NEXTGEN PROTEINS

Bioconversion of Underutilized Resources into Next Generation Proteins for Food and Feed

Project start: 01 October 2019 **Project duration: 48 months**

Deliverable No D4.3

Deliverable Title: "Report on the effect of feeding alternative proteins on productive traits, physiological indicators, gut health and product quality and safety in chickens and turkeys"

Lead author/editor UNIBO

Due Date of Submission: January 31st, 2023 (extended to March 11th, 2023)
Submission Date: March 13th, 2023

This Project has received funding from the European Union's Horizon 2020 Research and Innovation programme under grant agreement no. 862704.
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0 Document Information

Document Data

Work package related	Work package 4: Alternative protein application in feed products
Task related	Task 4.2: Dose response and field trials – terrestrial animals (chicken, turkey) Task 4.4: Health, welfare and physiological indicators of animals in dose-response trials (subtask 4.4.1: Gut health, welfare and physiological indicators in poultry)
Type	Report
Dissemination level	e.g. PU (public) or CO (confidential)
Keywords	broiler chicken, turkey, microalgae meal, insect meal, single-cell protein, performance, meat quality, foot-pad dermatitis, plasma, gut, metabolome, microbiota.

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Document history

Document version #	**Date**	**Notes/Change**	**Status**
V. 0.9	2.3.2023	UNIBO	draft
V. 1	12.3.2023	Admin team	reviewed
V. 2	15.3.2023	Accepted by Coordinator (BÖS)	final

Table of content

1 Executive summary

This deliverable summarizes the main results concerning the effects of the dietary substitution of soybean with alternative protein sources, namely microalgae meal, insect meal and single-cell proteins meal, on productive traits, physiological indicators, gut health and product quality and safety in broiler chickens and turkeys. In the different trials presented in this deliverable, the effect of feeding alternative protein sources was assessed through a multi-disciplinary approach that included the evaluation of growth performance, incidence and severity of foot-pad dermatitis as welfare indicator, breast meat quality traits, nutrient digestibility, plasma and cecal metabolome, and cecal microbiota.

2 Overview of activities carried out at Month 40

		Microalgae meal VAXA	**Insect meal MUTATEC**	**Insect meal MUTATEC**	**SCP meal ARBIOM**
	Specie	***Broiler chicken***	***Broiler chicken***	***Turkey***	***Broiler chicken***
	n. trials	***3***	***2***	***1***	***2***
Performed activities	**Growth performance**				
	Slaughter yields				
	Welfare indicators				
	Meat quality				
	Plasma metabolomics				
	Cecum metabolomics				
	Cecum microbiota				
Extra activities	**Digestibility assay**				
	Body weight uniformity				
	Sensorial analysis				

Activity completed | Activity not performed | Ongoing activity

3 Introduction

The growth of world population is increasing the protein demand on a global scale either for feed or food applications. Soybean is the most important and widely used protein source in livestock feeding, primarily because of its high crude protein content as well as the optimal balance and availability of essential amino acids (Beski et al., 2015). The EU production of conventional proteins crops including soybean is limited by sub-optimal climatic conditions, allowing to cover no more than 30% of the internal feed protein demand (Kim et al., 2019). Such deficit is usually bridged with imports from third countries, making the EU strongly dependent on the international supply of high protein content feed sources (FEFAC, 2022). For instance, recent reports have shown that more than 95% of the soybean meal used in EU has foreign origin (FEFAC, 2022). It is evident that such scenario imposes major social, economic, and environmental issues that will be further exacerbated by the expected population growth and the concomitant increase in protein demand.
The poultry sector is one of the pillars of livestock production, with chicken and turkey meat that are widely appreciated by consumers (Mottet and Tempio, 2017). Although modern poultry production is considered relatively efficient and sustainable being characterized by low emissions per unit of production output (GLEAM, 2018), feeding and related activities are important factors able to influence the overall sustainability of the poultry sector (Zampiga et al., 2021). Within this context, Ritchie and Roser (2021) pointed out that over one-third (37%) of global soy is used for poultry production. Therefore, the identification of novel, more sustainable protein sources, such as microalgae, insect and single-cell proteins, that might replace soybean in broiler and turkey diets is of paramount importance to enhance the sustainability and resilience of this crucial livestock sector.

4 Use of microalgae meal as alternative to soybean in broiler chicken diets

4.1 Trial #1

Aim

The aim of this study was to investigate the effects of the dietary substitution of soybean with dehydrated microalgae meal (MM; Arthrospira spp. - provided by VAXA) during the first stages of the rearing cycle (up to 22 d of bird age) on the growth performance of broiler chickens.

Materials and Methods

A total of 1,000 one-d-old male Ross 308 chicks were divided into 4 experimental groups (10 replicate pens/group with 25 birds each) receiving, during the starter (0-12 d) and grower (13-22 d) phases, either a conventional soybean-based diet (CON group) or the same diet including MM at low (LM group: 5% in both phases), intermediate (IM group: 10 and 9%, respectively), or high dosages (HM group: 15 and 14%, respectively). From 23 d onwards, all groups received the same conventional soybean-based diet up to slaughter age (47 d). The feed was provided in mash form and for ad libitum consumption. All diets were isoenergetic and with a similar amino acid profile, which was optimized maintaining the same ratio of total essential amino acids to total lysine. Birds were weighed on a pen basis at placement (0 d), at 22 d and at slaughter (47 d). Similarly, feed consumption was determined at 22 and 47 d. Mortality was monitored daily. Dead birds were recorded and weighed to calculate the mortality rate and to adjust the productive performance data. Body weight (BW), daily weight gain (DWG), daily feed intake (DFI) and feed conversion ratio (FCR) were obtained accordingly. The results have been reported for the following periods: 0-22 d, 23-47 d and 0-47 d. At 47 d, all birds were processed in a commercial slaughterhouse. Data were analyzed by means of one-way ANOVA and Tukey post-hoc test, while polynomial contrasts were used to assess linear and quadratic responses.

Results

Performance results are reported in **Table 1**. Average chick BW at placement showed no substantial difference among groups. At 22 d, BW was linearly reduced and FCR significantly worsened as the dietary inclusion of MM increased (931 vs. 850 vs. 709 vs. 462 g, and 1.539 vs. 1.656 vs. 1.783 vs. 2.312 for CON, LM, IM and HM groups, respectively; $P<0.001$). CON and LM groups presented similar DFI from 0 to 22 d, which was significantly higher if compared to that of IM and HM (62.0 vs. 60.5 vs. 53.7 vs. 43.7 g/bird/d, respectively; $P<0.001$). At 47 d, CON and LM groups exhibited comparable BW, while IM and HM showed lower values (3,455 vs. 3,446 vs. 3,221 vs. 2,802 g, respectively; $P<0.001$). The same trend was also observed for DWG and DFI in the overall trial period (71.9 vs. 71.6 vs. 66.6 vs. 57.7 g/bird day, and 130.5 vs. 127.4 vs. 117.3 vs. 103.9 g/bird day, respectively for CON, LM, MM and HM; $P<0.001$; linear: $P<0.001$). No significant difference in FCR was observed in the overall period of the trial (0-47 d). Similarly, mortality was not substantially affected by the dietary treatments.

Table 1. Growth performance of broiler chickens fed a conventional soybean-based diet (CON) or diets with different dosages of microalgae meal (LM, IM and HM) up to 22 d of age.

Parameter	Experimental groups				SEM	*P*-value	Response	
	CON	LM	IM	HM			Linear	Quadratic
			0-22 d					
Chick BW (g)	42.6	42.2	42.4	42.3	0.10	0.61	0.51	0.60
BW (g)	931 A	850 B	709 C	462 D	29.5	**<0.001**	**<0.001**	**<0.001**
DWG (g/bird/day)*	40.3 A	36.6 B	30.1 C	18.9 D	1.34	**<0.001**	**<0.001**	**<0.001**
DFI (g/bird/day)*	62.0 A	60.5 A	53.7 B	43.7 C	1.22	**<0.001**	**<0.001**	**<0.001**
FI (kg/bird)*	1.364 A	1.330 A	1.181 B	0.961 C	0.03	**<0.001**	**<0.001**	**<0.001**
FCR*	1.539 D	1.656 C	1.783 B	2.312 A	0.05	**<0.001**	**<0.001**	**<0.001**
Mortality (%)	0.8	0.4	0.8	0.8	0.02	n.s.	n.s.	n.s.
			23-47 d					
BW (g/bird)	3,455 A	3,446 A	3,221 B	2,802 C	45.6	**<0.001**	**<0.001**	**<0.001**
DWG (g/bird/day)*	101.0 A	104.0 A	100.3 A	93.6 B	0.77	**<0.001**	**<0.001**	**<0.001**
DFI (g/bird/day)*	193.7 A	189.3 A	176.2 B	159.5 C	2.38	**<0.001**	**<0.001**	**<0.01**
FI (kg/bird)*	4.843 A	4.733 A	4.406 B	3.988 C	0.06	**<0.001**	**<0.001**	**<0.01**
FCR*	1.921 A	1.822 B	1.757 BC	1.704 C	0.02	**<0.001**	**<0.001**	n.s.
Mortality (%)	0.4	1.7	0.5	0.0	0.01	0.09	n.s.	n.s.
			0-47 d					
BW (g/bird)	3,455 A	3,446 A	3,221 B	2,802 C	45.6	**<0.001**	**<0.001**	**<0.001**
DWG (g/bird/day)*	71.9 A	71.6 A	66.6 B	57.7 C	0.98	**<0.001**	**<0.001**	**<0.001**
DFI (g/bird/day)*	130.5 A	127.4 A	117.3 B	103.9 C	1.80	**<0.001**	**<0.001**	**<0.001**
FI (kg/bird)*	6.209 A	6.066 A	5.591 B	4.951 C	0.08	**<0.001**	**<0.001**	**<0.001**
FCR*	1.818	1.781	1.762	1.799	0.01	n.s.	n.s.	0.08
Mortality (%)	1.2	2.0	2.2	1.8	0.02	n.s.	n.s.	n.s.

*: corrected for mortality.
A, B: P<0.01
n.s.: not significant.

4.2 Trial #2

Aim

The aim of this trial was to evaluate the effects of the dietary replacement of soybean with dehydrated microalgae meal (MM; *Arthrospira spp.* - provided by VAXA) during grower and finisher phases on productive performance, occurrence of footpad dermatitis (FPD), breast meat quality traits, plasma and cecal metabolomic profile of broiler chickens.

Materials and Methods

A total of 1,000 d-old male Ross 308 chicks was divided into 5 experimental groups each composed by 8 replicates of 25 birds. For the starter phase, all groups received the same commercial corn-wheat-soybean basal diet. Then, the CON group received a commercial diet with soybean as main protein source in all feeding phases. The groups F3 and F6 received the CON diet during starter (0-14 d) and grower (15-28 d) phases while, during the finisher phase (29-41 d), they received the CON diet with 3% and 6% of MM, respectively. GF3 and GF6 groups were fed the CON diet during the starter phase, and then the grower and finisher feed (i.e. from 15 to 41 d) with 3 and 6% of MM, respectively. The feed was provided in mash form and for ad libitum consumption. All diets were isoenergetic and with a similar amino acid profile, which was optimized maintaining the same ratio of total essential amino acids to total lysine. Body weight (BW) was determined on a pen basis at placement, at the end of each feeding phase, and at slaughter. Similarly, feed intake (FI) was assessed on a pen basis at each diet switch and at the end of the trial (14, 28, and 41 d). The number and weight of dead birds were recorded daily and used to calculate the mortality rate and to correct daily weight gain (DWG), daily feed intake (DFI) and feed conversion ratio (FCR). At 41 d, all birds were processed in a commercial slaughterhouse and carcass, breast, leg and wing yields were assessed on all birds. Similarly, the incidence and severity of FPD were evaluated on all birds through a 3-point scale: 0 – no lesion, 1 – mild lesions, 2 – severe lesions (Ekstrand et al., 1998). Technological traits of breast meat, including pHu, color profile, water holding capacity and tenderness, were evaluated on 15 breasts per experimental groups. Similarly, breast meat proximate composition was assessed on the same 15 breasts per group for the groups CON, GF3 and GF6 (AOAC, 1990).
During the finisher phase, all diets were supplemented with titanium dioxide (3 kg/ton), which was used as indigestible marker to evaluate ileal amino acid (AA) digestibility at 41 d of age. A total of 16 birds per group (2 birds per replicate pen) was selected to collect the ileal content, which was then pooled and freeze-dried to obtain at least 3 pools of 8 g dried ileal content/group to be used for AA quantification. Amino acid digestibility was calculated according to the formula proposed by Kluth and Rodehutscord (2006): $Apparent\ digestibility\ (\%) = \{1 - TiO_2\ feed / TiO_2\ ileal \times AA\ ileal / AA\ feed\} \times 100$, where TiO_2 *feed* and TiO_2 *ileal* are the concentrations of titanium dioxide detected in feed and ileal content, while *AA feed* and *AA ileal* represent the quantity of the specific AA respectively in the feed and ileal content. Plasma and cecal content samples were collected at 21 and 41 d from 9 birds/group and then subjected to microbiota (16S Amplicon Sequencing) and metabolome (^{1}H-Nuclear Magnetic Resonance) analysis. Data concerning performance results and metabolomics were analyzed by means of one-way ANOVA and Tukey post-hoc test. Contrasts were applied where appropriate to further explore the data. Microbiota results were analyzed through Student t-test.

Results

As expected, no significant difference was observed during starter phase (**Table 2**). In the grower phase, the use of MM significantly impaired BW, DWG and FCR regardless of the dosage (1,396 vs. 1,296 g, 70.8 vs. 63.5 g/bird/d, and 1,594 vs. 1.794, respectively for CON and MM-fed groups; P<0.01). The same general trend was observed during the finisher phase (BW: 2,541 vs. 2,424 g; DWG: 89.5 vs. 82.5 g/bird/d; FCR: 1.971 vs. 2.124, respectively for CON and MM; P<0.01). The MM dosage had negative effects on DWG and FCR from 29 to 41 d, while broilers fed MM in grower and finisher phases exhibited higher DWG and DFI than those receiving it only during the finisher one. Considering the productive performance in the overall rearing cycle, final BW was significantly lower in groups F6 and GF6 compared to CON, while F3 and GF3 groups showed intermediate values (2,541 vs. 2,454 vs. 2,412 vs. 2,445 vs. 2,384 g, respectively for CON, F3, F6, GF3 and GF6; P<0.05). GF6 exhibited the highest FCR, while F3 did not present significant differences compared to CON (1.785 vs. 1.810 vs. 1.834 vs. 1.886 vs. 1.934, respectively for CON, F3, F6, GF3 and GF6; P<0.01). DFI was not significantly affected by the dietary treatment. Overall, it emerged that the use of MM had negative effects on BW, DWG and FCR (2,541 vs. 2,424 g, 59.6 vs. 57.4 g/bird/d, and 1.785 vs. 1.866, respectively for CON and MM; P<0.05). The tested dosages (3 vs. 6%) exerted no relevant effect, while the duration of MM administration influenced feed consumption and FCR, which were higher when MM was provided during both grower and finisher phases rather than only during the finisher one (105.8 vs. 108.3 g/bird/d and 1.822 vs. 1.910, respectively).

Table 2. Growth performance of broiler chickens fed a conventional soybean-based diet (CON) or diets with different dosages of microalgae meal (3 or 6%) during finisher (F) or grower and finisher (GF) phases.

Parameter	Experimental groups					SEM	*P*-value	Contrasts					
								Diet		MM dosage		Duration MM administration	
	CON	F3	F6	GF3	GF6			CON	MM	3%	6%	F	GF
			Starter (0-13 d)										
Chick BW (g)	37.7	37.3	38.0	37.5	38.0	0.11	n.s.						
BW (g)	325	330	331	330	333	2.46	n.s.						
DWG (g/bird/d)*	22.0	22.5	22.4	22.5	22.6	0.19	n.s.						
DFI (g/bird/d)*	36.0	36.4	36.2	36.2	36.4	0.17	n.s.						
FI (kg/bird)*	0.467	0.473	0.470	0.471	0.473	0.01	n.s.						
FCR*	1.634	1.622	1.617	1.610	1.611	0.01	n.s.						
Mortality (%)	0.50	0.50	0.50	0.50	0.50	0.01	n.s.						
			Grower (14-28 d)							Grower (14-28 d)			
BW (g/bird)	1,379 AB	1,392 AB	1,415 A	1,308 BC	1,284 C	12.3	**<0.001**	1,396 A	1,296 B	1,308	1,284		
DWG (g/bird/d)*	69.6 A	70.6 A	72.2 A	64.6 B	62.5 B	0.79	**<0.001**	70.8 A	63.5 B	64.6	62.5		
DFI (g/bird/d)*	113.4	111.0	113.4	114.5	113.0	0.59	n.s.	112.6	113.8	114.5	113.0		
FI (kg/bird)*	1.701	1.665	1.700	1.718	1.695	0.01	n.s.	1.689	1.706	1.718	1.695		

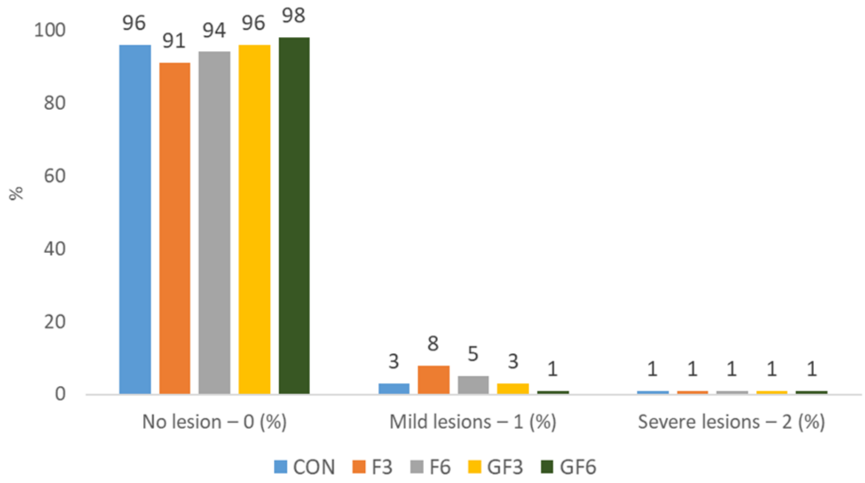
FCR (g feed/g bird)*	1.636 B	1.574 B	1.573 B	1.777 A	1.811 A	0.02	**<0.001**	1,594 B	1.794 A	1.777	1.811			
Mortality (%)	2.00	1.02	0.52	0.50	0.50	0.01	n.s.	1.18	0.50	0.50	0.50			
			Finisher (29-41 d)								**Finisher (29-41 d)**			
BW (g/bird)	2,541 A	2,454 AB	2,412 B	2,445 AB	2,384 B	15.6	**<0.01**	2,541 A	2,424 B	2,450	2,398	2,433	2,414	
DWG (g/bird/d)*	89.5 A	81.8 B	76.4 C	87.5 A	84.6 AB	0.91	**<0.001**	89.5 A	82.5 B	84.6 A	80.5 B	79.1 B	86.0 A	
DFI (g/bird/d)*	176.1 AB	171.5 AB	166.9 B	180.3 A	181.0 A	1.39	**<0.01**	176.1	174.9	175.9	174.0	169.2 B	180.2 A	
FI (kg/bird)*	2.289 AB	2.230 AB	2.170 B	2.344 A	2.353 A	0.02	**<0.01**	2.289	2.274	2.287	2.262	2.200	2.348	
FCR (g feed/g bird)*	1.971 B	2.102 AB	2.188 A	2.063 AB	2.143 A	0.02	**<0.01**	1.971 B	2.124 A	2.083 b	2.165 a	2.145	2.103	
Mortality (%)	0.57	1.50	1.52	0.00	0.00	0.01	n.s.	0.57	0.76	0.75	0.76	1.51 a	0.00 b	
			Overall period (0-41 d)								**Overall period (0-41 d)**			
BW (g/bird)	2,541 A	2,454 AB	2,412 B	2,445 AB	2,384 B	15.6	**<0.01**	2,541 A	2,424 B	2,450	2,398	2,433	2,414	
DWG (g/bird/d)*	59.6 a	58.7 ab	57.6 ab	57.5 ab	56.0 b	0.38	**0.03**	59.6 a	57.4 b	58.1	56.8	58.1	56.8	
DFI (g/bird/d)*	106.2	106.1	105.5	108.4	108.1	0.50	n.s.	106.2	107.1	107.3	106.8	105.8 b	108.3 a	
FI (kg/bird)*	4.457 AB	4.368 AB	4.341 B	4.532 A	4.521 AB	0.02	**<0.01**	4.457	4.440	4.450	4.431	4.354 B	4.526 A	
FCR (g feed/g bird)*	1.785 B	1.810 B	1.834 AB	1.886 AB	1.934 A	0.02	**<0.01**	1.785 b	1.866 a	1.848	1.884	1.822 B	1.910 A	
Mortality (%)	3.00	3.00	2.52	1.00	1.00	0.02	n.s.	3.00	1.88	2.00	1.76	2.76	1.00	

BW: body weight; DWG: daily weight gain; DFI: daily feed intake; FI: feed intake; FCR: feed conversion ratio; n.s.: not significant.

*: corrected for mortality. A, B: P<0.01; a,b: P<0.05

Slaughter yields and the incidence and severity of FPD (**Figure 1**) were substantially similar among experimental groups.

__Figure 1__**. Incidence and severity of footpad dermatitis in 41-d-old broiler chickens fed a conventional soybean-based diet (CON) or diets with different dosages of microalgae meal (3 or 6%) during finisher (F) or grower and finisher (GF) phases.**



n: CON =152; F3 = 149; F6 = 170; GF3 = 164; GF6 = 168. X^2 *P*-value = n.s.

