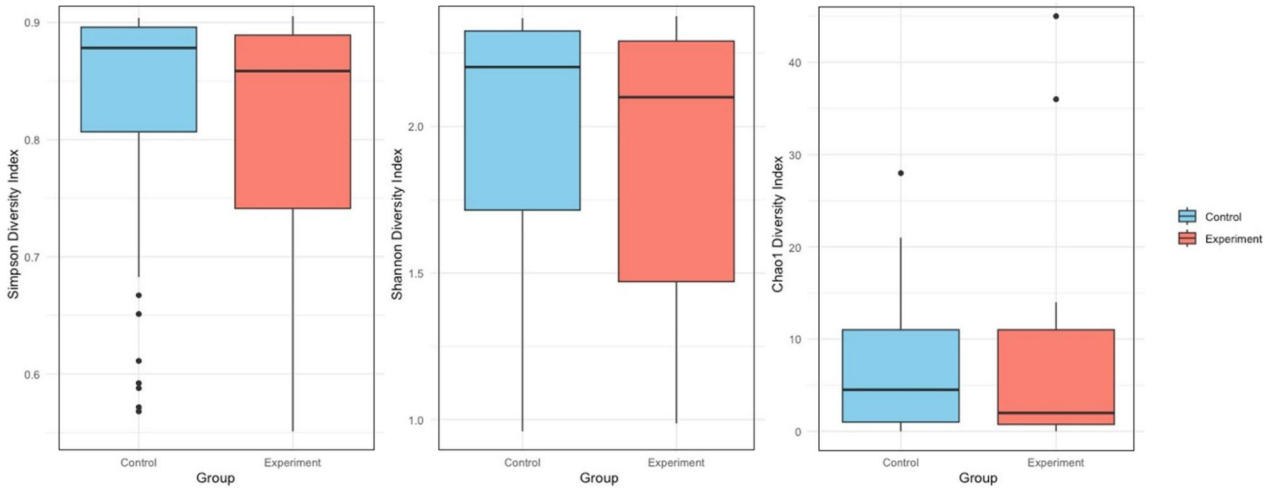

Figure 2. Analysis of the microbiota α-diversity indicators in the caecal digesta (Simpson, Shannon and Chao1) of an indigenous slow-growing chicken breed fed a control or a soybean meal-free experimental diet over the period 39–174 days of age. Mean of 147 and 174 days of age slaughtering.