The most relevant outcome regarding breast meat quality traits concerned the color profile (**Table 3**). For both raw and cooked meat, the dietary inclusion of MM significantly reduced the lightness (P<0.01) while increased redness and yellowness (P<0.01). Particularly for the latter, the effect was particularly pronounced resulting in breasts with very intense pigmentation. Moreover, both the dosage and duration had positive effects on meat pigmentation. The other meat quality traits were only slightly affected by the dietary treatments, with drip loss that was lower in GF6 compared to CON and F3 (1.87 vs. 1.87 vs. 1.75 vs. 1.61 vs. 1.54, respectively for CON, F3, F6, GF3 and GF6; P<0.05; **Table 4**). Proximate composition was not substantially affected by MM administration (**Table 5**).

Table 3. Color profile of raw and cooked breast meat (*n*=15 breasts/group) of broiler chickens fed a conventional soybean-based diet (CON) or diets with different dosages of microalgae meal (3 or 6%) during finisher (F) or grower and finisher (GF) phases.

	Parameter	Experimental groups					SEM	*P*-value	Contrasts					
									Diet		MM dosage		MM duration	
		CON	F3	F6	GF3	GF6			CON	MM	3%	6%	F	GF
Raw meat	Lightness (L*)	57.9 a	56.6 ab	54.5 bc	54.3 c	52.6 c	0.31	**<0.001**	57.9 A	54.5 B	55.5 A	53.5 B	55.6 A	53.4 B
	Redness (a*)	1.67 b	1.65 b	2.69 a	2.72 a	3.13 a	0.10	**<0.001**	1.67 B	2.55 A	2.19 B	2.91 A	2.17 B	2.93 A
	Yellowness (b*)	6.77 d	12.7 c	17.0 b	17.2 b	20.1 a	0.59	**<0.001**	6.77 B	16.7 A	15.0 B	18.5 A	14.9 B	18.6 A
Cooked meat	Lightness (L*)	83.7 a	83.4 ab	82.4 bc	82.4 bc	81.8 c	0.17	**<0.001**	83.7 A	82.5 B	82.9 a	82.1 b	82.9 a	82.1 b
	Redness (a*)	2.15 B	2.44 B	3.10 A	3.14 A	3.55 A	0.09	**<0.001**	2.15 B	3.06 A	2.80 B	3.32 A	2.77 B	3.35 A
	Yellowness (b*)	13.6 D	17.5 C	20.7 B	20.3 B	23.5 A	0.43	**<0.001**	13.6 B	20.5 A	18.9 B	22.1 A	19.1 b	21.9 a

A, B: P<0.01; a,b: P<0.05

Table 4. Technological properties of breast meat (*n*=15 breasts/group) of broiler chickens fed a conventional soybean-based diet (CON) or diets with different dosages of microalgae meal (3 or 6%) during finisher (F) or grower and finisher (GF) phases.

Parameter	Experimental groups					SEM	*P*-value	Contrasts					
								Diet		MM dosage		MM duration	
	CON	F3	F6	GF3	GF6			CON	MM	3%	6%	F	GF
pHu	5.76	5.73	5.74	5.74	5.79	0.01	n.s.	5.76	5.75	5.74	5.77	5.74	5.77
Drip loss (%)	1.87 a	1.87 a	1.75 ab	1.61 ab	1.54 b	0.04	**<0.05**	1.87	1.69	1.74	1.65	1.81	1.57
Cooking loss (%)	23.4	22.7	22.5	24.0	22.8	0.23	n.s.	23.4	23.0	23.3	22.6	22.5	23.4
Shear force (kg)	2.1	2.2	2.1	2.1	1.9	0.04	n.s.	2.1	2.1	2.2	2.0	2.2	2.0

a,b: P<0.05

Table 5. Proximate composition of breast meat (*n*=15 breasts/group) of broiler chickens fed a conventional soybean-based diet (CON) or diets with different dosages of microalgae meal (3 or 6%) during grower and finisher (GF) phases.

	CON	GF3	GF6	SEM	*P*-value
Moisture (%)	75.47	74.94	75.13	0.12	n.s.
Crude protein (%)	23.03	22.67	23.05	0.12	n.s.
Total fat (%)	1.76	1.79	1.51	0.06	0.08
Ash (%)	1.35	1.41	1.54	0.03	0.07

Compared to CON, the evaluation of AA digestibility evidenced a significant reduction in digestibility coefficients in F3 and, even more pronounced, in F6 (total AA digestibility: 81.1 vs. 77.8 vs. 72.2%, respectively for CON, F3 and F6; P<0.001). The digestibility values for each analyzed AA are shown in **Table 6**.

Table 6. Ileal amino acid digestibility in 41 d-old broilers fed soybean-based diets (CON) or diets with 3 or 6% microalgae meal during finisher phase (F3 and F6, respectively).

	CON		F3		F6		SEM	*P*-value
MET	89.5	A	88.0	B	85.6	C	0.56	**<0.001**
CYS	71.0	A	67.8	B	58.2	C	1.87	**<0.001**
MET+CYS	82.5	A	80.6	B	76.5	C	0.86	**<0.001**
LYS	83.3	A	80.9	B	77.1	C	0.89	**<0.001**
THR	72.8	A	70.4	B	63.4	C	1.38	**<0.001**
TRP	74.3	A	69.5	B	61.4	C	1.82	**<0.001**
ARG	87.1	A	83.3	B	79.8	C	1.02	**<0.001**
ILE	79.2	A	75.0	B	68.4	C	1.52	**<0.001**
LEU	80.9	A	77.8	B	72.4	C	1.21	**<0.001**
VAL	77.0	A	72.9	B	65.5	C	1.64	**<0.001**
HIS	81.4	A	78.0	B	71.5	C	1.41	**<0.001**
PHE	83.3	A	80.3	B	74.8	C	1.21	**<0.001**
GLY	73.3	A	69.8	B	62.4	C	1.56	**<0.001**
SER	76.4	A	72.6	B	65.4	C	1.56	**<0.001**
PRO	81.6	A	78.8	B	73.7	C	1.14	**<0.001**
ALA	77.1	A	73.9	B	67.0	C	1.45	**<0.001**
ASP	78.1	A	73.8	B	67.6	C	1.48	**<0.001**
GLU	86.1	A	82.8	B	78.4	C	1.08	**<0.001**
TOTAL	**81.1**	**A**	**77.8**	**B**	**72.2**	C	**1.26**	<0.001

To as concern the metabolomic profile of plasma at 21 d, a total of 9 metabolites (listed in **Table 7**) out of 60 identified showed significant difference among experimental groups. At 41 d, the number of molecules whose concentration was significantly affected by the dietary treatments was 10 (**Table 8**). Some of these molecules can play a role on energy and protein metabolism as well as on antioxidant status.

Table 7. Concentration (mmol/L) of plasma metabolites (identified through the ^{1}H-NMR analysis) showing significant differences in 21-d-old broilers fed soybean-based diets (CON) or diets with different dosages of microalgae meal (3 or 6%).

Metabolite (mmol/L)	CON		GF3		GF6		SEM	*P*-value	Linear Trend *P*-value
Sarcosine	4.13E-02	B	6.04E-02	AB	9.47E-02	A	3.97E-03	**<0.001**	**<0.001**
Methionine	1.60E-01	B	1.57E-01	B	2.38E-01	A	9.23E-03	**<0.001**	**<0.001**
Histidine	1.62E-01	AB	1.81E-01	A	1.32E-01	B	6.50E-03	**<0.01**	**0.05**
2,3-butanediol	1.62E-01	B	3.07E-01	A	3.15E-01	A	2.33E-02	**<0.01**	**<0.01**
myo-Inositol	7.95E-01	AB	9.73E-01	A	7.27E-01	B	3.50E-02	**<0.01**	n.s.
3-Hydroxyisobutyrate	4.03E-02	b	4.72E-02	ab	5.16E-02	a	1.61E-03	**0.01**	**<0.01**
Leucine	3.17E-01	b	3.66E-01	a	3.70E-01	a	8.58E-03	**0.02**	**0.01**
Citramalate	1.00E-01	b	1.07E-01	ab	1.21E-01	a	3.33E-03	**0.03**	**<0.01**
Acetone	1.35E-02	b	1.79E-02	a	1.71E-02	ab	7.31E-04	**0.03**	**0.04**

A,B : P<0.01; a. b: P<0.05. n.s.: not significant.

Table 8. Concentration (mmol/L) of plasma metabolites (identified through the ^{1}H-NMR analysis) showing significant differences in 41-d-old broilers fed soybean-based diets (CON) or diets with different dosages of microalgae meal (3 or 6%) during finisher (F) or grower and finisher (GF) phases.

Metabolite (mmol/L)	CON		F3		F6		GF3		GF6		SEM	*P*-value
Histidine	1.68E-01	A	1.44E-01	B	1.34E-01	BC	1.35E-01	BC	1.20E-01	C	2.66E-03	**<0.001**
Citramalate	8.67E-02	C	8.46E-02	C	1.06E-01	AB	9.26E-02	BC	1.10E-01	A	2.09E-03	**<0.001**
Sarcosine	4.83E-02	B	6.24E-02	B	7.84E-02	A	5.75E-02	B	8.38E-02	A	2.18E-03	**<0.001**
Methionine	2.33E-01	B	2.64E-01	AB	3.04E-01	A	2.80E-01	A	2.89E-01	A	5.63E-03	**<0.001**
Arginine	5.48E-01	A	4.61E-01	AB	3.73E-01	B	4.51E-01	AB	3.79E-01	B	1.56E-02	**<0.01**
Uridine	1.39E-02	B	2.35E-02	A	2.23E-02	A	2.03E-02	AB	2.45E-02	A	9.51E-04	**<0.01**
myo-Inositol	8.70E-01	AB	7.90E-01	B	8.14E-01	B	9.71E-01	A	8.04E-01	B	1.80E-02	**<0.01**
Creatine	1.23E-01	A	1.16E-01	AB	9.65E-02	AB	8.35E-02	AB	7.64E-02	B	4.95E-03	**<0.01**
3-Hydroxyisobutyrate	3.36E-02	B	3.92E-02	AB	4.23E-02	AB	3.77E-02	AB	4.63E-02	A	1.18E-03	**<0.01**
Valine	3.32E-01	b	3.59E-01	ab	3.75E-01	a	3.30E-01	b	3.54E-01	ab	4.73E-03	**0.01**

A,B : P<0.01; a. b: P<0.05.

Regarding the metabolomic profile of the cecal content at 21 d, out of 71 molecules, 23 of them exhibited significant differences among experimental groups (**Table 9**). At 41 d, the concentration of 20 cecal metabolites was significantly affected by the dietary treatments (**Table 10**).

Table 9. Concentration (mmol/L) of cecal metabolites (identified through the [1]H-NMR analysis) showing significant differences in 21-d-old broilers fed soybean-based diets (CON) or diets with different dosages of microalgae meal (3 or 6%).

Metabolite (mmol/L)	CON		GF3		GF6		SEM	P-value	Linear trend P-value
Phenylalanine	6.63E-03	A	4.71E-03	B	4.55E-03	B	2.45E-04	**<0.001**	**<0.001**
Glycine	8.91E-03	A	7.25E-03	AB	5.77E-03	B	3.64E-04	**<0.001**	**<0.001**
Malonate	1.10E-03	B	1.94E-03	B	3.46E-03	A	2.84E-04	**<0.001**	**<0.001**
1,3-Diaminopropane	1.31E-04	B	5.58E-04	B	2.06E-03	A	2.15E-04	**<0.001**	**<0.001**
Alpha-ketoisovaleric acid	1.22E-03	A	8.69E-04	AB	4.52E-04	B	8.42E-05	**<0.001**	**<0.001**
3-Methyl-2-oxovalerate	1.86E-03	A	1.27E-03	B	7.39E-04	B	1.15E-04	**<0.001**	**<0.001**
Propionate	3.35E-02	B	5.81E-02	A	7.26E-02	A	4.07E-03	**<0.001**	**<0.001**
Cholate	5.65E-04	B	7.87E-04	A	9.15E-04	A	4.06E-05	**<0.001**	**<0.001**
Tyrosine	4.41E-03	A	3.10E-03	B	2.83E-03	B	2.29E-04	**<0.01**	**<0.01**
Phenylacetate	1.50E-03	A	8.97E-04	AB	3.53E-04	B	1.57E-04	**<0.01**	**<0.01**
Acetate	2.89E-01	B	4.19E-01	AB	5.33E-01	A	2.92E-02	**<0.01**	**<0.001**
Isoleucine	7.13E-03	A	4.88E-03	B	4.65E-03	B	3.32E-04	**<0.01**	**<0.01**
Dimethylamine	1.14E-04	B	1.41E-04	B	2.08E-04	A	1.20E-05	**<0.01**	**<0.001**
Methylamine	9.12E-05	B	2.16E-04	B	5.84E-04	A	6.35E-05	**<0.01**	**<0.001**
2.3-Butanediol	4.09E-04	B	6.71E-04	AB	8.53E-04	A	5.61E-05	**<0.01**	**<0.001**
Thymine	5.84E-04	A	4.38E-04	AB	3.37E-04	B	3.18E-05	**<0.01**	**<0.01**
Trimethylamine	6.75E-04	B	7.53E-04	B	1.03E-03	A	4.90E-05	**<0.01**	**<0.01**
Alanine	2.51E-02	A	2.40E-02	A	1.74E-02	B	1.14E-03	**<0.01**	**<0.01**
Valine	9.63E-03	A	8.29E-03	AB	6.78E-03	B	3.87E-04	**<0.01**	**<0.01**
Leucine	1.26E-02	A	9.51E-03	B	8.78E-03	B	5.51E-04	**<0.01**	**<0.01**
2-Oxoisocaproate	4.14E-03	A	2.81E-03	AB	2.35E-03	B	2.55E-04	**<0.01**	**<0.01**
Acetoacetate	7.60E-04	ab	1.10E-03	a	5.95E-04	b	8.19E-05	**0.03**	n.s.
Nicotinate	1.85E-03	b	2.25E-03	ab	2.69E-03	b	1.35E-04	**0.04**	**0.01**

A,B : P<0.01; a. b: P<0.05. n.s.: not significant.

Table 10. Concentration (mmol/L) of cecal metabolites (identified through the ^{1}H-NMR analysis) showing significant differences in 41-d-old broilers fed soybean-based diets (CON) or diets with different dosages of microalgae meal (3 or 6%) during finisher (F) or grower and finisher (GF) phases.

Metabolite (mmol/L)	CON		F3		F6		GF3		GF6		SEM	P-value
Taurine	1.51E-03	B	2.96E-03	B	2.87E-03	B	3.27E-03	AB	5.57E-03	A	2.97E-04	**<.001**
Malonate	2.52E-03	C	5.59E-03	BC	1.06E-02	A	5.04E-03	BC	7.61E-03	AB	4.59E-04	**<.001**
1,3-Diaminopropane	3.82E-04	D	4.73E-03	BC	1.17E-02	A	4.02E-03	C	8.01E-03	B	5.74E-04	**<.001**
Trimethylamine	5.49E-04	B	5.55E-04	B	6.95E-04	AB	5.91E-04	B	9.57E-04	A	3.58E-05	**<.001**
Methylamine	2.91E-04	C	2.22E-03	B	4.46E-03	A	1.54E-03	BC	2.89E-03	B	2.21E-04	**<.001**
Isovalerate	5.56E-03	A	3.40E-03	B	3.02E-03	B	2.92E-03	B	2.57E-03	B	2.22E-04	**<.001**
Methylmalonate	3.93E-04	B	1.35E-03	AB	8.64E-04	AB	5.47E-04	B	7.38E-04	AB	8.36E-05	**<0.01**
Glutamate	5.66E-02	A	5.10E-02	AB	3.75E-02	B	4.52E-02	AB	3.81E-02	B	1.92E-03	**<0.01**
Isobutyrate	1.11E-02	A	8.01E-03	AB	7.16E-03	B	7.93E-03	AB	5.98E-03	B	4.49E-04	**<0.01**
Dimethylamine	1.19E-04	B	1.52E-04	AB	1.62E-04	A	1.45E-04	AB	1.74E-04	A	5.12E-06	**<0.01**
Phenylacetate	2.29E-03	a	1.24E-03	ab	9.79E-04	b	7.35E-04	b	1.44E-03	ab	1.50E-04	**0.01**
Threonine	5.24E-03	ab	4.25E-03	b	6.16E-03	ab	5.47E-03	ab	6.84E-03	a	2.47E-04	**0.01**
N,N-Dimethylglycine	1.66E-04	b	1.96E-04	ab	2.38E-04	ab	2.16E-04	ab	2.98E-04	a	1.26E-05	**0.01**
Methanol	7.65E-04	b	6.68E-04	b	1.08E-03	ab	1.18E-03	ab	2.19E-03	a	1.56E-04	**0.01**
Pyruvate	5.36E-04	b	6.68E-04	ab	5.81E-04	b	1.54E-03	a	1.09E-03	ab	1.13E-04	**0.02**
Thymine	6.54E-04	a	5.64E-04	ab	4.36E-04	b	4.50E-04	ab	5.20E-04	ab	2.52E-05	**0.04**
Methylsuccinate	4.90E-03	ab	7.81E-03	ab	9.87E-03	a	4.22E-03	b	6.27E-03	ab	6.51E-04	**0.04**
N6-Acetyllysine	1.23E-03	b	3.39E-03	ab	6.18E-03	a	4.75E-03	ab	3.67E-03	ab	5.30E-04	**0.05**
Cholate	8.36E-04	a	6.54E-04	b	6.85E-04	ab	6.76E-04	ab	6.89E-04	ab	2.13E-05	**0.05**
Proline	5.48E-03	a	4.46E-03	ab	4.14E-03	b	4.31E-03	ab	4.69E-03	ab	1.56E-04	**0.05**

A,B : P<0.01; a. b: P<0.05.

Regarding the cecal microbiota, Firmicutes was the most represented phylum in all tested groups at both 21 and 42 days (**Table 11** and **Table 12**). At the end of the rearing cycle, the phylum Firmicutes showed a statistically significant decrease corresponding to an increase of Proteobacteria in GF6 group only.

Table 11. Mean relative abundance (%) at phylum level in cecal content of 21-d-old broilers fed soybean-based diets (CON) or diets with different dosages of microalgae meal (3 or 6%).

PHYLUM (%)	Experimental groups			*P*-value t-test		
	CON	GF3	GF6	CON vs GF3	CON vs GF6	GF3 vs GF6
Firmicutes	98.1	97.4	97.5	0.08	n.s.	n.s.
Proteobacteria	0.50	0.70	0.60	n.s.	n.s.	n.s.
Tenericutes	1.10	1.70	1.70	0.05	0.07	n.s.
Cyanobacteria	0.10	0.10	0.20	n.s.	n.s.	n.s.
Actinobacteria	0.20	0.20	0.00	n.s.	**0.04**	n.s.

n.s.: not significant.

Table 12. Mean relative abundance (%) at phylum level in cecal content of 41-d-old broilers fed soybean-based diets (CON) or diets with different dosages of microalgae meal (3 or 6%) during finisher (F) or grower and finisher (GF) phases.

PHYLUM (%)	Experimental groups					*P*-value t-test									
	CON	F3	F6	GF3	GF6	CON vs F3	CON vs F6	CON vs GF3	CON vs GF6	F3 vs F6	F3 vs GF3	F3 vs GF6	F6 vs GF3	F6 vs GF6	GF3 vs GF6
Firmicutes	96.7	97.1	96.8	96.9	95.1	n.s.	n.s.	n.s.	0.07	n.s.	n.s.	**0.02**	n.s.	**0.04**	**0.03**
Proteobacteria	1.50	1.10	1.30	1.20	3.20	n.s.	n.s.	n.s.	0.08	n.s.	n.s.	**0.02**	n.s.	**0.04**	**0.03**
Tenericutes	1.40	1.50	1.50	1.70	1.40	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Cyanobacteria	0.20	0.20	0.20	0.20	0.10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.05	n.s.	n.s.	n.s.
Actinobacteria	0.10	0.10	0.00	0.00	0.20	n.s.	0.08	n.s.	n.s.	**0.03**	n.s.	n.s.	n.s.	n.s.	n.s.
Bacteroidetes	0.10	0.00	0.00	0.00	0.10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

n.s.: not significant.

At the genus level (**Table 13** and **Table 14**), *Fecalibacterium*, followed by *Ruminococcus* and *Oscillospira*, were the most represented genera in all tested groups at both 21 and 42 days. At 21 days (**Table 13**), *Lactobacillus* significantly decreased in both groups GF3 and GF6. At the end of the rearing cycle (**Table 14**), the genera *Bacillus* and *Dehalobacterium* showed a statistically significant increase in group GF6, while *Streptococcus* showed the highest abundance in the control group.

Table 13. Mean relative abundance (%) at genus level in cecal content of 21-d-old broilers fed soybean-based diets (CON) or diets with different dosages of microalgae meal (3 or 6%).

GENUS	Experimental groups			*P*-value t-test		
	CON	GF3	GF6	CON vs GF3	CON vs GF6	GF3 vs GF6
Faecalibacterium	21.2	17.4	16.7	n.s.	0.08	n.s.
Oscillospira	6.60	6.80	8.40	n.s.	0.05	n.s.
Ruminococcus	5.60	7.30	6.70	n.s.	n.s.	n.s.
Ruminococcus	3.40	3.50	4.40	n.s.	n.s.	n.s.
Bacillus	2.40	3.50	3.80	n.s.	n.s.	n.s.
Lactobacillus	5.00	2.10	0.60	**0.04**	**<0.001**	n.s.
Coprococcus	0.70	1.30	1.10	n.s.	n.s.	n.s.
Clostridium	1.90	2.00	0.80	n.s.	n.s.	n.s.
Dorea	1.00	1.40	1.30	n.s.	n.s.	n.s.
Blautia	0.90	0.60	0.50	n.s.	n.s.	n.s.
Anaeroplasma	0.50	0.70	0.70	n.s.	n.s.	n.s.
Desulfovibrio	0.20	0.20	0.30	n.s.	0.07	n.s.
Coprobacillus	0.30	0.30	0.20	n.s.	n.s.	n.s.
Dehalobacterium	0.10	0.10	0.10	n.s.	n.s.	n.s.
Roseburia	0.00	0.10	0.20	n.s.	n.s.	n.s.
Bifidobacterium	0.20	0.10	0.00	n.s.	0.05	n.s.

n.s.: not significant.

Table 14. **Mean relative abundance (%) at genus level in cecal content of 41-d-old broilers fed soybean-based diets (CON) or diets with different dosages of microalgae meal (3 or 6%) during finisher (F) or grower and finisher (GF) phases.**

GENUS	Experimental groups					*P*-value t-test									
	CON	F3	F6	GF3	GF6	CON vs F3	CON vs F6	CON vs GF3	CON vs GF6	F3 vs F6	F3 vs GF3	F3 vs GF6	F6 vs GF3	F6 vs GF6	GF3 vs GF6
Faecalibacterium	15.5	12.2	12.3	12.8	13.8	n.s.	0.09	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Oscillospira	6.20	6.90	7.30	8.30	7.70	n.s.	n.s.	**0.04**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Ruminococcus	5.10	4.40	4.10	4.80	5.00	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Bacillus	1.00	0.90	1.20	1.20	2.60	n.s.	n.s.	n.s.	**0.02**	n.s.	n.s.	**0.02**	n.s.	0.06	**0.04**
Lactobacillus	1.10	1.40	1.30	2.20	1.60	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Coprococcus	1.10	1.20	1.20	1.10	1.30	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Clostridium	0.50	0.50	0.80	0.40	0.90	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Dorea	0.60	0.60	0.50	0.60	0.80	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.05	n.s.
Blautia	0.60	0.70	0.60	0.50	0.40	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Anaeroplasma	0.40	0.30	0.20	0.50	0.60	n.s.	0.10	n.s.	n.s.	n.s.	n.s.	0.05	0.06	**0.02**	n.s.
Streptococcus	1.90	0.30	0.30	0.80	0.50	**<0.01**	**<0.01**	**0.03**	**0.01**	n.s.	0.06	n.s.	**0.02**	n.s.	n.s.
Desulfovibrio	0.30	0.40	0.40	0.30	0.30	n.s.	**<0.01**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Coprobacillus	0.40	0.20	0.20	0.30	0.30	**0.05**	**<0.01**	n.s.	n.s.	n.s.	n.s.	n.s.	**0.03**	**0.03**	n.s.
Dehalobacterium	0.30	0.40	0.40	0.40	0.20	n.s.	n.s.	n.s.	**<0.01**	n.s.	n.s.	**<0.001**	n.s.	**<0.001**	**<0.01**
Roseburia	0.10	0.10	0.10	0.60	0.10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Turicibacter	0.10	0.10	0.10	0.10	0.30	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Bifidobacterium	0.00	0.00	0.00	0.00	0.10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

n.s.: not significant.

4.3 Trial #3

Aim

The goal of the trial #3 was to ascertain whether the dietary inclusion of some exogenous enzymes, namely muramidase (i.e. lysozyme) and protease, during grower and finisher phases could provide benefits to the growth performance of broiler chickens fed on diets with 6% microalgae meal (MM; *Arthrospira spp*. - provided by VAXA).

Materials and Methods

A total of 1,000 male d-old Ross 308 chicks were divided into 5 experimental groups each composed of 8 replicates with 25 birds each. For the starter phase, all groups received the same commercial corn-wheat-soybean basal diet. The CON group received a commercial diet with soybean as main protein source in all feeding phases, while M group was fed the CON diet with 6% MM in substitution for soybean during the grower (16-29 d) and finisher phase (30-42 d). Besides, M+Mu and M+P groups were subjected to the same dietary treatment of M group, but with the grower and finisher diets supplemented with muramidase (0.09%) and protease (0.1%), respectively. Finally, the M+Mu+P received the grower and finisher feeds with both the enzymes (0.09% muramidase and 0.1% protease). The feed was provided in mash form and for ad libitum consumption. All diets were isoenergetic and with a similar amino acid profile, which was optimized maintaining the same ratio of total essential amino acids to total lysine. Body weight (BW) was determined on a pen basis at placement, at the end of each feeding phase, and at slaughter. Similarly, feed intake (FI) was assessed on a pen basis at each diet switch and at the end of the trial (15, 29, and 42 d). The number and weight of dead birds were recorded daily and used to calculate the mortality rate and to correct performance data such as daily weight gain (DWG), daily feed intake (DFI) and feed conversion ratio (FCR). At 41 d, all birds were processed in a commercial slaughterhouse and slaughter yields, such as carcass, breast, legs and wings were assessed on all birds. Similarly, the incidence and severity of footpad dermatitis (FPD) were evaluated on all birds through a 3-point scale: 0 – no lesion, 1 – mild lesions, 2 – severe lesions (Ekstrand et al., 1998). Data were analyzed by means of one-way ANOVA and Tukey post-hoc test, while contrasts were applied where appropriate to further explore the data.

Results

As expected, no significant difference among groups was observed up to 15 d (**Table 15**). At 29 d, CON group showed the highest BW (1,573 vs. 1,373 vs. 1,398 vs. 1,444 vs. 1,393 g, respectively for CON, M, M+Mu, M+P, M+Mu+P; $P<0.001$) and the lowest FCR (1.403 vs. 1.586 vs. 1.575 vs. 1.547 vs. 1.578, respectively for CON, M, M+Mu, M+P, M+Mu+P; $P<0.001$). Among the tested enzymes, the inclusion of protease improved BW and DWG compared to M group (1,373 vs. 1,444 g and 65.8 vs. 70.2 g/bird/d, respectively for M and M+P; $P<0.05$) and tended to improve FCR (1.586 vs. 1.547, respectively for M and M+P; $P=0.10$). BW at slaughter was higher in CON compared to other experimental groups (2,859 vs. 2,586 vs. 2,605 vs. 2,649 vs. 2,630 g, respectively for CON, M, M+Mu, M+P, M+Mu+P; $P<0.001$), whereas DFI from 30 to 42 d (finisher phase) tended to be higher in M+Mu+P compared to M group (168.0 vs. 173.3 g/bird/d, respectively; $P=0.07$). In the overall trial period (0-42 d), the administration of 6% MM significantly impaired BW, DWG and FCR and the use of the tested enzymes did not provide significant benefits (2,859 vs. 2,586 vs. 2,605 vs. 2,649 vs. 2,630 g, 67.1 vs. 60.6 vs. 61.1 vs. 62.1 vs. 61.6 g/bird/d, and 1.554 vs. 1.654 vs. 1.675 vs. 1.644 vs. 1.667, respectively for CON, M, M+Mu, M+P, M+Mu+P; $P<0.001$), although some slight positive effects could be observed for protease. Slaughter yields were substantially similar among the experimental groups and in line to those expected for the broiler chicken genotype used in this trial. Finally, the incidence and the severity of footpad dermatitis were not significantly modified by the dietary treatments (**Figure 2**; P=0.13).

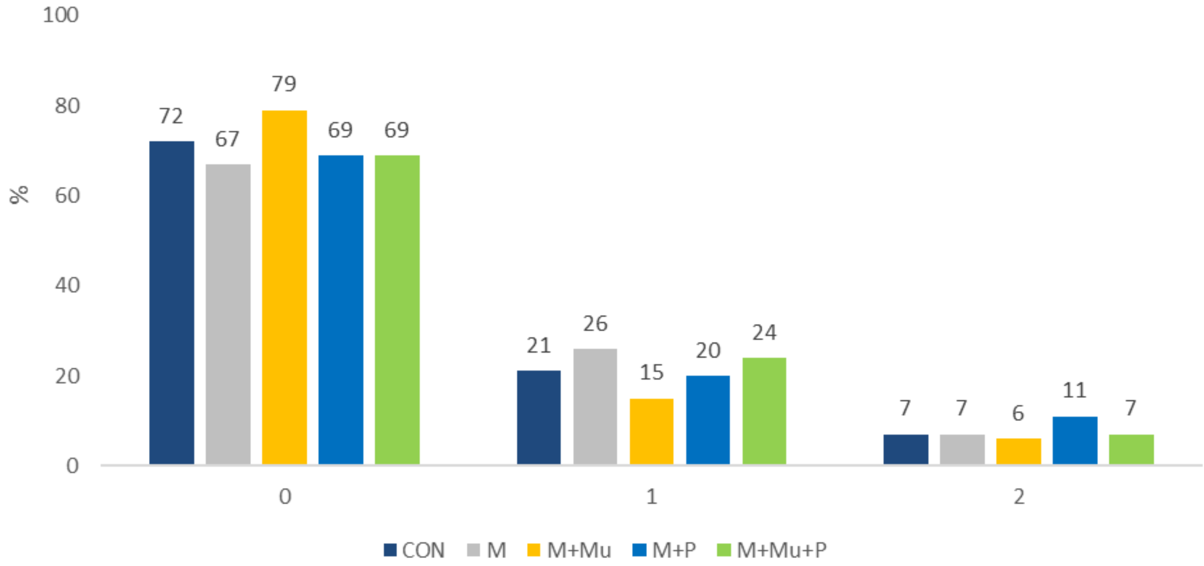
Table 15. Growth performance of broiler chickens fed a conventional soybean-based diet (CON) or diets containing 6% microalgae supplemented or not with exogenous enzymes during grower and finisher phases.

	Experimental groups					ANOVA		Contrasts			
Parameter	**CON**	**M**	**M+Mu**	**M+P**	**M+Mu+P**	**SE**	***P*-value**	**M vs. ENZ**	**M vs. M+Mu**	**M vs. M+P**	**M vs. M+Mu+P**
n	*8*	*7*	*8*	*8*	*8*						
			Starter (0-15 d)								
Chick BW (g)	37.70	37.90	38.10	38.00	38.00	0.50	0.51				
BW (g/bird)	441.30	452.70	446.80	459.20	443.80	17.4	0.23				
DWG (g/bird/d)*	27.00	27.60	27.20	28.00	27.00	1.14	0.28				
DFI (g/bird/d)*	37.20	38.00	37.80	38.50	37.80	1.32	0.34				
FI (kg/bird)*	0.558	0.570	0.567	0.577	0.57	0.01	0.34				
FCR*	1.377	1.379	1.391	1.373	1.401	0.05	0.88				
Mortality (%)	0.93	0.53	0.46	0.46	0.46	0.08	0.96				
			Grower (16-29 d)								
BW (g/bird)	1,573 A	1,373 B	1,398 B	1,444 B	1,393 B	54.4	<0.001	n.s.	n.s.	**0.03**	n.s.
DWG (g/bird/d)*	80.7 A	65.8 B	67.8 B	70.2 B	67.7 B	3.20	<0.001	0.06	n.s.	**0.02**	n.s.
DFI (g/bird/d)*	113.1 A	104.1 B	106.8 B	108.5 AB	106.8 B	3.93	<0.01	n.s.	n.s.	0.06	n.s.
FI (kg/bird)*	1.584 A	1.458 B	1.495 B	1.519 AB	1.495 B	0.06	<0.01	n.s.	n.s.	0.06	n.s.
FCR*	1.403 B	1.586 A	1.575 A	1.547 A	1.578 A	0.04	<0.001	n.s.	n.s.	0.10	n.s.
Mortality (%)	0.96	0.53	0.46	0.94	1.39	0.09	0.84	n.s.	n.s.	n.s.	n.s.
			Finisher (30-42 d)								
BW (g/bird)	2,859 A	2,586 B	2,605 B	2,649 B	2,630 B	82.0	<0.001	n.s.	n.s.	n.s.	n.s.

DWG (g/bird/d)*	98.5 a	92.8 ab	91.8 B	92.7 ab	94.8 ab	4.02	0.02	n.s.	n.s.	n.s.	n.s.
DFI (g/bird/d)*	172.3	168	170.7	169.3	173.3	5.39	0.34	n.s.	n.s.	n.s.	0.07
FI (kg/bird)*	2.240	2.184	2.219	2.201	2.253	0.07	0.34	n.s.	n.s.	n.s.	0.07
FCR*	1.751	1.812	1.862	1.827	1.831	0.08	0.12	n.s.	n.s.	n.s.	n.s.
Mortality (%)	0.48	1.14	1.44	0.00	0.48	0.07	0.28	n.s.	n.s.	n.s.	n.s.
			Overall period (0-42 d)								
BW (g/bird)	2,859 A	2,586 B	2,605 B	2,649 B	2,630 B	82	<0.001	n.s.	n.s.	n.s.	n.s.
DWG (g/bird/d)*	67.1 A	60.6 B	61.1 B	62.1 B	61.6 B	1.95	<0.001	n.s.	n.s.	n.s.	n.s.
DFI (g/bird/d)*	103	99.1	100.7	101.2	101.4	2.94	0.25	n.s.	n.s.	n.s.	n.s.
FI (kg/bird)*	4.381	4.211	4.282	4.297	4.315	0.12	0.21	n.s.	n.s.	n.s.	n.s.
FCR*	1.554 B	1.654 A	1.675 A	1.644 A	1.667 A	0.04	<0.001	n.s.	n.s.	n.s.	n.s.
Mortality (%)	2.31	2.12	2.31	1.39	2.31	0.11	0.96	n.s.	n.s.	n.s.	n.s.

CON: commercial diet with soybean as main protein source in all feeding phases, M: CON diet with 6% microalgae meal in substitution for soybean during grower (16-29 d) and finisher phase (30-42 d); M+Mu: M diet + muramidase (0.09%); M+P: M diet + protease (0.1%). M+Mu+P: M diet + muramidase (0.09%) + protease (0.1%); ENZ: all groups receiving the enzyme supplementation.
*: Corrected for mortality.
A,B : P<0.01; a,b: P<0.05.

<u>Figure 2</u>. Incidence and severity of footpad dermatitis in 42-d-old broiler chickens fed a conventional soybean-based diet (CON) or diets containing 6% microalgae supplemented or not with exogenous enzymes during grower and finisher phases.



CON: commercial diet with soybean as main protein source in all feeding phases, M: CON diet with 6% microalgae meal in substitution for soybean during grower (16-29 d) and finisher phase (30-42 d); M+Mu: M diet + muramidase (0.09%); M+P: M diet + protease (0.1%). M+Mu+P: M diet + muramidase (0.09%) + protease (0.1%).
n: CON: 192, M: 178; M+Mu: 194; M+P: 195; M+Mu+P: 196.
X^2 *P*-value = n.s.

4. Use of insect meal as alternative to soybean in broiler chicken diets

4.1 Trial #1

Aim

The aim of this trial was to investigate the digestibility of dry matter (dDM), crude protein (dCP) and total lipids (dEE), as well as the apparent metabolizable energy (AME, Mj/kg DM) and AME corrected for nitrogen (AMEn; Mj/kg DM), of the insect meal (*Hermetia illucens*) provided by MUTATEC when included in broiler chicken diets.

Materials and Methods

One-day-old male broiler chickens (Ross 308) were raised in a floor pen until day 20. Chicks were fed ad libitum a commercial starter diet from 1 to 10 d (225 g/kg of crude protein (CP); 13.10 MJ/kg of AME) and a grower diet from 11 to 26 d (203 g/kg of CP; 12.65 MJ/kg AME). On day 21, a total of 28 birds were chosen on the basis of the average live weight (body weight, LW; 490.10 ± 72.89 g) and randomly distributed in metabolic cages. Seven cages were assigned to each of the two dietary treatments (2 birds per cage). A basal diet was formulated (corn-soybean meal) and the experimental diet was obtained by substituting 250 g/kg (w/w) of the basal diet with the insect meal. In order to calculate nutrient digestibility, an indigestible marker (titanium oxide, TiO_2, 5 g/kg) was included in all experimental diets. The adaptation period to the experimental diets was performed from 26 to 31 d. From 32 to 35 d, daily feed consumption data and excreta were collected from each cage. The total amount of fresh excreta per cage were weighed daily and frozen at −20°C. The excreta collected over the 4 d were pooled per cage (using the same % for each replicate, in order to have a final sample of around 500 g on fresh matter basis) for further analysis. On day 35, all birds were euthanized by intravenous injection of sodium pentobarbital and the content of the lower half of the ileum (from Meckel's diverticulum to a point 40 mm proximal to the ileo-caecal junction) was collected and pooled for each cage. Both the pooled excreta and the ileal contents were lyophilized, grounded to pass through a 0.5 mm sieve, and stored at −20°C in airtight containers until laboratory analyses. The AME values for insect meal were evaluated on the basis of the total collection method, while the AMEn values were determined by correction of AME for zero nitrogen (N) retention. The apparent total tract digestibility coefficient (ATTDC) of dietary nutrient, and the apparent ileal digestibility coefficient (AIDC) of amino acids were also determined. The AME values of the insect meal were calculated using the following formula with appropriate corrections made according to the differences in DM content:

$$AME\ diet\ (MJ/kg) = [(feed\ intake \times gross\ energy\ diet) - (excreta\ output \times gross\ energy\ excreta)]\ /\ feed\ intake$$

$$AME\ insect\ meal\ (MJ/kg) = [AME\ of\ insect\ larvae\ meal\ diet - (AME\ basal\ diet \times 0.75)]\ /\ 0.25$$

The correction for zero nitrogen (N) retention was calculated using a factor of 36.54 KJ per gram N retained in the body in order to estimate the N-corrected apparent metabolisable energy (AMEn). N-retention was calculated as follows:

$$N\ retention = [(feed\ intake \times N\ diet) - (excreta\ output \times N\ excreta)]\ /\ feed\ intake\ (kg)$$

The ATTDC of the dietary nutrient was calculated as follows:

$$ATTDC\ X\ diet = [(total\ X\ ingested - total\ X\ excreted) / total\ X\ ingested]$$

$$ATTDC\ X\ insect\ larvae\ meal = [ATTDC\ X\ of\ insect\ larvae\ meal\ diet - (ATTDC\ X\ of\ basal\ diet \times 0.75)] / 0.25.$$

where X represents DM, CP, or EE.

Results

The digestibility coefficients for DM, CP, and EE were 62, 80 and 98%, respectively. The values of AME and AMEn were 13.14 and 11.76 Mj/kg DM. Overall, these values are in line with those generally reported for this type of meal.

4.2 Trial #2

Aim

The objective of trial #2 was to assess the effects of the partial substitution of soybean meal with insect meal (IM; *Hermetia illucens*; provided by MUTATEC) in association with muramidase (lysozyme) supplementation on growth performance, meat quality, incidence of footpad dermatitis (FPD), plasma and cecal metabolome and microbiota of broilers.

Materials and Methods

A total of 1,000 d-old male Ross 308 chicks was divided into 6 experimental groups each composed of 7 replicate pens with 25 birds. For the starter phase, all groups received the same commercial corn-wheat-soybean basal diet. Then, for the grower and finisher phase, the experimental diets were as follows: CON = commercial basal diet formulated according to the current nutritional recommendations for broiler chickens (corn-wheat-soybean based); CON + E = CON diet + muramidase supplementation (0.1%); 9%IM = CON diet with 9% of insect meal in grower and finisher phases (in partial substitution for soybean meal); 18%IM = CON diet with 18% of insect meal in grower and finisher phases (in partial substitution for soybean meal); 9%IM + E = CON diet with 9% of insect meal in grower and finisher phases (in partial substitution for soybean meal) + muramidase supplementation (0.1%); 18%IM + E = CON diet with 18% of insect meal in grower and finisher phases (in partial substitution for soybean meal) + muramidase supplementation (0.1%). All diets were isoenergetic and with a similar amino acid profile, which was optimized maintaining the same ratio of total essential amino acids to total lysine. Body weight (BW) was determined on a pen basis at placement, at the end of each feeding phase, and at slaughter. Similarly, feed intake (FI) was assessed on a pen basis at each diet switch and at slaughter (14, 28, 44 d). The number and weight of dead birds were recorded daily and used to calculate the mortality rate and to correct performance data such as daily weight gain (DWG), daily feed intake (DFI) and feed conversion ratio (FCR). At 44 d, all birds were individually weighed and processed in a commercial slaughterhouse. Slaughter yields, such as carcass, breast, leg and wing yields were assessed on all birds. Similarly, the incidence and severity of FPD were evaluated on all birds through a 3-point scale: 0 – no lesion, 1 – mild lesions, 2 – severe lesions (Ekstrand et al, 1998). Technological traits of breast meat, including pHu, color profile, water holding capacity and tenderness, were assessed on 15 breasts per experimental group, while breast meat proximate composition was determined on the same 15 breasts per group on the groups CON, 9%IM and 18%IM (AOAC, 1990). Plasma and cecal content samples were collected at 21 and 41 d from 14 birds/group and then subjected to microbiota (16S Amplicon Sequencing) and metabolomic (^{1}H-Nuclear Magnetic Resonance) analysis. Data were analyzed through a two-way ANOVA considering as the main effects the dietary inclusion level of insect meal (0, 9 and 18%) and muramidase (0 and 0.1%) as well as their interaction. Tukey post-hoc test was used to separate the means.