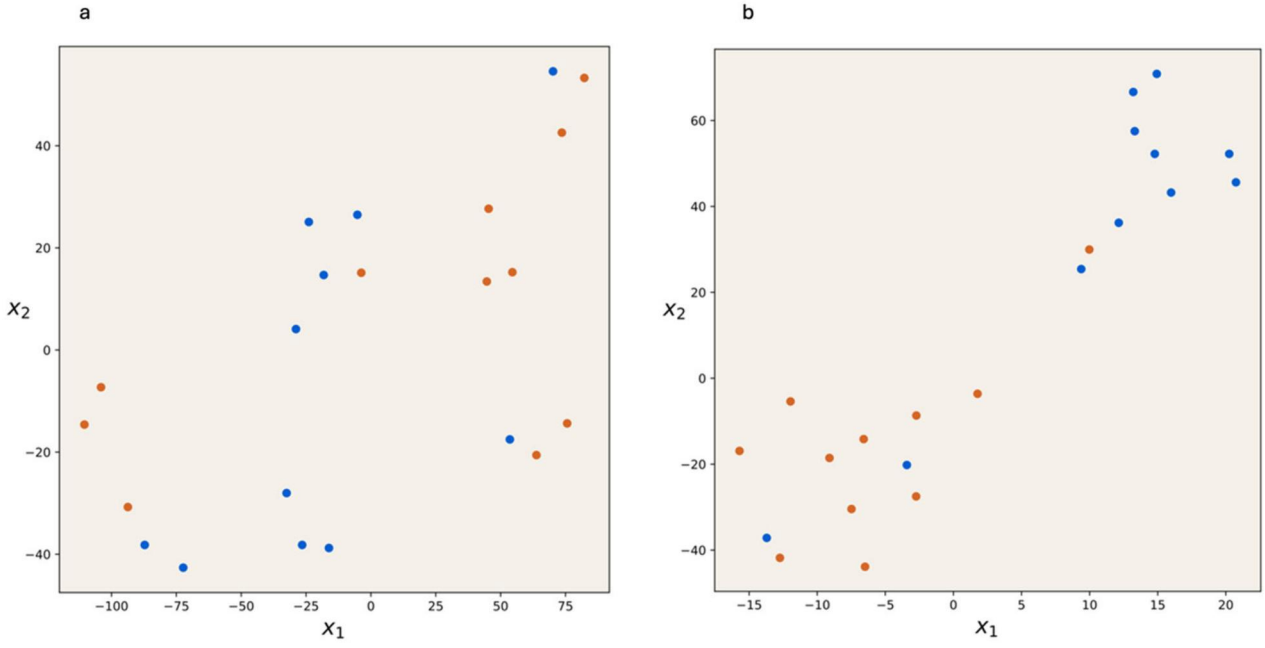

Figure 3. Total amplicon sequence variant of bacteria present in the gut of an indigenous slow-growing chicken breed fed a control or a soybean meal-free experimental diet over the period 39–174 days of age using *t*-distributed stochastic neighbor embedding (*t*-SNE) to map high-dimensional behavioural vectors to a two-dimensional. Each point corresponds to a bacteria family. Points (observations) are colour coded according to dietary treatment (C = orange, EXP = blue). Left panel represents the analysis of all the bacteria families isolated, the right panel represents only the bacteria families with different concentrations between the two experimental treatments.

chickens. Similarly, *Odoribacter*, which negatively correlated with isobutyric acid production, was higher in EXP chickens compared to C chickens, leading to lower levels of isobutyric acid in EXP chickens.

Discussion

Finding alternatives to soybean meal in animal feed is increasingly important. This study compared a soy-free diet to a standard commercial one, finding only slight differences in growth and no significant impact on organ or gut health. Slow-growing poultry breeds, like the Bianca di Saluzzo, adapted well to alternative ingredients such as fava beans and pea protein, maintaining good performance and gut health. These alternatives meet the rising demand for organic and environmentally friendly poultry products (Guarino Amato and Castellini 2022). The results obtained