Results

As expected, no significant difference among groups was observed at the end of the starter phase (**Table 16**). As for the grower phase (15-28 d; **Table 17**), no significant interaction between insect meal and muramidase was observed. However, BW, DWG and DFI were reduced as the dietary inclusion of insect meal increased (1,554 vs. 1,451 vs. 1,314 g; 80.1 vs. 73.1 vs. 62.9 g/bird/d and 120.0 vs. 115.5 vs. 109.2 g/bird/d, respectively for 0, 9 and 18%; P<0.001), whereas FCR increased (1.498 vs. 1.583 vs. 1.741, respectively for 0, 9 and 18%; P<0.001). The dietary administration of muramidase had no relevant effects on growth performance parameters. At slaughtering, BW was significantly reduced by increasing dosages of IM (3,207 vs. 3,004 vs. 2,492 g, respectively for 0, 9 and 18%; P<0.001; **Table 18**). A significant interaction between insect meal and muramidase administration was observed for DWG and FCR during the finisher phase (**Table 18)**. The highest value of DWG was obtained by CON and CON+E groups, followed by 9%IM, 9%IM+E and 18%IM+E, and then by 18IM (104.4 vs. 104.4 vs. 98.8 vs. 69.2 vs. 97.4 vs. 78.8 g/bird/d, respectively for CON, CON+E, 9%IM, 18%IM, 9%IM+E and 18%IM+E). The highest FCR was found in 18%IM, while the lowest in CON (2.365 vs. 1.781, respectively; P<0.001). The supplementation of muramidase significantly improved FCR in groups fed diets with 18% of insect meal (2.365 vs. 2.065, respectively for 18%IM and 18%IM+E; P<0.001) but not in those with 9% or with soybean as the main protein source (9%IM and CON, respectively). No significant interaction was detected for DFI, which was however affected by IM administration (188.8 vs. 185.0 vs. 162.0 g/bird/d, respectively for 0, 9 and 18%; P<0.001). In the overall trial period (**Table 19**), IM inclusion reduced DWG (70.8 vs. 66.4 vs. 54.5 g/bird/d, respectively for 0, 9%, 18%, respectively; P<0.001), while DFI and FCR were lower in 18% compared to both 0 and 9%, which in turn showed comparable values (115.4 vs. 112.4 vs. 102.3 g/bird/d, and 1.630 vs. 1.695 vs. 1.884, respectively for 0, 9, and 18%; P<0.001). A significant interaction between the tested factors was observed on FCR. In particular, CON, CON+E, 9%IM, and 9%IM+E showed comparable FCR values (1.610 vs. 1.650 vs. 1.716 vs. 1.673, respectively; P<0.01), while muramidase supplementation improved FCR in birds fed diets with 18% insect meal (1.939 vs. 1.807, respectively for 18IM and 18IM+E). Mortality was not affected by the dietary treatment.

Table 16. Productive performance of broiler chickens from 0 to 14 d.

	CON	CON +E	9%IM	18%IM	9%IM+E	18%IM+E	SEM	*P*-value
n	*7*	*7*	*6*	*7*	*6*	*5*		
Chick BW (g)	44.3	43.8	44.1	43.9	44.0	44.0	0.87	n.s.
BW (g)	438.0	429.0	434.7	433.9	430.6	431.7	17.8	n.s.
DWG (g/bird/d)*	27.9	27.4	27.9	27.7	27.6	27.5	1.29	n.s.
DFI (g/bird/d)*	36.1	36.0	36.3	35.8	35.3	35.9	1.25	n.s.
FI (kg/bird)*	0.505	0.504	0.508	0.501	0.495	0.502	0.01	n.s.
FCR*	1.293	1.313	1.303	1.294	1.280	1.304	0.04	n.s.
Mortality (%)	1.19	0.60	0.69	1.19	0.00	1.67	0.08	n.s.

BW: body weight; DWG: daily weight gain; DFI: daily feed intake; FI: feed intake; FCR: feed conversion ratio. * corrected for mortality. n.s.: not significant.

Table 17. Productive performance of broiler chickens fed grower and finisher diets with different dosages of insect meal (0, 9 or 18%) and supplemented or not with muramidase from 15 to 28 d.

	n	BW (g)	DWG (g/bird/d)*	DFI (g/bird/d)*	FI (kg/bird)*	FCR	Mortality (%)
Main effects							
Insect meal							
0%	14	1,554 A	80.1 A	120.0 A	1.679 A	1.498 C	0.60
9%	12	1,451 B	73.1 B	115.5 B	1.617 B	1.583 B	0.69
18%	12	1,314 C	62.9 C	109.2 C	1.529 C	1.741 A	1.40
Enzyme							
NO (0%)	20	1,434	71.4	115.2	1.613	1.625	1.04
YES (0.1%)	18	1,458	73.6	115.1	1.612	1.575	0.70
Interactions							
CON	7	1,551	79.5	118.4	1.658	1.490	1.19
CON+E	7	1,556	80.7	121.5	1.701	1.505	0.00
9%IM	6	1,435	71.9	116.8	1.635	1.625	0.00
18%IM	7	1,316	63.0	110.7	1.550	1.760	1.79
9%IM+E	6	1,466	74.2	114.3	1.600	1.540	1.39
18%IM+E	5	1,310	62.7	107.2	1.501	1.714	0.87
SEM		55.9	3.02	3.75	0.05	0.08	0.09
***P*-value**							
Insect meal		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	n.s.
Enzyme		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Insect meal*Enzyme		n.s.	n.s.	0.08	0.08	n.s.	n.s.

BW: body weight; DWG: daily weight gain; DFI: daily feed intake; FI: feed intake; FCR: feed conversion ratio.
CON: commercial diet with soybean as main protein source in all feeding phases, CON+E: CON diet supplemented with muramidase (0.1%); 9%IM: CON diet with 9% insect meal; 18%IM: diet with 18% insect meal; 9%IM+E: 9%IM diet + muramidase (0.1%); 18%IM+E: 18%IM diet + muramidase (0.1%). * corrected for mortality; A, B: P<0.01.

Table 18. **Productive performance of broiler chickens fed grower and finisher diets with different dosages of insect meal (0, 9 or 18%) and supplemented or not with muramidase from 29 to 44 d.**

	n	BW (g)	DWG (g/bird/d)*	DFI (g/bird/d)*	FI (kg/bird)*	FCR	Mortality (%)
Main effects							
Insect meal							
0%	14	3,207 A	104.4 A	188.8 A	3.022 A	1.811 B	0.00
9%	12	3,004 B	98.1 B	185.0 A	2.960 A	1.887 B	1.95
18%	12	2,492 C	73.2 C	162.0 B	2.592 B	2.240 A	1.97
Enzyme							
NO (0%)	20	2,877	90.4	177.8	2.845	2.019	0.95
YES (0.1%)	18	2,963	95.0	180.6	2.890	1.917	1.55
Interactions							
CON	7	3,205	104.4 A	185.7	2.972	1.781 C	0.00
CON+E	7	3,210	104.4 A	192.0	3.072	1.842 BC	0.00
9%IM	6	3,001	98.8 B	186.9	2.990	1.893 BC	0.76
18%IM	7	2,441	69.2 C	162.1	2.594	2.365 A	2.08
9%IM+E	6	3,007	97.4 B	183.2	2.931	1.881 BC	3.14
18%IM+E	5	2,564	78.8 B	161.7	2.588	2.065 B	1.82
SEM		101.6	4.80	7.20	0.11	0.14	0.10
***P*-value**							
Insect meal		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	n.s.
Enzyme		n.s.	0.09	n.s.	n.s.	0.08	n.s.
Insect meal*Enzyme		n.s.	**<0.01**	n.s.	n.s.	**<0.01**	n.s.

BW: body weight; DWG: daily weight gain; DFI: daily feed intake; FI: feed intake; FCR: feed conversion ratio.
CON: commercial diet with soybean as main protein source in all feeding phases, CON+E: CON diet supplemented with muramidase (0.1%); 9%IM: CON diet with 9% insect meal; 18%IM: diet with 18% insect meal; 9%IM+E: 9%IM diet + muramidase (0.1%); 18%IM+E: 18%IM diet + muramidase (0.1%). * corrected for mortality; A, B: P<0.01.

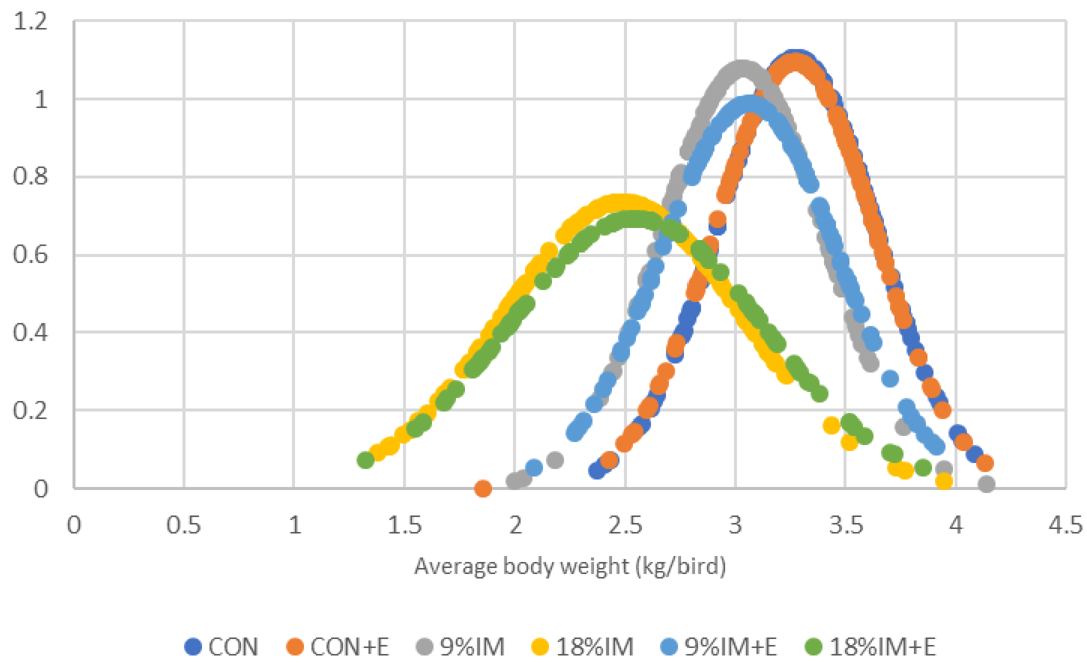
<u>Table 19</u>. Productive performance of broiler chickens fed grower and finisher diets with different dosages of insect meal (0, 9 or 18%) and supplemented or not with muramidase from 0 to 44 d.

	n	BW (g)	DWG (g/bird/d)*	DFI (g/bird/d)*	FI (kg/bird)*	FCR	Mortality (%)
Main effects							
Insect meal							
0%	14	3,207 A	70.8 A	115.4 A	5.206 A	1.630 B	1.49
9%	12	3,004 B	66.4 B	112.4 A	5.079 A	1.695 B	2.78
18%	12	2,492 C	54.5 C	102.3 B	4.622 B	1.884 A	4.51
Enzyme							
NO (0%)	20	2,877	63.2	109.9	4.963	1.757	2.92
YES (0.1%)	18	2,962	65.4	110.8	5.002	1.702	2.78
Interactions							
CON	7	3,205	70.6	113.6	5.135	1.610 C	2.38
CON+E	7	3,210	71.0	117.1	5.277	1.650 C	0.60
9%IM	6	3,001	66.4	113.8	5.133	1.716 BC	1.39
18%IM	7	2,441	53.2	102.8	4.645	1.939 A	4.76
9%IM+E	6	3,007	66.3	111.0	5.025	1.673 C	4.17
18%IM+E	5	2,564	56.3	101.7	4.591	1.807 B	4.17
SEM		101.6	2.33	3.70	0.15	0.06	0.13
***P*-value**							
Insect meal		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	n.s.
Enzyme		n.s.	n.s.	n.s.	n.s.	**0.04**	n.s.
Insect meal*Enzyme		n.s.	n.s.	0.08	0.09	**<0.01**	n.s.

BW: body weight; DWG: daily weight gain; DFI: daily feed intake; FI: feed intake; FCR: feed conversion ratio.
CON: commercial diet with soybean as main protein source in all feeding phases, CON+E: CON diet supplemented with muramidase (0.1%); 9%IM: CON diet with 9% insect meal; 18%IM: diet with 18% insect meal; 9%IM+E: 9%IM diet + muramidase (0.1%); 18%IM+E: 18%IM diet + muramidase (0.1%). * corrected for mortality; A, B: P<0.01.

Regarding the coefficient of variation (CV) of individual BW, CON group showed similar values compared to CON+E and 9%IM (11.0 vs. 11.1 vs. 12.2%, respectively; **Figure 3**). When compared to all other groups, CV of individual BW distribution was higher for the group receiving 18% of IM, with or without muramidase supplementation (21.8 and 22.6%; P<0.01). Muramidase supplementation did not significantly improve BW uniformity regardless of the IM inclusion level.

Figure 3. Individual BW distribution of 44-d-old broiler chickens fed grower and finisher diets with different dosages of insect meal (0, 9 or 18%) and supplemented or not with muramidase.

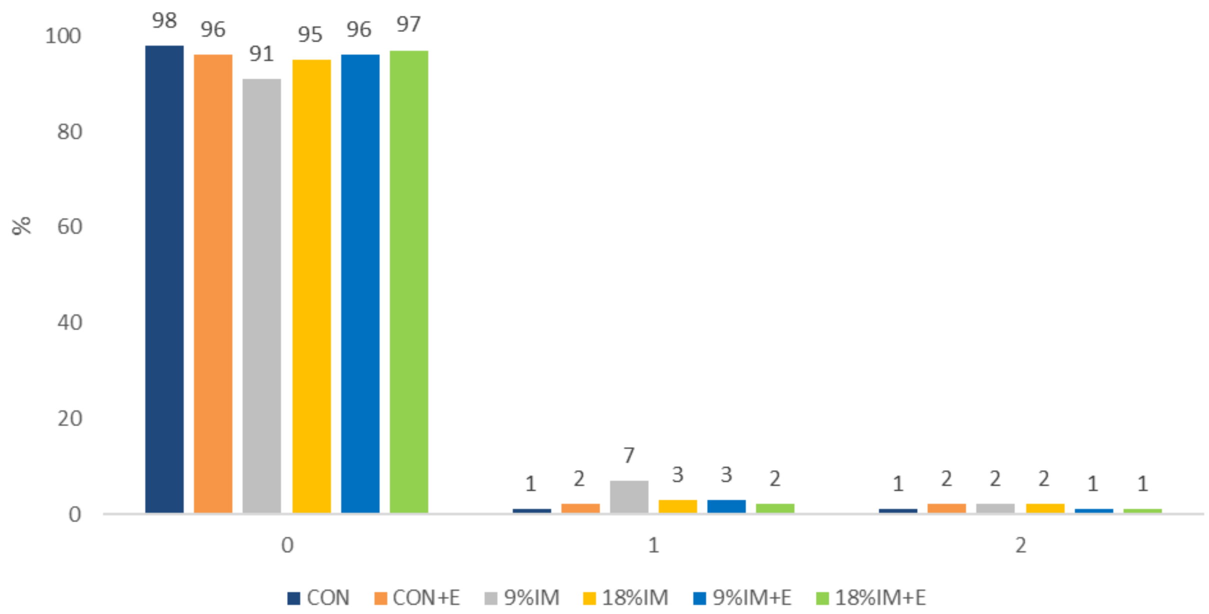


	CV (%)	*P*-value					
		***vs.* CON**	***vs.* CON+E**	***vs.* 9%IM**	***vs.* 18%IM**	***vs.* 9%IM+E**	***vs.* 18%IM+E**
CON	11.0	1.00	n.s.	n.s.	<0.001	0.05	<0.001
CON+E	11.1	-	1.00	n.s.	<0.001	0.06	<0.001
9%IM	12.2	-	-	1.00	<0.001	n.s.	<0.001
18%IM	21.8	-	-	-	1.00	<0.001	n.s.
9%IM+E	13.2	-	-	-	-	1.00	<0.001
18%IM+E	22.6	-	-	-	-	-	1.00

CON: commercial diet with soybean as main protein source in all feeding phases, CON+E: CON diet supplemented with muramidase (0.1%); 9%IM: CON diet with 9% insect meal; 18%IM: diet with 18% insect meal; 9%IM+E: 9%IM diet + muramidase (0.1%); 18%IM+E: 18%IM diet + muramidase (0.1%). CV: coefficient of variation. n.s.: not significant.

Slaughter yields were substantially similar among the experimental groups and in line with those expected for the broiler chicken genotype used in this trial. Finally, the incidence and the severity of FPD were not significantly affected by the dietary treatments (**Figure 4**).

Figure 4. Incidence and severity of footpad dermatitis in 44-d-old broiler chickens fed grower and finisher diets with different dosages of insect meal (0, 9 or 18%) and supplemented or not with muramidase.



CON: commercial diet with soybean as main protein source in all feeding phases, CON+E: CON diet supplemented with muramidase (0.1%); 9%IM: CON diet with 9% insect meal; 18%IM: diet with 18% insect meal; 9%IM+E: 9%IM diet + muramidase (0.1%); 18%IM+E: 18%IM diet + muramidase (0.1%).
n: CON: 135; CON+E: 137; 9%IM: 136; 18%IM: 127; 9%IM+E: 131; 18%IM+E: 126; X^2 *P*-value = n.s.

As for meat quality parameters (**Table 20**), no significant effect of the dietary treatment was observed on meat pH, redness, cooking loss, shear force, TBARS and carbonyls content. Lightness showed the highest value in 9%IM group and the lowest one in CON, while yellowness was increased by muramidase supplementation. A significant interaction was found for drip loss, with 18%IM+E group exhibiting higher drip loss compared to 18%IM and 9%IM+E. Moisture, total fat and ash were similar among experimental groups, while crude protein content was lower in 9%IM if compared to CON, with 18%IM showing intermediate value (**Table 21**).

Table 20. Technological properties of breast meat (*n* = 15 breasts/group) of 44-d-old broiler chickens fed grower and finisher diets with different dosages of insect meal (0, 9 or 18%) and supplemented or not with muramidase.

	n	pHu	Lightness L*	Redness a*	Yellowness b*	Drip loss (%)	Cooking loss (%)	Shear force (kg)	TBARS (mg MDA/kg meat)	Carbonyls (nmol/mg protein)
Main effects										
Insect meal										
0%	30	5.74	58.36	1.54	7.14	2.02	19.5	1.78	0.27	2.13
9%	30	5.65	59.10	1.37	7.41	1.82	20.1	1.86	0.61	2.29
18%	30	5.64	57.67	1.76	7.29	2.25	19.6	1.93	0.42	2.07
Enzyme										
NO (0%)	45	5.69	57.83	1.60	6.88 b	2.10	19.8	1.80	0.49	2.10
YES (0.1%)	45	5.66	58.92	1.52	7.68 a	1.96	19.6	1.92	0.38	2.24
Interactions										
CON	15	5.78	57.24 c	1.59	6.78	2.14 ab	19.1	1.56	0.24	2.27
CON+E	15	5.70	59.48 ab	1.50	7.50	1.89 ab	19.8	2.00	0.31	1.99
9%IM	15	5.64	59.78 a	1.28	7.08	2.38 ab	20.2	1.95	0.79	1.98
18%IM	15	5.64	56.46 bc	1.93	6.79	1.77 b	20.1	1.88	0.42	2.01
9%IM+E	15	5.65	58.41 abc	1.46	7.74	1.25 b	20.0	1.78	0.44	2.59
18%IM+E	15	5.64	58.87 abc	1.60	7.79	2.72 a	19.1	1.97	0.41	2.14
SEM		0.01	0.29	0.06	0.35	0.09	0.23	0.04	0.05	0.07
***P*-value**										
Insect meal		0.07	0.10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Enzyme		n.s.	n.s.	n.s.	**<0.01**	n.s.	n.s.	n.s.	n.s.	n.s.
Insect meal*Enzyme		n.s.	**<0.05**	n.s.	n.s.	**<0.05**	n.s.	n.s.	n.s.	n.s.

CON: commercial diet with soybean as main protein source in all feeding phases, CON+E: CON diet supplemented with muramidase (0.1%); 9%IM: CON diet with 9% insect meal; 18%IM: diet with 18% insect meal; 9%IM+E: 9%IM diet + muramidase (0.1%); 18%IM+E: 18%IM diet + muramidase (0.1%). A,B: P < 0.01. a,b: P<0.05. n.s.: not significant.

<u>Table 21</u>. Proximate composition of breast meat (*n* = 15 breasts/group) of 44-d-old broiler chickens fed grower and finisher diets with different dosages of insect meal (0, 9 or 18%).

	CON		9%IM		18%IM		SEM	*P*-value
n	14		15		14			
Moisture (%)	75.47		75.51		75.36		0.15	n.s.
Crude protein (%)	23.03	A	22.22	B	22.92	AB	0.13	0.01
Total fat (%)	1.76		1.79		1.87		0.06	0.74
Ash (%)	1.35		1.28		1.32		0.03	0.62

CON: commercial diet with soybean as main protein source in all feeding phases, 9%IM: CON diet with 9% insect meal; 18%IM: diet with 18% insect meal; A,B: $P < 0.01$.

Extensive variations were observed for plasma metabolomic profile at 21 d of bird age in response to the dietary administration of IM and muramidase. The list of plasma metabolites whose concentration was affected by the tested experimental factors in shown in **Table 22.** Analogously, the molecules showing variations at 44 d are reported in **Table 23.** Similarly, also the cecal metabolome, both at 21 and 44 d (**Table 24** and **Table 25**, respectively), exhibited significant changes according to the dietary treatments. Overall, the use of IM, associated or not with muramidase, determined variations in plasma and cecal metabolites that play relevant physiological roles in bird metabolism (e.g. energy and protein homeostasis) and antioxidant status, or that can be associated with fermentation processes in the digestive tract. Such results can provide information on the metabolic consequences induced by IM administration, either associated or not with muramidase.

Table 22. **Concentration (mmol/L) of plasma metabolites (identified through the [1]H-NMR analysis) showing significant differences in 21-d-old broilers fed diets with different dosages of insect meal (0, 9 or 18%) and supplemented or not with muramidase.**

Metabolite	Insect meal (IM)						Muramidase (MUR)		Interaction												SEM	*P*-value		
	0%		9%		18%		NO (0%)	YES (0.1%)	CON		CON+E		9%IM		18%IM		9%IM+E		18%IM+E			INS	MUR	IM × MUR
Hydroxyisobutyrate	4.97E-02	C	7.35E-02	B	9.43E-02	A	7.40E-02	7.05E-02	4.92E-02		5.02E-02		7.80E-02		9.47E-02		6.90E-02		9.38E-02		1.42E-02	**<0.001**	n.s.	n.s.
Betaine	1.46E+00		3.72E+00		4.79E+00		3.35E+00	3.26E+00	1.44E+00	c	1.49E+00	c	3.63E+00	b	4.99E+00	a	3.82E+00	b	4.57E+00	a	4.63E-01	**<0.001**	n.s.	**0.04**
Citramalate	1.14E-01		2.45E-01		3.93E-01		2.53E-01	2.44E-01	1.18E-01	c	1.09E-01	c	2.57E-01	b	3.84E-01	a	2.32E-01	b	4.02E-01	a	3.24E-02	**<0.001**	n.s.	0.05
N,N-Dimethylglycine	2.45E-01	C	5.72E-01	B	8.00E-01	A	5.57E-01	5.14E-01	2.43E-01		2.46E-01		5.83E-01		8.45E-01		5.61E-01		7.51E-01		1.11E-01	**<0.001**	n.s.	n.s.
Valine	3.26E-01	C	4.70E-01	B	6.68E-01	A	4.85E-01	4.87E-01	3.36E-01		3.16E-01		4.71E-01		6.48E-01		4.69E-01		6.90E-01		5.57E-02	**<0.001**	n.s.	n.s.
Beta-Alanine	1.40E-01	A	7.60E-02	B	5.73E-02	B	9.62E-02	8.71E-02	1.48E-01		1.33E-01		7.56E-02		6.54E-02		7.64E-02		4.86E-02		3.70E-02	**<0.001**	n.s.	n.s.
TMAO	5.72E-02	A	3.53E-02	B	2.75E-02	B	3.93E-02	4.10E-02	5.75E-02		5.70E-02		3.36E-02		2.68E-02		3.69E-02		2.82E-02		1.45E-02	**<0.001**	n.s.	n.s.
Isoleucine	1.53E-01	B	1.58E-01	B	1.90E-01	A	1.66E-01	1.68E-01	1.58E-01		1.48E-01		1.57E-01		1.82E-01		1.59E-01		1.98E-01		2.48E-02	**<0.001**	n.s.	n.s.
Arginine	5.48E-01	A	4.20E-01	B	3.38E-01	B	4.48E-01	4.25E-01	5.66E-01		5.29E-01		4.36E-01		3.43E-01		4.05E-01		3.33E-01		1.39E-01	**<0.001**	n.s.	n.s.
Asparagine	5.43E-01	A	4.14E-01	B	4.13E-01	B	4.78E-01	4.37E-01	5.83E-01		5.03E-01		4.50E-01		4.00E-01		3.78E-01		4.28E-01		1.02E-01	**<0.001**	0.07	0.10
Tyrosine	3.02E-01	B	3.32E-01	B	4.34E-01	A	3.57E-01	3.53E-01	3.14E-01		2.90E-01		3.13E-01		4.43E-01		3.50E-01		4.25E-01		9.42E-02	**<0.001**	n.s.	n.s.
Sarcosine	6.27E-02	B	8.42E-02	A	8.37E-02	A	7.59E-02	7.78E-02	6.00E-02		6.55E-02		8.14E-02		8.62E-02		8.71E-02		8.10E-02		1.85E-02	**<0.001**	n.s.	n.s.
Glutamine	2.53E+00	A	2.20E+00	B	2.20E+00	B	2.40E+00	2.22E+00	2.64E+00		2.42E+00		2.34E+00		2.21E+00		2.06E+00		2.19E+00		2.91E-01	**<0.001**	**<0.01**	n.s.
Proline	5.76E-01	B	6.34E-01	A	6.76E-01	A	6.46E-01	6.10E-01	6.04E-01		5.47E-01		6.40E-01		6.95E-01		6.29E-01		6.57E-01		7.76E-02	**<0.001**	**0.04**	n.s.
Histidine	1.07E-01	B	1.14E-01	B	1.45E-01	A	1.22E-01	1.22E-01	1.11E-01		1.03E-01		1.16E-01		1.39E-01		1.12E-01		1.52E-01		3.13E-02	**<0.001**	n.s.	n.s.
Lysine	1.30E-01	A	7.79E-02	B	8.16E-02	B	1.01E-01	9.24E-02	1.45E-01		1.14E-01		8.11E-02		7.56E-02		7.47E-02		8.81E-02		5.13E-02	**<0.001**	n.s.	n.s.
Glucose	2.45E+01	A	2.28E+01	AB	2.07E+01	B	2.30E+01	2.24E+01	2.46E+01		2.43E+01		2.32E+01		2.10E+01		2.25E+01		2.03E+01		3.38E+00	**<0.001**	n.s.	n.s.
Dimethylamine	2.23E-02	A	1.30E-02	AB	5.30E-03	B	1.48E-02	1.24E-02	2.82E-02		1.63E-02		1.06E-02		5.68E-03		1.54E-02		4.89E-03		1.56E-02	**<0.01**	n.s.	n.s.
Dimethyl-Sulfone	2.59E-01	A	1.71E-01	AB	1.04E-01	B	1.75E-01	1.83E-01	2.78E-01		2.40E-01		1.45E-01		1.02E-01		1.96E-01		1.07E-01		1.46E-01	**<0.001**	n.s.	n.s.
Glycine	1.29E+00	B	1.36E+00	B	1.51E+00	A	1.40E+00	1.37E+00	1.32E+00		1.27E+00		1.34E+00		1.54E+00		1.38E+00		1.47E+00		2.03E-01	**<0.001**	n.s.	n.s.
Leucine	2.94E-01	B	2.94E-01	B	3.43E-01	A	3.19E-01	3.01E-01	3.12E-01		2.77E-01		2.94E-01		3.52E-01		2.94E-01		3.33E-01		5.27E-02	**<0.001**	n.s.	n.s.
Ascorbate	1.07E-01	A	9.57E-02	A	7.15E-02	B	8.60E-02	9.71E-02	9.54E-02		1.18E-01		9.73E-02		6.52E-02		9.40E-02		7.83E-02		3.48E-02	**<0.001**	n.s.	n.s.
Fumarate	2.35E-02	A	2.23E-02	A	1.79E-02	B	2.01E-02	2.24E-02	2.16E-02		2.55E-02		2.13E-02		1.75E-02		2.33E-02		1.83E-02		6.17E-03	**<0.001**	n.s.	n.s.
Citrate	4.57E-01	A	4.21E-01	AB	3.70E-01	B	4.44E-01	3.88E-01	4.87E-01		4.27E-01		4.40E-01		4.06E-01		4.01E-01		3.31E-01		9.21E-02	**<0.001**	**<0.01**	n.s.
Threonine	2.23E+00	A	2.03E+00	AB	1.86E+00	B	1.95E+00	2.14E+00	2.21E+00		2.26E+00		1.87E+00		1.76E+00		2.19E+00		1.97E+00		4.04E-01	**<0.001**	**0.03**	n.s.
Succinate	1.67E-01	A	1.41E-01	AB	1.15E-01	B	1.28E-01	1.55E-01	1.57E-01		1.77E-01		1.23E-01		1.03E-01		1.59E-01		1.26E-01		5.68E-02	**<0.001**	**0.04**	n.s.
Glycerol	4.62E-01	A	3.64E-01	AB	2.85E-01	B	3.46E-01	3.97E-01	3.75E-01		5.48E-01		3.44E-01		3.19E-01		3.84E-01		2.48E-01		1.93E-01	**<0.001**	n.s.	0.07

Phenylalanine	2.58E-01	B	2.32E-01	B	2.34E-01	B	2.41E-01	2.41E-01	2.54E-01	2.62E-01	2.31E-01	2.38E-01	2.32E-01	2.29E-01	3.27E-02	**<0.001**	n.s.	n.s.
2-Aminobutyrate	4.58E-02	B	4.48E-02	B	5.63E-02	A	4.78E-02	5.00E-02	4.77E-02	4.38E-02	4.58E-02	4.98E-02	4.38E-02	6.32E-02	1.44E-02	**<0.001**	n.s.	0.06
myo-Inositol	9.55E-01	a	9.59E-01	a	8.10E-01	b	8.79E-01	9.40E-01	9.25E-01	9.85E-01	9.06E-01	8.06E-01	1.01E+00	8.14E-01	2.08E-01	**0.01**	n.s.	n.s.
2-Hydroxybutyrate	1.75E-02	b	2.33E-02	a	2.19E-02	ab	2.23E-02	1.94E-02	1.90E-02	1.61E-02	2.49E-02	2.31E-02	2.18E-02	2.06E-02	7.69E-03	**0.02**	0.094	n.s.
Alanine	2.40E+00	a	2.17E+00	b	2.27E+00	ab	2.34E+00	2.22E+00	2.52E+00	2.28E+00	2.15E+00	2.35E+00	2.19E+00	2.18E+00	3.03E-01	**0.02**	0.067	n.s.
Methanol	1.20E-01	a	1.09E-01	ab	9.60E-02	b	1.09E-01	1.07E-01	1.20E-01	1.19E-01	1.08E-01	9.96E-02	1.10E-01	9.21E-02	3.15E-02	**0.02**	n.s.	n.s.
Creatine	1.19E-01		1.03E-01		9.86E-02		9.91E-02	1.15E-01	1.18E-01	1.21E-01	9.37E-02	8.59E-02	1.12E-01	1.12E-01	3.85E-02	n.s.	0.069	n.s.
trans-4-Hydroxy-L-proline	1.78E-01		1.54E-01		1.61E-01		1.75E-01	1.54E-01	1.88E-01	1.68E-01	1.58E-01	1.79E-01	1.51E-01	1.42E-01	4.26E-02	n.s.	**0.03**	n.s.
Acetone	1.92E-02		1.91E-02		2.13E-02		2.14E-02	1.83E-02	1.91E-02	1.93E-02	2.07E-02	2.43E-02	1.76E-02	1.81E-02	4.95E-03	n.s.	**<0.01**	0.06
Acetate	2.95E-02		2.56E-02		2.94E-02		3.39E-02	2.23E-02	3.81E-02	2.10E-02	2.98E-02	3.37E-02	2.14E-02	2.48E-02	2.04E-02	n.s.	**0.01**	n.s.

CON: commercial diet with soybean as main protein source in all feeding phases, CON+E: CON diet supplemented with muramidase (0.1%); 9%IM: CON diet with 9% insect meal; 18%IM: diet with 18% insect meal; 9%IM+E: 9%IM diet + muramidase (0.1%); 18%IM+E: 18%IM diet + muramidase (0.1%).
A,B: P < 0.01. a,b: P<0.05. n.s.: not significant.

<u>Table 23</u>. Concentration (mmol/L) of plasma metabolites (identified through the [1]H-NMR analysis) showing significant differences in 44-d-old broilers fed diets with different dosages of insect meal (0, 9 or 18%) and supplemented or not with muramidase.

Metabolite	Insect meal (IM)						Muramidase (MUR)		Interaction												SEM	*P*-value		
	0%		9%		18%		NO (0%)	YES (0.1%)	CON		CON+E		9%IM		18%IM		9%IM+E		18%IM+E			IM	MUR	IM x MUR
Betaine	1.27E+00	C	3.44E+00	B	4.79E+00	A	3.16E+00	3.17E+00	1.31E+00		3.44E+00		4.74E+00		1.23E+00		3.44E+00		4.83E+00		5.65E-01	**<0.001**	n.s.	n.s.
Citramalate	8.65E-02	C	1.80E-01	B	2.97E-01	A	1.85E-01	1.90E-01	8.62E-02		1.74E-01		2.95E-01		8.67E-02		1.86E-01		2.98E-01		4.13E-02	**<0.001**	n.s.	n.s.
Histidine	1.57E-01	B	1.70E-01	B	2.86E-01	A	2.07E-01	2.02E-01	1.64E-01		1.69E-01		2.88E-01		1.50E-01		1.72E-01		2.84E-01		4.21E-02	**<0.001**	n.s.	n.s.
Valine	4.09E-01	C	5.07E-01	B	6.78E-01	A	5.43E-01	5.19E-01	4.18E-01		5.19E-01		6.94E-01		4.00E-01		4.96E-01		6.62E-01		6.82E-02	**<0.001**	n.s.	n.s.
Sarcosine	4.56E-02	C	6.77E-02	B	8.42E-02	A	6.34E-02	6.82E-02	4.57E-02		6.72E-02		7.74E-02		4.55E-02		6.82E-02		9.10E-02		1.38E-02	**<0.001**	n.s.	n.s.
N,N-Dimethylglycine	1.81E-01	B	3.45E-01	A	3.65E-01	A	2.81E-01	3.13E-01	1.82E-01		3.26E-01		3.35E-01		1.79E-01		3.63E-01		3.95E-01		7.64E-02	**<0.001**	0.066	n.s.
3-Hydroxyisobutyrate	3.72E-02	C	4.75E-02	B	6.81E-02	A	5.01E-02	5.18E-02	3.68E-02		4.41E-02		6.94E-02		3.76E-02		5.09E-02		6.68E-02		1.30E-02	**<0.001**	n.s.	n.s.
Isoleucine	1.92E-01	B	1.98E-01	B	2.48E-01	A	2.19E-01	2.06E-01	1.98E-01		2.08E-01		2.52E-01		1.86E-01		1.88E-01		2.44E-01		2.85E-02	**<0.001**	**0.04**	n.s.
Beta-Alanine	1.56E-01	A	9.31E-02	B	5.75E-02	C	9.86E-02	1.06E-01	1.43E-01		8.88E-02		6.36E-02		1.70E-01		9.75E-02		5.14E-02		5.20E-02	**<0.001**	n.s.	n.s.
Arginine	6.07E-01	A	4.59E-01	B	3.61E-01	C	4.69E-01	4.82E-01	6.13E-01		4.37E-01		3.55E-01		6.00E-01		4.80E-01		3.67E-01		1.43E-01	**<0.001**	n.s.	n.s.
TMAO	3.06E-02	A	2.13E-02	B	1.73E-02	B	2.32E-02	2.30E-02	3.18E-02		2.30E-02		1.48E-02		2.94E-02		1.96E-02		1.99E-02		8.05E-03	**<0.001**	n.s.	n.s.
Glutamine	2.51E+00	A	2.23E+00	B	2.05E+00	B	2.28E+00	2.25E+00	2.43E+00		2.32E+00		2.09E+00		2.59E+00		2.13E+00		2.01E+00		3.03E-01	**<0.001**	n.s.	n.s.
Ascorbate	1.41E-01	A	1.32E-01	A	9.34E-02	B	1.24E-01	1.20E-01	1.46E-01		1.42E-01		8.56E-02		1.37E-01		1.23E-01		1.01E-01		3.84E-02	**<0.001**	n.s.	n.s.
Serine	1.72E+00	B	1.90E+00	AB	2.09E+00	A	1.95E+00	1.85E+00	1.79E+00		1.95E+00		2.11E+00		1.64E+00		1.84E+00		2.07E+00		3.08E-01	**<0.001**	n.s.	n.s.
Formate	1.51E-01	B	1.55E-01	B	2.30E-01	A	2.05E-01	1.52E-01	1.75E-01		1.77E-01		2.64E-01		1.27E-01		1.34E-01		1.96E-01		7.64E-02	**<0.001**	**<0.01**	n.s.
Glucose	2.43E+01	A	2.22E+01	B	2.12E+01	B	2.26E+01	2.25E+01	2.43E+01		2.22E+01		2.14E+01		2.42E+01		2.22E+01		2.10E+01		2.76E+00	**<0.001**	n.s.	n.s.
myo-Inositol	1.20E+00	A	1.15E+00	A	9.73E-01	B	1.13E+00	1.09E+00	1.28E+00		1.16E+00		9.37E-01		1.12E+00		1.13E+00		1.01E+00		2.29E-01	**<0.01**	n.s.	n.s.
Proline	5.66E-01	B	5.84E-01	B	6.54E-01	A	5.97E-01	6.05E-01	5.74E-01		5.87E-01		6.29E-01		5.57E-01		5.81E-01		6.78E-01		9.22E-02	**<0.01**	n.s.	n.s.
Citrate	4.98E-01	A	4.38E-01	AB	4.04E-01	B	4.37E-01	4.56E-01	4.78E-01		4.15E-01		4.18E-01		5.19E-01		4.60E-01		3.90E-01		9.49E-02	**<0.01**	n.s.	n.s.
Threonine	1.47E+00	A	1.28E+00	B	1.24E+00	B	1.30E+00	1.37E+00	1.47E+00		1.20E+00		1.22E+00		1.47E+00		1.36E+00		1.26E+00		2.48E-01	**<0.01**	n.s.	n.s.
trans-4-Hydroxy-L-proline	1.85E-01	A	1.52E-01	B	1.59E-01	B	1.74E-01	1.56E-01	1.93E-01		1.62E-01		1.68E-01		1.77E-01		1.42E-01		1.49E-01		3.81E-02	**<0.01**	**0.03**	n.s.
Asparagine	3.76E-01	A	2.94E-01	B	2.74E-01	B	3.45E-01	2.84E-01	4.29E-01		3.20E-01		2.88E-01		3.24E-01		2.67E-01		2.61E-01		1.22E-01	**<0.01**	**0.02**	n.s.
3-Hydroxybutyrate	1.65E-01	B	2.56E-01	AB	2.93E-01	A	2.48E-01	2.28E-01	1.82E-01		2.59E-01		3.03E-01		1.48E-01		2.54E-01		2.83E-01		1.51E-01	**<0.01**	n.s.	n.s.
Leucine	3.86E-01	B	3.81E-01	B	4.26E-01	A	4.12E-01	3.83E-01	3.91E-01		4.05E-01		4.41E-01		3.81E-01		3.57E-01		4.11E-01		5.79E-02	**<0.01**	**0.02**	n.s.
Tyrosine	4.21E-01	b	4.53E-01	ab	5.07E-01	a	4.57E-01	4.65E-01	3.94E-01		4.66E-01		5.09E-01		4.49E-01		4.40E-01		5.05E-01		1.18E-01	**0.03**	n.s.	n.s.
Glycine	1.59E+00	ab	1.67E+00	a	1.52E+00	b	1.59E+00	1.59E+00	1.57E+00		1.66E+00		1.55E+00		1.62E+00		1.68E+00		1.48E+00		2.15E-01	**0.04**	n.s.	n.s.
Phenylalanine	2.69E-01		2.54E-01		2.54E-01		2.63E-01	2.56E-01	2.60E-01	AB	2.56E-01	AB	2.71E-01	A	2.77E-01	A	2.52E-01	AB	2.37E-01	B	2.98E-02	n.s.	n.s.	**<0.01**

Table 24. Concentration (mmol/L) of cecal metabolites (identified through the [1]H-NMR analysis) showing significant differences in 21-d-old broilers fed diets with different dosages of insect meal (0, 9 or 18%) and supplemented or not with muramidase.