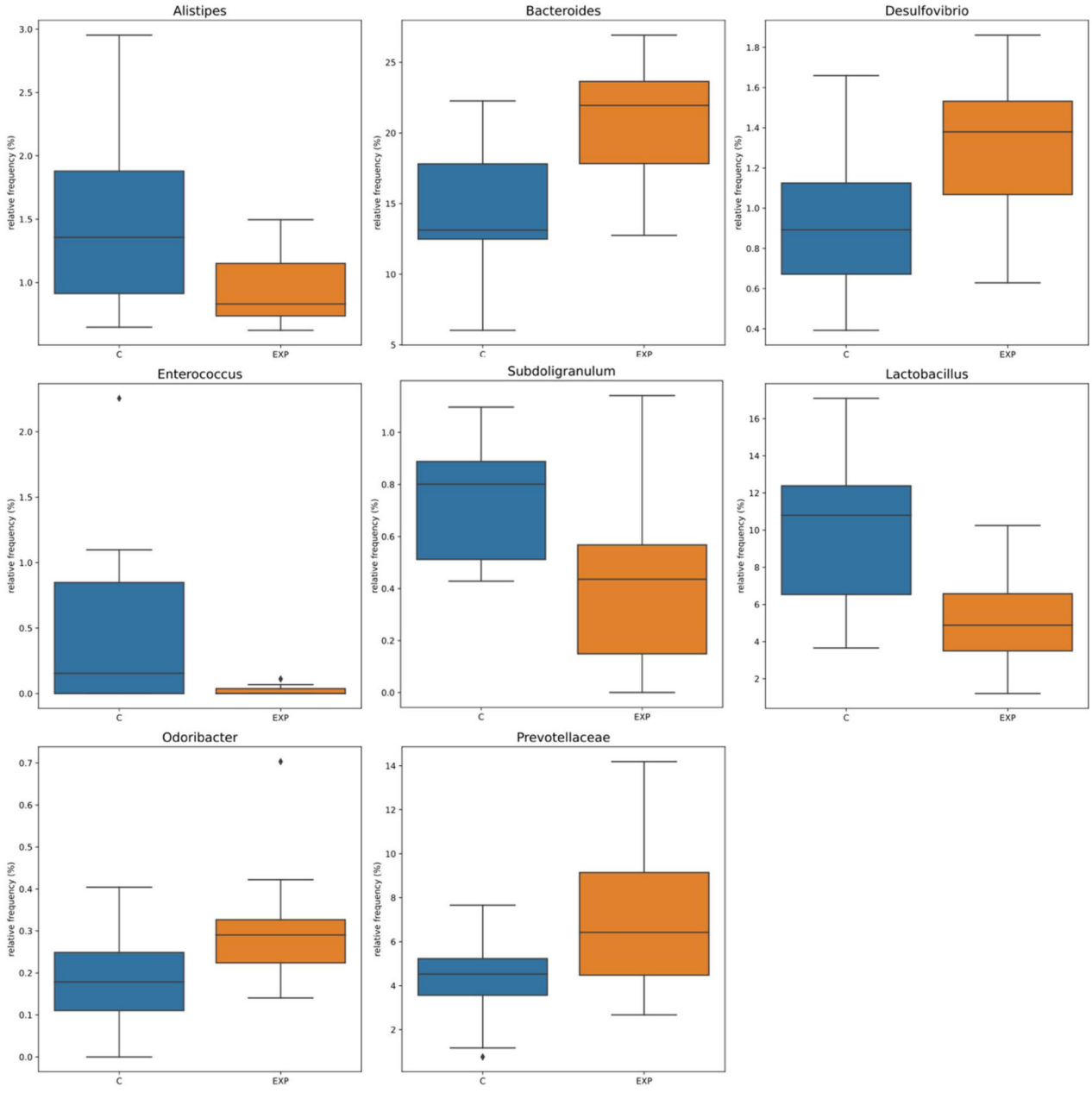

Figure 4. Significantly different relative frequency of total amplicon sequence variant of bacteria present in the gut of an indigenous slow-growing chicken breed fed a control or a soybean meal-free experimental diet over the period 39–174.

indicate that the EXP diet did not cause significant alterations in the main histopathological and morphometric parameters examined, suggesting good tolerability of the alternative soy-free diet. However, it is important to consider some particulars in the results that could have relevant implications.

The inclusion of peas and other alternative plant ingredients in poultry diets has garnered increasing interest, with results generally aligning with our study, showing no significant pathological changes in the liver and spleen of chickens. Studies have shown that the incorporation of field peas does not lead to adverse effects on the health of these organs, maintaining their integrity and function in chickens (Röhe et al. 2017). Furthermore, the impact of alternative protein sources on liver health is particularly noteworthy. The inclusion of various plant ingredients, including peas, has been found to have no significant detrimental effects on liver health in poultry (Kirn et al. 2024). This is crucial for poultry producers who are increasingly seeking sustainable and cost-effective feed options without compromising animal welfare or health. In terms of growth performance, broilers fed diets containing peas have demonstrated comparable results to those fed traditional protein sources. Studies indicate that there are no significant differences in

Table 5. The volatile fatty acids (mg/g faeces) in the gut of an indigenous slow-growing chicken breed fed a control or a soybean meal-free experimental diet over the periods 39–147 and 39–174 days of age (means, $n = 12$).