Metabolite	Insect meal (IM)						Muramidase (MUR)				Interaction												SEM	*P*-value		
	0%		9%		18%		NO (0%)		YES (0.1%)		CON		CON+E		9%IM		18%IM		9%IM+E		18%IM+E			IM	MUR	IM × MUR
Malonate	1.61E-03	B	2.06E-03	B	3.42E-03	A	2.60E-03	a	2.13E-03	b	1.84E-03		1.38E-03		2.17E-03		3.78E-03		1.96E-03		3.07E-03		1.04E-03	**<0.001**	**0.04**	n.s.
Tyramine	4.35E-03	A	1.92E-03	B	1.99E-03	B	2.48E-03		3.02E-03		3.70E-03		4.99E-03		1.76E-03		1.98E-03		2.08E-03		2.01E-03		1.54E-03	**<0.001**	n.s.	n.s.
Alanine	1.39E-02	A	1.06E-02	B	9.38E-03	B	1.17E-02		1.09E-02		1.45E-02		1.32E-02		1.10E-02		9.69E-03		1.03E-02		9.06E-03		3.25E-03	**<0.001**	n.s.	n.s.
Glutamate	5.07E-02		4.06E-02		3.38E-02		4.28E-02		4.07E-02		4.52E-02	ab	5.62E-02	a	4.42E-02	abc	3.89E-02	bc	3.70E-02	bc	2.88E-02	c	1.40E-02	**<0.001**	n.s.	**0.01**
Creatine	3.98E-04	B	5.60E-04	B	8.28E-04	A	6.32E-04		5.58E-04		4.30E-04		3.65E-04		6.53E-04		8.14E-04		4.67E-04		8.42E-04		3.66E-04	**<0.001**	n.s.	n.s.
Dimethyl sulfone	9.32E-04	A	6.72E-04	AB	3.95E-04	B	6.26E-04		7.07E-04		8.95E-04		9.69E-04		6.13E-04		3.71E-04		7.32E-04		4.20E-04		5.08E-04	**<0.01**	n.s.	n.s.
Leucine	4.60E-03	A	3.33E-03	B	2.84E-03	B	3.78E-03		3.40E-03		5.12E-03		4.09E-03		3.17E-03		3.04E-03		3.48E-03		2.64E-03		1.73E-03	**<0.01**	n.s.	n.s.
N,N-Dimethylglycine	4.13E-04	B	5.65E-04	B	1.31E-03	A	9.13E-04		6.15E-04		4.15E-04		4.10E-04		5.65E-04		1.76E-03		5.65E-04		8.71E-04		9.36E-04	**<0.01**	n.s.	n.s.
Trimethylamine	7.63E-04	B	1.04E-03	B	1.53E-03	A	1.17E-03		1.05E-03		9.45E-04		5.81E-04		1.03E-03		1.53E-03		1.05E-03		1.53E-03		7.58E-04	**<0.01**	n.s.	n.s.
Uracil	2.51E-03	A	2.37E-03	A	1.86E-03	B	2.27E-03		2.23E-03		2.40E-03		2.62E-03		2.54E-03		1.86E-03		2.20E-03		1.87E-03		6.63E-04	**<0.01**	n.s.	n.s.
Sarcosine	2.05E-04	B	2.54E-04	A	2.65E-04	A	2.47E-04		2.36E-04		1.91E-04		2.20E-04		2.68E-04		2.84E-04		2.41E-04		2.47E-04		6.80E-05	**<0.01**	n.s.	n.s.
Butyrate	1.02E-01	A	7.67E-02	AB	6.05E-02	B	7.37E-02		8.61E-02		1.01E-01		1.04E-01		6.34E-02		5.68E-02		9.00E-02		6.42E-02		4.70E-02	**<0.01**	n.s.	n.s.
Isobutyrate	6.35E-03		5.84E-03		8.41E-03		7.09E-03		6.65E-03		5.65E-03	ab	7.06E-03	ab	7.53E-03	a	8.09E-03	a	4.15E-03	b	8.74E-03	a	3.05E-03	**<0.01**	n.s.	**<0.01**
3-Hydroxybutyrate	1.80E-03	A	1.15E-03	B	1.15E-03	b	1.24E-03		1.49E-03		1.73E-03		1.87E-03		9.11E-04		1.07E-03		1.39E-03		1.22E-03		8.54E-04	**<0.01**	n.s.	n.s.
Glycerol	3.99E-03	a	3.55E-03	ab	2.89E-03	b	3.71E-03		3.24E-03		4.24E-03		3.73E-03		3.54E-03		3.36E-03		3.56E-03		2.43E-03		1.34E-03	**0.01**	n.s.	n.s.
Valine	4.12E-03	a	3.33E-03	ab	2.93E-03	b	3.59E-03		3.33E-03		4.56E-03		3.69E-03		3.15E-03		3.08E-03		3.51E-03		2.78E-03		1.50E-03	**0.01**	n.s.	n.s.
Glucose	1.97E-02	a	1.61E-02	ab	1.17E-02	b	1.55E-02		1.62E-02		2.10E-02		1.84E-02		1.33E-02		1.23E-02		1.90E-02		1.12E-02		1.01E-02	**0.02**	n.s.	n.s.
Methanol	2.47E-03		1.75E-03		1.48E-03		1.89E-03		1.91E-03		3.13E-03	a	1.81E-03	ab	1.43E-03	b	1.10E-03	b	2.07E-03	ab	1.86E-03	ab	1.33E-03	**0.02**	n.s.	**<0.01**
Galactose	4.35E-03	b	8.57E-03	a	5.86E-03	ab	6.76E-03		5.75E-03		4.35E-03		4.36E-03		1.00E-02		5.89E-03		7.08E-03		5.82E-03		5.63E-03	**0.02**	n.s.	n.s.
Succinate	3.14E-02	a	2.18E-02	ab	1.32E-02	b	2.24E-02		2.19E-02		3.81E-02		2.47E-02		1.57E-02		1.34E-02		2.79E-02		1.29E-02		2.43E-02	**0.02**	n.s.	n.s.
2-Aminobutyrate	7.51E-04	a	3.82E-04	b	4.48E-04	ab	4.86E-04		5.68E-04		6.84E-04		8.18E-04		2.54E-04		5.20E-04		5.09E-04		3.76E-04		5.24E-04	**0.02**	n.s.	n.s.
Fucose	5.09E-04		5.54E-04		9.57E-04		6.19E-04		7.27E-04		7.46E-04	ab	2.72E-04	b	3.22E-04	b	7.89E-04	ab	7.85E-04	ab	1.12E-03	a	6.88E-04	**0.03**	n.s.	**0.03**
Propionate	6.22E-02	b	6.49E-02	ab	8.15E-02	a	6.79E-02		7.11E-02		5.99E-02		6.45E-02		7.29E-02		7.09E-02		5.68E-02		9.20E-02		3.01E-02	**0.04**	n.s.	0.08
Lysine	5.29E-03	a	4.60E-03	ab	3.51E-03	b	4.46E-03		4.47E-03		4.94E-03		5.63E-03		4.74E-03		3.71E-03		4.45E-03		3.31E-03		2.66E-03	**<0.05**	n.s.	n.s.
Taurine	3.03E-02	ab	1.21E-02	b	3.50E-02	a	2.47E-02		2.70E-02		3.49E-02		2.58E-02		1.55E-02		2.37E-02		8.76E-03		4.64E-02		1.83E-03	**<0.05**	n.s.	n.s.
Tyrosine	1.52E-03		1.19E-03		1.29E-03		1.47E-03	a	1.20E-03	b	1.76E-03		1.28E-03		1.33E-03		1.32E-03		1.05E-03		1.26E-03		5.80E-04	0.103	**0.03**	n.s.

Methylsuccinate	3.20E-03	3.53E-03	4.46E-03	4.23E-03		3.23E-03		3.20E-03	b	3.20E-03	b	3.32E-03	b	6.18E-03	a	3.74E-03	ab	2.74E-03	b	2.27E-03	n.s.	n.s.	**<0.01**
Nicotinate	2.99E-03	2.74E-03	2.63E-03	2.74E-03		2.84E-03		2.66E-03	ab	3.33E-03	a	2.97E-03	ab	2.58E-03	ab	2.52E-03	b	2.68E-03	ab	6.88E-04	n.s.	n.s.	**0.01**
Carnitine	5.37E-04	8.23E-04	7.69E-04	5.32E-04	b	8.87E-04	a	3.44E-04		7.29E-04		6.12E-04		6.40E-04		1.03E-03		8.97E-04		7.01E-04	n.s.	**0.02**	n.s.
Betaine	1.68E-03	1.48E-03	1.51E-03	1.72E-03		1.39E-03		1.65E-03	ab	1.71E-03	ab	1.82E-03	a	1.69E-03	ab	1.13E-03	b	1.33E-03	ab	5.44E-04	n.s.	**<0.01**	0.04
Glutarate	5.94E-03	5.96E-03	5.26E-03	6.35E-03	a	5.09E-03	b	6.46E-03		5.42E-03		6.07E-03		6.53E-03		5.85E-03		3.99E-03		2.85E-03	n.s.	**0.05**	n.s.
3-Phenylpropionate	5.55E-03	5.80E-03	5.43E-03	5.04E-03	b	6.15E-03	a	4.92E-03		6.18E-03		5.02E-03		5.18E-03		6.58E-03		5.69E-03		2.06E-03	n.s.	**0.02**	n.s.
Glycine	5.18E-03	5.21E-03	5.07E-03	5.47E-03	a	4.84E-03	b	5.55E-03		4.81E-03		5.58E-03		5.30E-03		4.85E-03		4.85E-03		1.40E-03	n.s.	**0.04**	n.s.

CON: commercial diet with soybean as main protein source in all feeding phases, CON+E: CON diet supplemented with muramidase (0.1%); 9%IM: CON diet with 9% insect meal; 18%IM: diet with 18% insect meal; 9%IM+E: 9%IM diet + muramidase (0.1%); 18%IM+E: 18%IM diet + muramidase (0.1%).
A,B: P < 0.01. a,b: P<0.05. n.s.: not significant.

Table 25. Concentration (mmol/L) of cecal metabolites (identified through the [1]H-NMR analysis) showing significant differences in 44-d-old broilers fed diets with different dosages of insect meal (0, 9 or 18%) and supplemented or not with muramidase.

Metabolite	Insect meal (IM)			Muramidase (MUR)		Interaction						SEM	*P*-value		
	0%	9%	18%	NO (0%)	YES (0.1%)	CON	CON+E	9%IM	18%IM	9%IM+E	18%IM+E		IM	MUR	IM × MUR
Taurine	2.03E-03	2.77E-03	5.68E-03	3.97E-03	3.01E-03	2.00E-03 b	2.07E-03 b	2.77E-03 b	7.16E-03 a	2.78E-03 b	4.19E-03 b	2.35E-03	**<0.001**	0.06	**0.03**
Alpha-ketoisovalerate	1.30E-03 A	7.73E-04 B	6.51E-04 B	9.23E-04	8.90E-04	1.36E-03	1.23E-03	7.31E-04	6.74E-04	8.16E-04	6.28E-04	4.81E-04	**<0.001**	n.s.	n.s.
Dimethylamine	1.34E-04 B	1.66E-04 B	2.05E-04 A	1.65E-04	1.71E-04	1.29E-04	1.38E-04	1.70E-04	1.95E-04	1.61E-04	2.14E-04	5.17E-05	**<0.001**	n.s.	n.s.
N,N-Dimethylglycine	1.67E-04 B	3.02E-04 B	4.97E-04 A	2.94E-04	3.50E-04	1.52E-04	1.82E-04	3.22E-04	4.09E-04	2.82E-04	5.86E-04	2.50E-04	**<0.001**	n.s.	n.s.
3-Methyl-2-oxovalerate	1.78E-03 A	1.18E-03 B	1.10E-03 B	1.41E-03	1.30E-03	1.83E-03	1.73E-03	1.14E-03	1.25E-03	1.22E-03	9.60E-04	5.63E-04	**<0.001**	n.s.	n.s.
Trimethylamine	5.75E-04	7.19E-04	1.13E-03	7.72E-04	8.45E-04	5.99E-04	5.52E-04	8.34E-04	8.84E-04	6.03E-04	1.38E-03	4.46E-04	**<0.001**	n.s.	**<0.01**
2-Oxoglutarate	9.82E-03 A	7.23E-03 B	5.36E-03 B	7.29E-03	7.65E-03	1.01E-02	9.50E-03	6.98E-03	4.74E-03	7.47E-03	5.99E-03	3.47E-03	**<0.001**	n.s.	n.s.
Betaine	1.05E-03 B	1.46E-03 AB	1.83E-03 A	1.48E-03	1.41E-03	1.09E-03	1.01E-03	1.55E-03	1.81E-03	1.37E-03	1.85E-03	6.68E-04	**<0.001**	n.s.	n.s.
Succinate	1.16E-02 B	1.57E-02 B	5.92E-02 A	2.97E-02	2.80E-02	1.04E-02	1.29E-02	1.47E-02	6.40E-02	1.67E-02	5.45E-02	4.89E-02	**<0.01**	n.s.	n.s.
3-Phenylpropionate	4.69E-03 A	3.92E-03 AB	3.11E-03 B	3.69E-03	4.12E-03	4.50E-03	4.87E-03	3.88E-03	2.70E-03	3.96E-03	3.51E-03	1.52E-03	**<0.01**	n.s.	n.s.
Fumarate	5.50E-04	6.61E-04	8.31E-04	6.22E-04	7.39E-04	5.64E-04 B	5.35E-04 B	6.88E-04 B	6.16E-04 B	6.34E-04 B	1.05E-03 A	3.19E-04	**<0.01**	0.10	**<0.01**
1,3-Dihydroxyacetone	5.35E-04 B	5.36E-04 B	7.26E-04 A	6.06E-04	5.92E-04	5.23E-04	5.46E-04	5.75E-04	7.20E-04	4.97E-04	7.32E-04	2.61E-04	**<0.01**	n.s.	n.s.
Phenylalanine	3.59E-03	2.82E-03	2.90E-03	3.38E-03	2.83E-03	4.17E-03 A	3.01E-03 B	2.55E-03 B	3.42E-03 AB	3.09E-03 AB	2.39E-03 B	9.99E-04	**<0.01**	**0.01**	**<0.01**
Carnitine	2.13E-04 b	2.84E-04 b	7.64E-04 a	3.81E-04	4.59E-04	1.46E-04	2.80E-04	1.75E-04	8.23E-04	3.93E-04	7.05E-04	7.37E-04	**0.01**	n.s.	n.s.
Acetate	4.46E-01 a	3.84E-01 ab	3.43E-01 b	3.87E-01	3.96E-01	4.32E-01	4.60E-01	3.90E-01	3.38E-01	3.79E-01	3.48E-01	1.34E-01	**0.02**	n.s.	n.s.
O-Acetylcarnitine	1.40E-04 b	1.53E-04 ab	1.94E-04 a	1.66E-04	1.59E-04	1.39E-04	1.41E-04	1.57E-04	2.01E-04	1.49E-04	1.87E-04	7.27E-05	**0.02**	n.s.	n.s.
Alanine	2.14E-02 a	1.75E-02 ab	1.74E-02 b	2.02E-02 a	1.73E-02 b	2.24E-02	2.03E-02	1.82E-02	1.99E-02	1.68E-02	1.49E-02	6.06E-03	**0.03**	0.03	n.s.
Glycerol	5.08E-03 a	3.76E-03 b	4.21E-03 ab	4.63E-03	4.07E-03	5.23E-03	4.93E-03	4.26E-03	4.38E-03	3.25E-03	4.04E-03	1.81E-03	**0.03**	n.s.	n.s.
Methionine	2.75E-03	2.14E-03	2.14E-03	2.31E-03	2.38E-03	2.60E-03	2.91E-03	1.94E-03	2.38E-03	2.34E-03	1.90E-03	9.79E-04	0.05	n.s.	n.s.
Nicotinate	2.25E-03	2.54E-03	2.19E-03	2.33E-03	2.32E-03	2.27E-03 ab	2.23E-03 ab	2.73E-03 a	1.98E-03 b	2.35E-03 ab	2.39E-03 ab	5.49E-04	**0.04**	n.s.	**0.03**
Proline	5.41E-03	6.19E-03	6.31E-03	6.07E-03	5.87E-03	5.83E-03	4.99E-03	6.40E-03	5.98E-03	5.98E-03	6.65E-03	1.45E-03	**0.05**	n.s.	n.s.
Aspartate	1.48E-02	1.65E-02	1.33E-02	1.50E-02	1.47E-02	1.39E-02	1.56E-02	1.80E-02	1.32E-02	1.50E-02	1.34E-02	4.93E-03	0.06	n.s.	n.s.
Thymine	8.83E-04	1.03E-03	1.45E-03	1.13E-03	1.11E-03	9.61E-04	8.05E-04	1.04E-03	1.40E-03	1.03E-03	1.49E-03	9.20E-04	0.07	n.s.	n.s.
Sarcosine	1.88E-04	2.14E-04	2.15E-04	2.03E-04	2.09E-04	1.86E-04	1.90E-04	2.27E-04	1.97E-04	2.02E-04	2.33E-04	4.83E-05	0.07	n.s.	0.06
Glucose	2.34E-02	1.84E-02	1.52E-02	1.74E-02	2.06E-02	2.20E-02	2.49E-02	1.40E-02	1.63E-02	2.29E-02	1.41E-02	1.41E-02	0.10	n.s.	n.s.
Glutamate	3.67E-02	3.55E-02	3.09E-02	3.78E-02	3.10E-02	4.42E-02 A	2.92E-02 B	4.04E-02 AB	2.88E-02 B	3.06E-02 B	3.30E-02 AB	1.06E-02	0.10	**<0.01**	**<0.01**

Valine	5.58E-03	5.02E-03	5.89E-03	5.78E-03		5.21E-03		6.05E-03	b	5.11E-03	b	4.69E-03	b	6.61E-03	b	5.35E-03	b	5.16E-03	a	1.65E-03	n.s.	n.s.	**<0.05**
Isoleucine	3.21E-03	2.90E-03	3.43E-03	3.44E-03		2.91E-03		3.57E-03	ab	2.84E-03	ab	2.71E-03	b	4.04E-03	a	3.08E-03	ab	2.82E-03	ab	1.17E-03	n.s.	0.04	**0.04**
Glutarate	2.28E-03	2.82E-03	2.82E-03	2.29E-03	b	2.99E-03	a	1.97E-03		2.60E-03		2.76E-03		2.14E-03		2.88E-03		3.49E-03		1.36E-03	n.s.	**0.02**	n.s.
Ribose	1.99E-02	2.18E-02	2.22E-02	1.96E-02	b	2.30E-02	a	1.88E-02		2.10E-02		2.14E-02		1.87E-02		2.23E-02		2.57E-02		5.93E-03	n.s.	**0.01**	n.s.
Leucine	5.06E-03	5.10E-03	5.59E-03	5.37E-03		5.13E-03		5.22E-03	ab	4.90E-03	ab	4.35E-03	b	6.54E-03	a	5.85E-03	ab	4.64E-03	ab	1.95E-03	n.s.	n.s.	**<0.01**
Isovalerate	3.44E-03	3.18E-03	2.97E-03	3.10E-03		3.30E-03		3.31E-03		3.58E-03		3.66E-03		2.33E-03		2.70E-03		3.62E-03		1.57E-03	n.s.	n.s.	**0.03**
Glycine	8.45E-03	7.84E-03	8.52E-03	8.89E-03	a	7.65E-03	b	8.99E-03		7.91E-03		7.77E-03		9.93E-03		7.92E-03		7.11E-03		2.77E-03	n.s.	**0.04**	n.s.

CON: commercial diet with soybean as main protein source in all feeding phases, CON+E: CON diet supplemented with muramidase (0.1%); 9%IM: CON diet with 9% insect meal; 18%IM: diet with 18% insect meal; 9%IM+E: 9%IM diet + muramidase (0.1%); 18%IM+E: 18%IM diet + muramidase (0.1%).

A,B: P < 0.01. a,b: P<0.05. n.s.: not significant.

At both 21 and 44 d, Firmicutes was the most represented phylum in all experimental groups (**Table 26** and **Table 27**). At the end of the rearing cycle, the phylum Firmicutes showed a statistically significant decrease as the dietary concentration of insect meal increased, which was associated with an increase of Bacteroidetes and Proteobacteria (**Table 27**).

Table 26. Mean relative abundance (%) at phylum level in cecal content of 21-d-old broilers fed diets with different dosages of insect meal (0, 9 or 18%) and supplemented or not with muramidase.

PHYLUM (%)	Insect meal			Muramidase		Interaction						*P*-value		
	0%	9%	18%	NO (0%)	YES (0.1%)	CON	CON+E	9%IM	18%IM	9%IM+E	18%IM+E	Insect Meal	Muramidase	Insect meal x Muramidase
Bacteroidetes	13.7	18.3	16.6	17.5	14.9	16.15 ab	11.19 b	23.18 a	13.02 b	13.43 b	20.08 ab	n.s.	n.s.	**<0.01**
Firmicutes	84.7	79.7	80.9	80.4	83.1	82.1 ab	87.2 a	74.8 b	84.2 ab	84.6 a	77.6 ab	0.10	n.s.	**<0.01**
Verrucomicrobia	0.03	0.21	0.26	0.26	0.08	0.00	0.07	0.29	0.48	0.14	0.04	n.s.	n.s.	n.s.
Proteobacteria	0.40	0.61	0.85	0.70	0.54	0.62	0.18	0.68	0.79	0.53	0.90	n.s.	n.s.	n.s.
Actinobacteria	0.16	0.15	0.13	0.10	0.19	0.14	0.17	0.08	0.07	0.23	0.19	n.s.	**<0.001**	n.s.
Tenericutes	1.00	0.98	1.20	1.06	1.06	0.94	1.06	0.95	1.29	1.01	1.11	n.s.	n.s.	n.s.
Cyanobacteria	0.07	0.08	0.10	0.09	0.08	0.05	0.09	0.07	0.14	0.08	0.07	n.s.	n.s.	n.s.

CON: commercial diet with soybean as main protein source in all feeding phases, CON+E: CON diet supplemented with muramidase (0.1%); 9%IM: CON diet with 9% insect meal; 18%IM: diet with 18% insect meal; 9%IM+E: 9%IM diet + muramidase (0.1%); 18%IM+E: 18%IM diet + muramidase (0.1%). a,b: P<0.05. n.s.: not significant.

Table 27. Mean relative abundance (%) at phylum level in cecal content of 44-d-old broilers fed diets with different dosages of insect meal (0, 9 or 18%) and supplemented or not with muramidase.

PHYLUM	Insect meal			Muramidase		Interaction						*P*-value		
	0%	9%	18%	NO (0%)	YES (0.1%)	CON	CON+E	9%IM	18%IM	9%IM+E	18%IM+E	Insect Meal	Muramidase	Insect meal x Muramidase
Bacteroidetes	7.41 b	12.0 a	11.9 a	11.93	8.96	8.98	5.84	15.7	11.16	8.33	12.70	**0.04**	0.08	0.10
Firmicutes	88.5 A	80.9 B	78.8 B	80.86	84.60	87.5	89.6	76.7	78.43	85.04	79.20	**<0.001**	0.05	n.s.
Verrucomicrobia	1.33	2.89	2.76	2.79	1.86	0.85	1.80	3.46	4.05	2.32	1.46	n.s.	n.s.	n.s.
Proteobacteria	1.16 B	2.22 AB	3.55 A	2.70	1.92	1.33	0.98	2.42	4.35	2.02	2.75	**<0.001**	0.08	n.s.
Actinobacteria	0.11 B	0.46 B	1.82 A	0.43	1.17	0.08	0.14	0.13	1.07	0.78	2.57	**<0.01**	n.s.	n.s.
Tenericutes	1.11 a	0.95 a	0.64 b	0.86	0.94	1.08	1.13	0.98	0.50	0.92	0.77	**0.02**	n.s.	n.s.
Cyanobacteria	0.15 b	0.29 a	0.18 b	0.21	0.20	0.13	0.16	0.30	0.19	0.28	0.17	**0.01**	n.s.	n.s.