	Diet (*D*)		Age (*A*)			*p* Value		
	C	EXP	147 d	174 d	SEM	*D*	*A*	*D* × *A*
Citric acid	0.67	1.11	1.02	0.75	0.153	.004	.108	.640
Succinic acid	0.69	1.62	1.20	1.12	0.216	.033	.856	.976
Formic acid	0.91	0.65	0.77	0.69	0.091	.045	.635	.867
Acetic acid	6.12	5.56	6.94	4.74	1.501	.852	.465	.230
Propionic acid	0.92	0.76	0.81	0.89	0.0413	.059	.478	.837
Butyric acid	0.58	0.69	0.59	0.68	0.0817	.462	.535	.586
Isobutyric acid	8.23	5.28	6.33	7.18	0.513	.004	.408	.433
Valeric acid	1.12	2.53	2.02	1.83	0.314	.025	.226	.251
Lactic acid	0.75	0.81	0.21	0.78	0.258	.218	.269	.332
Total	19.1	17.5	19.8	16.9	1.566	.596	.358	.118

C: control; EXP: soybean meal-free experimental diet; SEM: standard error of mean.

growth performance or FCR when peas are included in the diet (Laudadio and Tufarelli 2010).

Regarding histomorphometry measures of the jejunum, the lack of significant differences between diets is to be considered a positive result, indicating that the EXP diet does not significantly alter the intestinal structure of the birds. Partial replacement of soybean meal protein with chickpea protein in broiler diets resulted in reduced intestinal villus length, increased villous thickness and decreased absorptive surface area in the duodenum and jejunum. This was accompanied by a reduced villus length to Cd ratio in the jejunum indicating possible disturbances in intestinal metabolism and structure (Danek-Majewska et al. 2022). Similarly, in another study, feeding chickens a diet containing peas resulted in altered proteolytic activities in the intestinal content due to the presence of the pea-derived Bowman–Birk protease inhibitor, which forms complexes with chicken proteases, thereby affecting digestion and potentially leading to digestive disorders (Moreau et al. 2024). The absence of differences in our study may be attributed to the great resilience and adaptability of local slow-growing chickens. The trend towards morphological changes in response to age in our study, although not significant, could also reflect natural physiological changes that the diet does not seem to influence markedly.

Moreover, although AST levels remained within the physiological range, we observed a 21.5% reduction in liver AST levels in subjects fed the C diet compared to those in the EXP diet. This finding is noteworthy because AST is crucial for amino acid metabolism, and its reduced levels may suggest a modest alteration in hepatic metabolism. A decrease in AST levels could potentially impair the efficiency of the transamination

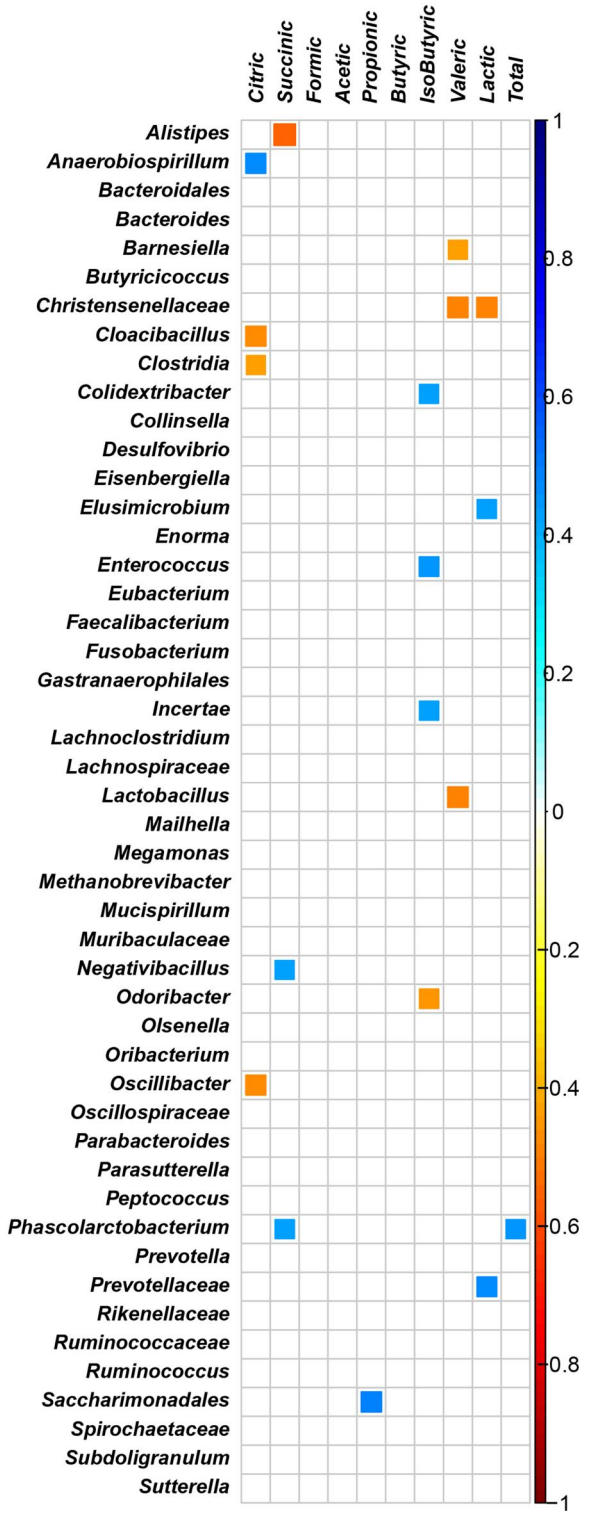

Figure 5. Correlation plot showing Spearman's correlation between microbial ASVs and VFAs of an indigenous chicken breed fed a control or a soybean meal-free experimental diet over the period 39–174 days of age. Only significant associations between ASVs and VFAs are shown (FDR < 0.05). The intensity of the colours represents the degree of correlation, where blue represents a positive degree of correlation and red represents a negative correlation.

process, thereby affecting protein turnover. Tesseraud (1995) emphasises that amino acid availability and metabolism are crucial for protein turnover, while MacDonald and Swick (1981) underscore the role of enzymes like AST in maintaining amino acid balance in chickens. A decrease in AST activity could lead to an imbalance, potentially reducing the rate of protein synthesis and increasing protein degradation, as the organism tries to compensate for the reduced transamination capacity. Hernandez et al. (2013) explain that diet can influence protein metabolism in chickens by affecting enzymes like AST, which are key to protein turnover. Tesseraud et al. (1996) add that this enzyme activity is vital for growth, as reduced AST can hinder protein turnover and, in turn, chicken growth, highlighting its importance in poultry production (Chang et al. 2024).

Studying the results on microbiota and VFAs, it is particularly interesting that in chickens fed the EXP diet, a significant reduction in formic acid levels was observed compared to the C group. This decrease could indicate an improvement in metabolic efficiency or a different pathway for metabolising organic acids, suggesting that the experimental diet may positively influence acid metabolism in chickens. The reduction in formic acid levels could be linked to better metabolic efficiency. It is known that organic acids like formic acid influence lipid metabolism, as seen in studies where they modulate the expression of genes related to fat metabolism, thereby improving metabolic processes in the liver (Wang et al. 2022; Qiu et al. 2023). Moreover, the increase in the presence of succinic acid by +132% and citric acid by +65% in the EXP group, which are key intermediates in the tricarboxylic acid cycle and play a crucial role in cellular metabolism and energy production. Their integration into animal feed has been explored for potential benefits on growth performance, intestinal health and overall metabolic efficiency. Research by Wang et al. (2024) suggests that succinic acid can improve growth performance of chickens by improving FCR and promoting better nutrient absorption. This is supported by the findings of Islam et al. (2012), who notes that organic acids, including citric acid, can lower intestinal pH, thereby inhibiting pathogenic bacteria and promoting beneficial microflora, which is critical for intestinal health and nutrient absorption. Furthermore, research suggests that succinic acid can act as an antimicrobial agent, reducing the prevalence of harmful bacteria in the gastrointestinal tract of chickens. This antimicrobial property not only supports gut health but also improves immune response, leading to better overall health and productivity in poultry (Dexlin et al. 2024). Wang et al. (2022) provide evidence that supplementation with succinic acid and other organic acids could improve the antioxidant status of chickens, which is critical for reducing oxidative stress and improving meat quality. This is especially important in free-range and outdoor farming systems, where chickens are often exposed to increased stressors and an uncontrolled environment that could compromise their health and productivity (Wang et al. 2009).