CON: commercial diet with soybean as main protein source in all feeding phases, CON+E: CON diet supplemented with muramidase (0.1%); 9%IM: CON diet with 9% insect meal; 18%IM: diet with 18% insect meal; 9%IM+E: 9%IM diet + muramidase (0.1%); 18%IM+E: 18%IM diet + muramidase (0.1%). A, B: P<0.01; a,b: P<0.05. n.s.: not significant.

At the genus level, *Fecalibacterium*, followed by *Bacteroides*, *Ruminococcus*, *Lactobacillus* and *Oscillospira*, were the most represented genera in all tested groups at both 21 and 42 days (**Table 28** and **Table 29**). At 21 days (**Table 28**), *Oscillospira* and *Ruminococcus* (Ruminococcaceae) significantly increased in response insect meal administration. At the end of trial (**Table 29**), the genera Ruminococcus (Ruminococcaceae) and *Bifidobacterium* increased significantly as the dietary concentration of insect meal increased, while *Dorea* and *Dehalobacterium* decreased.

Table 28. Mean relative abundance (%) at genus level in cecal content of 21-d-old broilers fed diets with different dosages of insect meal (0, 9 or 18%) and supplemented or not with muramidase.

Genus	Insect meal			Muramidase		Interaction						*P*-value		
	0%	9%	18%	NO (0%)	YES (0.1%)	CON	9IM	18IM	CON+E	9IM+E	18IM+E	Insect Meal	Muramidase	Insect meal x Muramidase
Bacteroides	7.83	11.84	10.10	11.18	8.66	10.62 ABC	16.48 A	6.44 BC	5.04 C	7.19 BC	13.76 AB	n.s.	n.s.	**<0.001**
Faecalibacterium	17.85	15.17	7.86	11.87	15.39	12.66 bc	14.53 bc	8.41 bc	23.04 a	15.81 ab	7.32 c	**<0.001**	**0.03**	**0.01**
Lactobacillus	4.33	3.09	3.95	1.40	6.17	2.50	1.04	0.66	6.15	5.13	7.24	n.s.	**<0.001**	n.s.
Ruminococcus (Lachnospiraceae)	6.61	4.82	7.93	6.63	6.28	7.65 ab	5.38 bc	6.85 abc	5.57 bc	4.27 c	9.01 a	0.00	n.s.	**0.02**
Akkermansia	0.03	0.21	0.26	0.26	0.08	0.00	0.29	0.48	0.07	0.14	0.04	n.s.	n.s.	n.s.
Bacillus	1.02	0.83	1.28	1.28	0.81	1.50	1.09	1.25	0.54	0.58	1.31	n.s.	**0.02**	n.s.
Oscillospira	3.86 B	4.34 AB	5.19 A	4.72	4.21	4.14	4.24	5.77	3.59	4.43	4.60	**<0.01**	n.s.	n.s.
Streptococcus	0.00	0.06	0.42	0.02	0.31	0.00	0.00	0.05	0.00	0.13	0.79	n.s.	0.08	n.s.
Ruminococcus (Ruminococcaceae)	2.00 B	2.62 AB	3.12 A	2.71	2.45	1.72 B	2.39 B	4.02 A	2.28 B	2.85 AB	2.22 B	**<0.01**	n.s.	**<0.01**
Dorea	1.21	1.18	1.29	1.22	1.24	1.30	1.24	1.12	1.13	1.13	1.46	n.s.	n.s.	n.s.
Coprobacillus	0.77 a	0.76 a	0.49 b	0.42	0.92	0.47	0.45	0.35	1.07	1.06	0.62	**0.04**	**<0.001**	n.s.
Bilophila	0.16 B	0.41 A	0.53 A	0.43	0.31	0.21	0.45	0.63	0.12	0.37	0.44	**<0.001**	n.s.	n.s.
Bifidobacterium	0.02	0.02	0.07	0.01	0.06	0.03 ab	0.00 b	0.00 b	0.01 b	0.04 ab	0.13 a	n.s.	**0.01**	**0.01**
Blautia	1.91 a	1.42 ab	0.87 b	0.85	1.95	1.01	0.86	0.68	2.82	1.98	1.06	**0.03**	**<0.01**	n.s.
Coprococcus	0.86	0.84	1.10	1.03	0.84	0.87	1.05	1.16	0.85	0.64	1.04	n.s.	n.s.	n.s.
Enterococcus	0.22	0.04	0.05	0.02	0.19	0.03	0.01	0.01	0.40	0.07	0.10	n.s.	n.s.	n.s.
Clostridium	0.34	0.55	0.51	0.35	0.58	0.35 b	0.33 b	0.38 b	0.34 b	0.76 a	0.65 ab	0.05	**<0.01**	**0.04**
Dehalobacterium	0.10	0.10	0.14	0.18	0.04	0.18	0.17	0.20	0.02	0.03	0.08	n.s.	**<0.001**	n.s.
Anaeroplasma	0.23	0.24	0.15	0.12	0.29	0.20	0.12	0.04	0.26	0.37	0.25	n.s.	**0.04**	n.s.

CON: commercial diet with soybean as main protein source in all feeding phases, CON+E: CON diet supplemented with muramidase (0.1%); 9%IM: CON diet with 9% insect meal; 18%IM: diet with 18% insect meal; 9%IM+E: 9%IM diet + muramidase (0.1%); 18%IM+E: 18%IM diet + muramidase (0.1%). A, B: P<0.01; a,b: P<0.05. n.s.: not significant.

Table 29. Mean relative abundance (%) at genus level in cecal content of 44-d-old broilers fed diets with different dosages of insect meal (0, 9 or 18%) and supplemented or not with muramidase.

Taxa	Insect meal			Muramidase		Interaction						P-value		
	0%	9%	18%	NO (0%)	YES (0.1%)	CON	9IM	18IM	CON+E	9IM+E	18IM+E	Insect Meal	Muramidase	Insect meal x Muramidase
Bacteroides	3.60	7.82	8.30	7.79	5.36	5.20 ab	11.6 a	6.59 ab	2.01 b	4.04 ab	10.02 ab	0.05	n.s.	**0.03**
Faecalibacterium	8.78	7.85	6.87	7.67	8.00	9.22	7.85	5.93	8.35	7.86	7.80	n.s.	n.s.	n.s.
Lactobacillus	5.13	6.94	11.3	5.12	10.5	2.45	3.42	9.49	7.81	10.5	13.25	n.s.	0.06	n.s.
Ruminococcus (Lachnospiraceae)	6.84	5.30	6.60	6.57	5.92	7.81	5.03	6.89	5.88	5.58	6.31	0.09	n.s.	n.s.
Akkermansia	1.33	2.89	2.76	2.79	1.86	0.85	3.46	4.05	1.80	2.32	1.46	n.s.	n.s.	n.s.
Bacillus	1.12	1.05	1.16	1.44	0.78	1.30	1.50	1.51	0.94	0.60	0.80	n.s.	**0.02**	n.s.
Oscillospira	3.34	3.29	3.77	3.59	3.34	3.30	3.50	3.97	3.38	3.08	3.56	n.s.	n.s.	n.s.
Streptococcus	2.17	1.82	2.12	0.91	3.16	0.96	1.18	0.59	3.37	2.47	3.65	n.s.	**<0.001**	n.s.
Ruminococcus (Ruminococcaceae)	0.61 B	1.49 AB	1.99 A	1.60	1.13	0.75	1.43	2.62	0.46	1.55	1.36	**<0.01**	n.s.	n.s.
Ruminococcus.1	1.39	2.00	2.08	2.14	1.51	1.60	2.43	2.40	1.19	1.58	1.76	n.s.	**0.04**	n.s.
Dorea	1.02 A	0.81 AB	0.49 B	0.78	0.76	1.06	0.74	0.55	0.98	0.88	0.43	**<0.01**	n.s.	n.s.
Coprobacillus	0.24	0.38	0.36	0.26	0.39	0.25	0.24	0.30	0.24	0.52	0.42	n.s.	**0.02**	n.s.
Bilophila	0.28 b	0.31 b	0.43 a	0.33	0.35	0.23	0.33	0.44	0.33	0.29	0.42	**0.03**	n.s.	n.s.
Bifidobacterium	0.04 B	0.39 AB	1.76 A	0.36	1.10	0.01	0.09	0.98	0.08	0.69	2.53	**<0.01**	n.s.	n.s.
Blautia	0.68	0.84	0.54	0.38	1.00	0.45	0.36	0.32	0.92	1.33	0.75	n.s.	**<0.001**	n.s.
Coprococcus	1.09	1.05	1.30	1.53	0.76	1.52	1.51	1.57	0.65	0.58	1.04	n.s.	**<0.001**	n.s.
Enterococcus	0.21 b	0.37 b	0.69 a	0.45	0.40	0.24	0.57	0.54	0.18	0.17	0.84	**0.04**	n.s.	n.s.
Clostridium	0.37	0.56	0.39	0.35	0.53	0.38 B	0.27 B	0.41 B	0.37 B	0.86 A	0.36 B	0.09	0.025	**<0.001**
Dehalobacterium	0.37 A	0.26 AB	0.13 B	0.31	0.20	0.46	0.30	0.16	0.29	0.21	0.11	**<0.001**	**<0.01**	n.s.
Roseburia	0.03	0.14	0.16	0.18	0.04	0.02	0.27	0.26	0.04	0.01	0.07	n.s.	**0.04**	n.s.
Turicibacter	0.02	0.03	0.11	0.10	0.00	0.03 B	0.05 B	0.20 A	0.00 B	0.00 B	0.01 B	**<0.001**	**<0.001**	n.s.

CON: commercial diet with soybean as main protein source in all feeding phases, CON+E: CON diet supplemented with muramidase (0.1%); 9%IM: CON diet with 9% insect meal; 18%IM: diet with 18% insect meal; 9%IM+E: 9%IM diet + muramidase (0.1%); 18%IM+E: 18%IM diet + muramidase (0.1%). A, B: P<0.01; a,b: P<0.05. n.s.: not significant.

5. Use of insect meal as alternative to soybean in turkey diets

Aim

The present study was performed to investigate the effects of the partial replacement of dietary soybean with insect meal (*Hermetia illucens*; provided by MUTATEC) in turkey diets on growth performance, meat quality and cecal metabolome and microbiota of heavy female turkeys.

Materials and Methods

A total of 1,512 one-day-old female poults (BUT Big 6) were divided into two experimental groups (9 replicates of 84 birds/each): CON, fed a conventional soybean-based diet in all feeding phases, and INS, which received CON diet up to 64 d and then CON diet with 5% of insect meal as a substitution for soybean until slaughter (105 d). All diets were isoenergetic and with a similar amino acid profile, which was optimized maintaining the same ratio of total essential amino acids to total lysine, and supplied in pelleted form. Body weight (BW) was determined on a pen basis at placement, at the end of each feeding phase, and at slaughter. Similarly, feed intake (FI) was assessed on a pen basis at each diet switch and at slaughter. The number and weight of dead birds were recorded daily and used to calculate the mortality rate and to correct performance data such as daily weight gain (DWG), daily feed intake (DFI) and feed conversion ratio (FCR). At 105 d, all birds were processed in a commercial slaughterhouse and slaughter yields, including carcass, breast, leg and wing yield were assessed on all birds. At slaughtering, cecal content samples were obtained from 15 birds per group and then subjected to microbiota (16S Amplicon Sequencing) and metabolome (^{1}H-Nuclear Magnetic Resonance) analysis. Moreover, 12 breasts/group were collected for the evaluation of proximate composition (AOAC, 1990) and the main meat technological properties (pH, color profile, water holding capacity and shear-force). Data were analyzed by means of one-way ANOVA and Tukey post-hoc test.

Results

As expected, CON and INS turkeys performed similarly up to 64 d **(Table 30)**. Likewise, no significant effect of the dietary treatment was observed from 65 to 78 d. At 93 d, BW was significantly higher in INS turkeys (8,756 vs. 8,821 g/bird for CON and INS, respectively; P<0.05). DWG and FCR tended to be improved by the administration of insect meal (141.3 vs. 144.7 g/bird/d and 2.501 vs. 2.434, for CON and INS, respectively P=0.09 and P=0.07). At market age (105 d), INS turkeys presented greater BW than CON (10,173 vs. 10,057 g/bird, respectively; P<0.05) as well as higher DFI in the finisher phase (94-105 d; 343.7 vs. 353.7 g/bird/d, respectively; P<0.05). Considering the overall period of the trial (0-105 d), INS turkeys presented higher DWG and lower FCR (96.2 vs. 95.2 g/bird/d, and 2.127 vs. 2.141, respectively; P<0.05). Mortality and DFI were similar between experimental groups.

Table 30. Growth performance of female turkeys receiving soybean-based diets in all feeding phases (CON group) or diets with insect meal form 65 d to slaughter (105 d; INS group).

Parameter	Group		SEM	*P*-value
	CON	INS		
Poult weight (g/bird)	57.44	57.12	0.60	0.283
Starter (0-22 d)				
BW (g/bird)	646.2	654.6	24.32	0.482
DWG (g/bird/d)*	26.76	27.16	1.09	0.461
DFI (g/bird/d)*	36.34	37.05	1.71	0.404
FI (kg/bird)*	0.799	0.815	0.04	0.404
FCR*	1.358	1.365	0.04	0.707
Mortality (%)	0.00	0.00	0.00	.
Grower I (23-50 d)				
BW (g/bird)	3023	3059	51.0	0.175
DWG (g/bird/d)*	84.81	85.86	1.71	0.231
DFI (g/bird/d)*	129.8	131.7	2.35	0.127
FI (kg/bird)*	3.635	3.688	0.07	0.127
FCR*	1.531	1.534	0.02	0.760
Mortality (%)	0.00	0.13	0.03	0.347
Grower II (51-64 d)				
BW (g/bird)	4796	4804	59.3	0.775
DWG (g/bird/d)*	126.6	124.6	4.34	0.335
DFI (g/bird/d)*	240.4	238.1	5.98	0.434
FI (kg/bird)*	3.366	3.333	0.08	0.434
FCR*	1.900	1.914	0.02	0.261
Mortality (%)	0.00	0.27	0.04	0.347
Grower III (65-78 d; insect inclusion in INS diet from D65 onwards)				
BW (g/bird)	6645	6661	78.3	0.671
DWG (g/bird/d)*	133.5	134.9	4.43	0.518
DFI (g/bird/d)*	298.5	296.8	3.95	0.401
FI (kg/bird)*	4.179	4.155	0.06	0.401
FCR*	2.236	2.202	0.06	0.276
Mortality (%)	0.00	0.00	0.00	.
Grower IV (79-93 d)				
BW (g/bird)	8756	8821	58.8	**0.047**
DWG (g/bird/d)*	141.3	144.7	3.67	0.089
DFI (g/bird/d)*	353.3	352.0	6.85	0.689
FI (kg/bird)*	5.300	5.280	0.10	0.689
FCR*	2.501	2.434	0.07	0.068
Mortality (%)	0.00	0.00	0.00	.
Finisher (94-105 d)				
BW (g/bird)	10057	10173	103.9	**0.045**
DWG (g/bird/d)*	108.65	112.7	5.57	0.161
DFI (g/bird/d)*	343.7	353.7	8.62	**0.040**

FI (kg/bird)*	4.125	4.244	0.10	**0.040**
FCR*	3.172	3.146	0.12	0.659
Mortality (%)	0.00	0.40	0.04	0.081
Entire trial (0-105 d)				
BW (g/bird)	10057	10173	103.9	**0.045**
DWG (g/bird/d)*	95.23	96.22	0.83	**0.034**
DFI (g/bird/d)*	203.9	204.7	1.93	0.393
FI (kg/bird)*	21.40	21.52	0.23	0.324
FCR*	2.141	2.127	0.01	**0.026**
Mortality (%)	0.00	0.79	0.06	0.104

*Corrected for mortality.

The dietary utilization of insect meal had no substantial effects on technological properties (**Table 31)** and proximate composition of turkey breast meat (**Table 32**). Indeed, only yellowness (b*) presented statistically significant variations in response to the dietary treatment (2.83 vs. 1.95 for CON and INS; P<0.05).

Table 31. Technological traits of breast meat (*n*=15 breasts/group) of female turkeys fed soybean-based diets in all feeding phases (CON group) or diets with insect meal form 65 d to slaughter (105d; INS group).

	CON	INS	SEM	*P*-value
Ultimate pH (pHu)	5.66	5.66	0.01	n.s.
Lightness - L*	50.57	49.59	0.27	n.s.
Redness - a*	3.89	3.88	0.15	ns
Yellowness - b*	2.83	1.95	0.22	<0.05
Drip loss (%)	0.89	0.85	0.02	n.s.
Cooking loss (%)	17.87	18.96	0.34	n.s.
Shear force (kg)	2.36	2.28	0.08	n.s.

n.s.: not significant.

Table 32. Proximate composition of breast meat (*n* = 15 breasts/group) of female turkeys fed soybean-based diets in all feeding phases (CON group) or diets with insect meal form 65 d to slaughter (105d; INS group).

	CON	INS	SEM	*P*-value
Moisture (%)	73.78	73.55	0.25	n.s.
Crude protein (%)	25.26	24.92	0.21	n.s.
Total fat (%)	1.24	1.26	0.07	n.s.
Ash (%)	1.47	1.45	0.13	n.s.

n.s.: not significant.

The [1]H-NMR analysis identified a total of 71 metabolites in the cecal content. This analysis revealed that some metabolites (**Table 33**), including glucose (P<0.05), malonate (P<0.05), isoleucine (P=0.06), betaine (P=0.07) and butyrate (P=0.10), exhibited higher concentrations in the cecal content of INS birds. Conversely, the cecal content of INS turkeys showed lower concentration of tyramine (P<0.05) and 3-phenylpropionate (P=0.10).

Table 33. Concentration (mmol/L) of cecal metabolites (identified through the [1]H-NMR analysis) showing differences (up to *P*<0.10) in female turkeys fed soybean-based diets in all feeding phases (CON group) or diets with insect meal form 65 d to slaughter (105d; INS group).

Metabolite	CON		INS		*P*-value
	Mean	SE	Mean	SE	
Tyramine	8.27E-04	8.96E-05	5.48E-04	7.44E-05	**0.03**
Glucose	1.02E-03	1.13E-04	1.53E-03	2.07E-04	**0.03**
Malonate	7.41E-04	7.53E-05	1.01E-03	9.07E-05	**0.03**
Isoleucine	4.21E-04	3.38E-05	5.24E-04	3.96E-05	0.06
Betaine	2.74E-04	2.02E-05	3.41E-04	2.94E-05	0.07
3-Phenylpropionate	1.08E-03	7.92E-05	8.88E-04	7.61E-05	0.10
Butyrate	7.89E-03	5.82E-04	1.03E-02	1.42E-03	0.10

As for the cecal microbiota (**Table 34**), Firmicutes, followed by Bacteroidetes, were the most abundant phyla in all the analyzed samples. However, no statistically significant difference was found. Analogously, no difference was detected at genus level (**Table 35**).

Table 34. Mean relative abundance (%) at phylum level in cecal content of 105-d-old female turkeys fed soybean-based diets in all feeding phases (CON group) or diets with insect meal form 65 d to slaughter (105d; INS group).

Phylum	CON	INS	*P*-value
Firmicutes	70.9	69.4	n.s.
Bacteroidetes	23.2	24.5	n.s.
Proteobacteria	2.99	3.17	n.s.
Tenericutes	1.52	1.79	n.s.
Actinobacteria	0.83	0.74	n.s.
Cyanobacteria	0.60	0.41	n.s.

n.s.: not significant

Table 35. Mean relative abundance (%) at genus level in cecal content of 105-d-old female turkeys fed soybean-based diets in all feeding phases (CON group) or diets with insect meal form 65 d to slaughter (105d; INS group).

Genus	CON	INS	*P*-value
Faecalibacterium	12.2	8.08	0.070
Bacteroides	9.33	7.83	n.s.
Oscillospira	3.39	3.25	n.s.
Parabacteroides	2.15	2.91	n.s.
Ruminococcus	4.66	4.37	n.s.
Blautia	1.90	1.98	n.s.
Lactobacillus	1.65	1.55	n.s.
AF12	1.22	1.38	n.s.
Helicobacter	0.78	0.72	n.s.
Desulfovibrio	0.65	0.78	n.s.
Odoribacter	0.61	0.75	n.s.
Sutterella	0.57	0.58	n.s.
Bifidobacterium	0.59	0.51	n.s.
Streptococcus	0.48	0.51	n.s.
Dorea	0.35	0.47	n.s.
Clostridium	0.37	0.33	n.s.
Coprobacillus	0.38	0.32	n.s.
Megamonas	0.47	0.17	0.073
Phascolarctobacterium	0.30	0.25	n.s.
Bilophila	0.28	0.18	n.s.
Coprococcus	0.13	0.14	n.s.
Butyricimonas	0.13	0.10	n.s.
Collinsella	0.11	0.03	n.s.

n.s.: not significant

6. Use of single-cell proteins meal as alternative to soybean in broiler chicken diets

6.1 Trial #1

Aim

This study was carried out to investigate the effects of the substitution of dietary soybean with single-cell proteins (SCP) from torula yeast (*Candida utilis* - provided by ARBIOM) on the growth performance of broiler chickens up to 21 d of age.