No significant differences were observed between the two treatments in the alpha diversity indices, Shannon and Chao1, in the chicken microbiota. These findings suggest that the overall microbial diversity within the intestinal tract of chickens was not significantly impacted by the treatments applied. Both the Shannon index, which measures species diversity and evenness, and the Chao1 index, which estimates species richness, showed no substantial variations, indicating a stable microbiota composition despite the differences in treatments. However, it is worth noting that other studies have shown that chickens fed soy-free diets exhibit greater microbial diversity as they age compared to those on soy-containing diets, as evidenced by higher Shannon diversity indices in the caecal content of adult birds on soy-free diets (Lourenco et al. 2019).

A particularly noteworthy finding is the positive correlation between lactic acid production and the presence of Prevotellaceae. This relationship is significant for poultry health and productivity, as Prevotellaceae, a family of bacteria within the phylum Bacteroidetes, are known to play a key role in carbohydrate metabolism. Their presence in the gut microbiota of chickens has been linked to increased fermentative activity, which may, in turn, boost lactic acid production (Lan et al. 2003; Adhikari and Kwon 2017). Lactic acid production by bacteria such as Prevotellaceae could lower the pH of the intestinal environment, inhibiting the growth of pathogenic bacteria and favouring that of beneficial microorganisms, improving intestinal health and feed efficiency, resulting in improved growth performance (Zhang et al. 2023). However, it is important to recognise that the relationship between Prevotellaceae and lactic acid production is complex and not yet fully understood. This relationship may be influenced by various factors, including diet, age and environmental conditions. While some studies have reported a positive correlation between the abundance of Prevotellaceae and increased lactic acid production, others have highlighted the variability of microbial communities and

their metabolic outputs depending on specific study conditions (Adamberg and Adamberg 2024). Additionally, the role of Prevotellaceae in lactic acid production should be considered within the broader context of the entire intestinal microbiota. For instance, other microbial groups, such as *Lactobacillus* spp. which were less abundant in the EXP group compared to the C group are also significant producers of lactic acid and may interact with Prevotellaceae to influence overall lactic acid levels in the gut (Olumuyide et al. 2020).

Conclusions

This study underscores the potential of alternative vegetable protein sources as viable substitutes for soybean meal in poultry diets, particularly for slow-growing chickens like the Bianca di Saluzzo breed. The soy-free diet resulted in minimal differences in growth and had no significant impacts on organ or gut health. The resilience of local breeds to dietary changes, together with the positive impact on gut microbiota and metabolites such as succinic acid and citric acid, highlights the potential benefits of integrating such alternative ingredients into the diet of slow-growing chickens. However, further studies may be needed to fully understand the long-term implications of these dietary modifications as the role of lactic acid production in the gut microbiota, particularly the relationship with the presence of Prevotellaceae, represents another area of future research, with the aim of optimising poultry health and productivity through nutrition. These findings contribute to the growing demand for organic and environmentally friendly poultry products by promoting sustainable feeding strategies that align with consumer preferences and industry trends.

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Edoardo Fiorilla: conceptualisation, data curation, formal analysis, investigation, methodology, writing – original draft, writing – review and editing. Ilario Ferrocino: investigation, writing – review and editing. Marta Gariglio: conceptualisation, investigation, methodology, writing – review and editing. Francesco Gai: conceptualisation, investigation, methodology, writing – review and editing, funding acquisition. Valeria Zambotto: investigation. Laura Ozella: formal analysis, writing – review and editing. Irene Franciosa: investigation, writing – review and editing. Marzia Giribaldi: investigation. Sara Antoniazzi: investigation. Federica Raspa: investigation. Eleonora Erika Cappone: investigation. Dmitri Fabrikov: investigation. Valentina Bongiorno: investigation. Dorotea Ippolito: investigation. Chiara Sferra: investigation. Maria Teresa Capucchio: investigation, writing – review and editing. Achille Schiavone: conceptualisation, investigation, methodology, writing – original draft, writing – review and editing, funding acquisition.

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Data availability statement

The data presented in this study are available upon request from the corresponding author. Raw 16S data are available at: 10.5281/zenodo.13898460.

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