Materials and Methods

A total of 560 one-day-old Ross 308 male chicks were divided into 4 experimental groups (10 replicate pens/group with 14 birds each) receiving, during the starter (0-12 d) and grower (13-22 d) phases, either a conventional soybean-based diet (CON group) or the same diet including SCP at low (SCP5 group: 5% in both phases), intermediate (SCP10 group: 10 and 9%, respectively), or high dosages (SCP15 group: 15 and 14%, respectively). From 23 d onwards, all groups received the same conventional soybean-based diet up to slaughter age (42 d). The feed was provided in mash form and for ad libitum consumption. All diets were isoenergetic and with a similar amino acid profile, which was optimized maintaining the same ratio of total essential amino acids to total lysine. Birds were weighed on a pen basis at placement (0 day), at 22 d and at slaughter (42 d). Similarly, feed intake was determined at 22 and 42 d. Mortality was monitored daily. Dead birds were recorded and weighed to calculate the mortality rate and to adjust the productive performance data for mortality. Body weight (BW), daily weight gain (DWG), daily feed intake (DFI) and feed conversion ratio (FCR) were obtained accordingly. The results were reported for the following periods: 0-22 d, 23-42 d and 0-42 d. At 42 d, all birds were processed in a commercial slaughterhouse. Data were analyzed by means of one-way ANOVA and Tukey post-hoc test, while polynomial contrasts were used to assess linear response.

Results

Performance results are shown in **Table 36**. At placement, chicks presented a comparable BW. After 21 d, BW, DWG and DFI were linearly reduced by SCP administration (890 vs. 775 vs. 635 vs. 529 g, 40.08 vs. 34.56 vs. 28.12 vs. 23.01 g/bird/d, and 57.20 vs. 53.80 vs. 48.49 vs. 43.68 g/bird/d, respectively for CON, SCP5, SCP10 and SCP15; P<0.001). Conversely, FCR increased linearly from CON to SCP15 (1.428 vs. 1.559 vs. 1.728 vs. 1.916, respectively for CON, SCP5, SCP10 and SCP15; P<0.001). At 42 d, BW exhibited a linear reduction trend (2914 vs. 2693 vs. 2432 vs. 2233 g, respectively for CON, SCP5, SCP10 and SCP15; P<0.001). CON group presented the highest DWG during the finisher phase, followed by SCP5, SCP10 and SCP15 (96.4 vs. 91.3 vs. 85.5 vs. 81.2 g, respectively; linear: P<0.001). From 22 to 42 d, DFI was similar between CON and SCP5 groups, while both SCP10 and SCP15 showed lower values compared to the other groups (238.1 vs. 235.7 vs. 217.8 vs. 212.2 g/bird/d, respectively for CON, SCP5, SCP10 and SCP15; linear: P<0.001). CON group presented significantly lower FCR compared to SCP15, with SCP5 and SCP10 achieving intermediate values (2.473 vs. 2.582 vs. 2.548 vs. 2.622, respectively for CON, SCP5, SCP10 and SCP15; P<0.001). In the overall trial period, CON group presented a significantly higher DWG than the other groups (68.33 vs. 63.05 vs. 56.86 vs. 52.12 g/bird/d, respectively for CON, SCP5, SCP10 and SCP15; P<0.001). DFI was similar between CON and

SCP5, which presented higher values than SCP10 and SCP15 (147.2 vs. 143.9 vs. 133.0 vs. 127.6 g/bird/d, respectively for CON, SCP5, SCP10 and SCP15; P<0.001). FCR exhibited a linear response, with CON presenting the lowest value and SCP15 the highest one (2.164 vs. 2.298 vs. 2.344 vs. 2.464, respectively for CON, SCP5, SCP10 and SCP15; P<0.001). Mortality was not affected in each feeding phase nor in the overall period of the trial.

<u>Table 36</u>. Growth performance of broiler chickens fed a conventional soybean-based diet (CON) or diets with different dosages of single-cell proteins (5%, SCP5; 10%, SCP10; and 15%, SCP15) up to 21 d of age.

Parameter	**Group**								**SEM**	***P*-value**	**Linear trend *P*-value**
	CON		**SCP5**		**SCP10**		**SCP15**				
Chick weight (g/bird)	43.15		43.43		43.02		43.30		1.08	n.s.	n.s.
Starter (0-14 d)											
BW (g/bird)	422.8	A	374.9	B	304.7	C	246.1	D	15.18	**<0.001**	**<0.001**
DWG (g/bird/d)*	26.89	A	23.50	B	18.62	C	14.41	D	1.07	**<0.001**	**<0.001**
DFI (g/bird/d)*	38.57	A	38.27	A	36.96	A	33.54	B	2.16	**<0.001**	**<0.001**
FI (kg/bird)*	0.540	A	0.536	A	0.517	A	0.470	B	0.03	**<0.001**	**<0.001**
FCR*	1.435	C	1.629	C	1.989	B	2.350	A	0.17	**<0.001**	**<0.001**
Mortality (%)	2.00		2.67		0.67		1.33		0.11	n.s.	n.s.
Grower (0-21 d)											
BW (g/bird)	890.3	A	775.3	B	635.1	C	528.8	D	39.19	**<0.001**	**<0.001**
DWG (g/bird/d)*	40.08	A	34.56	B	28.12	C	23.01	D	1.84	**<0.001**	**<0.001**
DFI (g/bird/d)*	57.20	A	53.80	B	48.49	C	43.68	D	2.12	**<0.001**	**<0.001**
FI (kg/bird)*	1.201	A	1.130	B	1.018	C	0.917	D	0.04	**<0.001**	**<0.001**
FCR*	1.428	C	1.559	C	1.728	B	1.916	A	0.13	**<0.001**	**<0.001**
Mortality (%)	2.00		3.33		0.67		1.33		0.11	n.s.	n.s.
Finisher (22-42 d)											
BW (g/bird)	2914	A	2693	B	2432	C	2233	D	111.4	**<0.001**	**<0.001**
DWG (g/bird/d)*	96.37	A	91.32	B	85.54	C	81.16	C	3.80	**<0.001**	**<0.001**
DFI (g/bird/d)*	238.1	A	235.7	A	217.8	B	212.2	B	8.68	**<0.001**	**<0.001**
FI (kg/bird)*	5.000	A	4.949	A	4.574	B	4.455	B	0.18	**<0.001**	**<0.001**
FCR§	2.473	b	2.582	ab	2.548	ab	2.622	a	0.11	**0.04**	**0.01**
Mortality (%)	0.00		0.00		0.00		0.00		0.00	.	.
Entire feeding trial (0-42 d)											
BW (g/bird)	2914	A	2693	B	2432	C	2233	D	111.4	**<0.001**	**<0.001**
DWG (g/bird/d)*	68.33	A	63.05	B	56.86	C	52.12	D	2.64	**<0.001**	**<0.001**
DFI (g/bird/d)*	147.2	A	143.9	A	133.0	B	127.6	B	4.55	**<0.001**	**<0.001**
FI (kg/bird)*	6.204	A	6.079	A	5.593	B	5.374	B	0.20	**<0.001**	**<0.001**
FCR*	2.164	C	2.298	B	2.344	AB	2.464	A	0.10	**<0.001**	**<0.001**
Mortality (%)	2.00		3.33		0.67		1.33		0.11	n.s.	n.s.

BW: body weight; DWG: daily weight gain; DFI: daily feed intake; FI: feed intake; FCR: feed conversion ratio.
* corrected for mortality; A, B: P<0.01; a, b: P<0.05.
n.s.: not significant.

6.2 Trial #2

Aim

This study was conducted to assess the effects of the substitution of dietary soybean with single-cell proteins (SCP) from torula yeast (*Candida utilis* - provided by ARBIOM) in the grower and finisher feeding phases on productive performance, occurrence of footpad dermatitis (FPD), breast meat quality traits, plasma and cecal metabolomic profile of broiler chickens.

Materials and Methods

A total of 720 d-old Ross 308 male chicks was divided into 4 experimental groups each composed of 10 replicates with 18 birds. The CON group received a commercial diet with soybean as the main protein source in all feeding phases. The groups SCP2, SCP4 and SCP6 were fed the CON diet during the starter phase (0-14 d), and then the CON diet with respectively 2, 4 or 6% of SCP as partial replacement for soybean during the grower and finisher phases (i.e. from 15 to 42 d). The feed was provided in mash form and for ad libitum consumption. All diets were isoenergetic and with a similar amino acid profile, which was optimized maintaining the same ratio of total essential amino acids to total lysine. Body weight (BW) was determined on a pen basis at placement, at the end of each feeding phase, and at slaughter. Similarly, feed intake (FI) was assessed on a pen basis at each diet switch and at slaughter (14, 29, 42 d). The number and weight of dead birds were recorded daily and used to calculate the mortality rate and to correct performance data such as daily weight gain (DWG), daily feed intake (DFI) and feed conversion ratio (FCR). At 42 d, all birds were processed in a commercial slaughterhouse and slaughter yields, such as carcass, breast, leg and wing yields were assessed on all birds. Similarly, the incidence and severity of FPD were evaluated on all birds through a 3-point scale: 0 – no lesion, 1 – mild lesions, 2 – severe lesions (Ekstrand et al., 1998). Proximate composition (AOAC, 1990) and technological traits of breast meat, including pHu, color profile, water holding capacity and tenderness, were evaluated on 12 breasts per experimental group. Plasma and cecal content samples were collected at 21 and 42 d on 9 birds/group and then subjected to microbiota (16S Amplicon Sequencing) and metabolome (^{1}H-Nuclear Magnetic Resonance) analysis. Data were analyzed by means of one-way ANOVA and Tukey post-hoc test, while polynomial contrasts were used to assess linear and quadratic responses.

Results

Chick BW was similar at placement (**Table 37**). Birds performed similarly during the starter phase, achieving a similar BW at the end of the starter phase (14 d; 434.5 vs. 420.8 vs. 429.9 vs. 430.1 g, respectively for CON, SCP2, SCP4 and SCP6; P=0.59). At 29 d, although a linear reduction response was observed by increasing the dietary dosage of SCP (P<0.05), no significant difference in BW among groups was identified (1665 vs. 1640 vs. 1616 vs. 1590 g, respectively for CON, SCP2, SCP4 and SCP6; P=0.10). DWG was significantly affected by the dietary treatments, with CON showing significantly higher value compared to SCP6, with SCP2 and SCP4 presenting intermediate values (81.64 vs. 80.29 vs. 78.40 vs. 76.96 g/bird/d, respectively for CON, SCP2, SCP4 and SCP6; P<0.05; linear trend: P<0.01). As for FCR, a linear trend could be observed even though no significant difference among groups emerged. In the finisher phase (30-42 d), no significant difference was observed for DWG and FCR, although CON birds presented higher DFI than SCP6 (191.1 vs. 188.7 vs. 186.6 vs. 183.7 g/bird/d, respectively for CON, SCP2, SCP4 and SCP6; P<0.05; linear: P<0.01). Final BW was not affected by the dietary treatments (3083 vs. 3058 vs. 2988 vs. 2942 g, respectively for CON, SCP2, SCP4 and SCP6; P=0.07), although a significant linear trend was observed (P<0.01). In the overall trial period, DWG tended (P=0.07) to be negatively affected by increasing dosages of SCP (72.15 vs. 71.55 vs. 69.83 vs. 68.79 g/bird/d, respectively for CON, SCP2, SCP4 and SCP6; linear: P<0.01). DFI was similar among groups, yet a significant linear trend was detected (109.1 vs. 108.0 vs. 106.9 vs. 106.3, g/bird/d, respectively for CON, SCP2, SCP4 and SCP6; linear: P<0.05). No significant difference was observed for FCR and mortality.

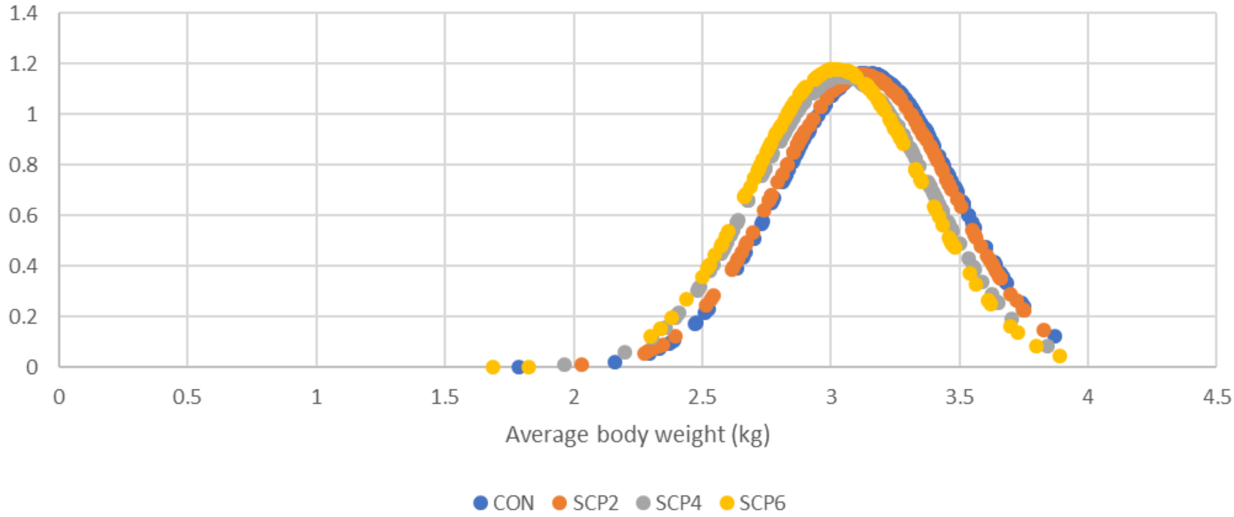
<u>Table 37</u>. Growth performance of broiler chickens fed a conventional soybean-based diet (CON) or diets with different dosages of single-cell proteins (2%, SCP2; 4%, SCP4; and 6%, SCP6) during grower and finisher phases.

Trait	Group								SEM	*P*-value		
	CON		SCP2		SCP4		SCP6			Group	Linear	Quadratic
Chick weight (g/bird)	41.63		41.53		41.43		41.63		0.79	n.s.	n.s.	n.s.
Starter (0-14 d)												
BW (g/bird)	434.5		420.8		429.9		430.1		22.46	n.s.	n.s.	n.s.
DWG (g/bird/d)*	27.95		27.33		28.08		27.89		1.59	n.s.	n.s.	n.s.
DFI (g/bird/d)*	36.36		35.90		35.12		36.14		1.63	n.s.	n.s.	n.s.
FI (kg/bird)*	0.509		0.503		0.492		0.506		0.02	n.s.	n.s.	n.s.
FCR*	1.302		1.313		1.255		1.298		0.05	0.09	n.s.	n.s.
Mortality (%)	0.56		1.11		3.89		1.67		0.11	n.s.	n.s.	n.s.
Grower (15-29 d)												
BW (g/bird)	1665		1640		1616		1590		67.06	0.10	**0.01**	n.s.
DWG (g/bird/d)*	81.64	a	80.29	ab	78.40	ab	76.96	b	3.43	**0.03**	**<0.01**	n.s.
DFI (g/bird/d)*	115.7		114.9		115.8		114.0		3.76	n.s.	n.s.	n.s.
FI (kg/bird)*	1.736		1.724		1.737		1.711		0.06	n.s.	n.s.	n.s.
FCR*	1.419		1.433		1.480		1.483		0.08	n.s.	n.s.	n.s.
Mortality (%)	0.56		0.59		0.56		0.00		0.07	n.s.	n.s.	n.s.
Finisher (30-42 d)												
BW (g/bird)	3083		3058		2988		2942		125.7	0.07	0.01	n.s.
DWG (g/bird/d)*	109.4		110.3		107.5		105.9		5.80	n.s.	n.s.	n.s.
DFI (g/bird/d)*	191.1	a	188.7	ab	186.6	ab	183.7	b	5.78	**0.05**	**<0.01**	n.s.
FI (kg/bird)*	2.484	a	2.452	ab	2.426	ab	2.388	b	0.08	**0.05**	**<0.01**	n.s.
FCR*	1.750		1.712		1.742		1.737		0.08	n.s.	n.s.	n.s.
Mortality (%)	0.63		0.00		0.00		0.63		0.06	n.s.	n.s.	n.s.
Entire feeding trial (0-42 d)												
BW (g/bird)	3083		3058		2988		2942		125.7	0.07	**0.01**	n.s.
DWG (g/bird/d)*	72.15		71.55		69.83		68.79		3.01	0.07	**0.01**	n.s.
DFI (g/bird/d)*	109.1		108.0		106.9		106.3		3.17	n.s.	**0.05**	n.s.
FI (kg/bird)*	4.729		4.679		4.655		4.604		0.12	n.s.	**0.03**	n.s.
FCR*	1.603		1.596		1.619		1.626		0.06	n.s.	n.s.	n.s.
Mortality (%)	1.67		1.67		4.44		1.67		0.12	n.s.	n.s.	n.s.

BW: body weight; DWG: daily weight gain; DFI: daily feed intake; FI: feed intake; FCR: feed conversion ratio.
* corrected for mortality; A, B: P<0.01; a, b: P<0.05.
n.s.: not significant.

Individual BW analysis revealed that the groups presented comparable BW uniformity (**Figure 5**). Analogously, the incidence and severity of FPD were similar among groups (**Figure 6**).

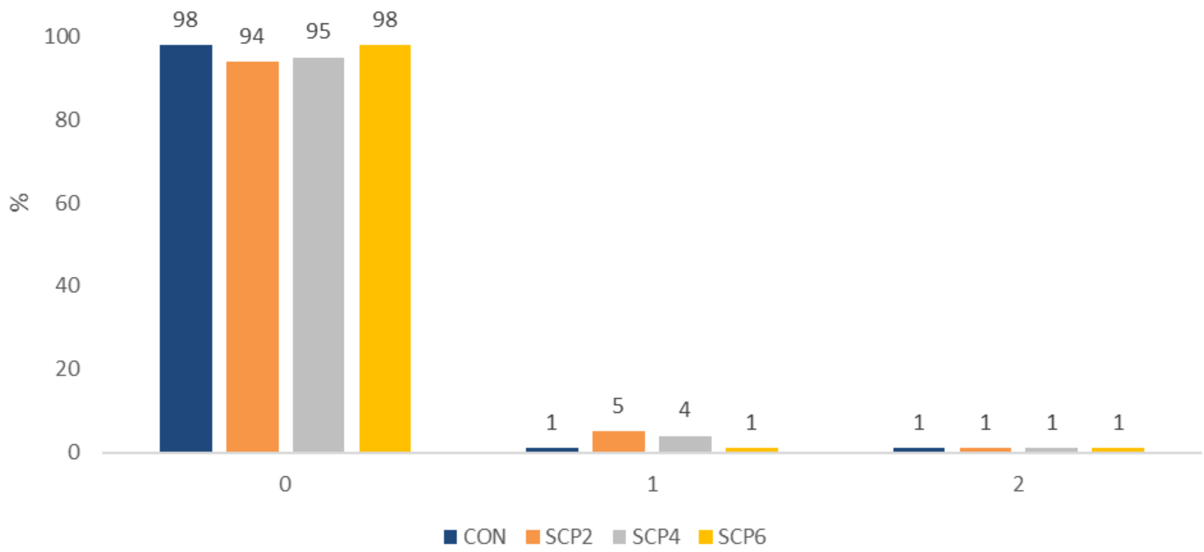
Figure 5. Individual BW distribution of broiler chickens at 42 d of age fed a conventional soybean-based diet (CON) or diets with different dosages of single-cell proteins (2%, SCP2; 4%, SCP4; and 6%, SCP6) during grower and finisher phases.



	CV (%)	*P*-value			
		vs. CON	*vs.* SCP2	*vs.* SCP4	*vs.* SCP6
CON	**10.9**	1.00	n.s.	n.s.	n.s.
SCP2	**11.1**	-	1.00	n.s.	n.s.
SCP4	**11.4**	-	-	1.00	n.s.
SCP6	**11.2**	-	-	-	1.00

n.s.: not significant.

Figure 6. Incidence and severity of footpad dermatitis in 42-d-old broiler chickens fed a conventional soybean-based diet (CON) or diets with different dosages of single-cell proteins (2%, SCP2; 4%, SCP4; and 6%, SCP6) during grower and finisher phases.



n: CON: 138; SCP2: 137; SCP4: 135; SCP6: 139. X^2 *P*-value = n.s.

Breast meat technological properties showed limited variations in response to the dietary treatments, with only the yellowness that increased as the dietary SCP level increased (7.67 vs. 8.57 vs. 9.11 vs. 10.38, respectively for CON, SCP2, SCP4 and SCP6; P<0.01; **Table 38**). Analogously, the proximate composition of breast meat was similar among experimental groups (**Table 39**).

Table 38. Technological traits of breast meat (*n*=12 breasts/group) of broiler chickens fed a conventional soybean-based diet (CON) or diets with different dosages of single-cell proteins (2%, SCP2; 4%, SCP4; and 6%, SCP6) during grower and finisher phases.

	CON	SCP2	SCP4	SCP6	SEM	*P*-value
Ultimate pH (pHu)	5.81	5.86	5.87	5.85	0.02	n.s.
Lightness - L*	55.83	54.59	55.02	53.86	0.34	n.s.
Redness - a*	2.65	2.64	2.58	2.09	0.17	n.s.
Yellowness - b*	7.67 B	8.57 AB	9.11 AB	10.38 A	0.22	**<0.01**
Drip loss (%)	3.24	2.75	2.24	2.89	0.19	n.s.
Cooking loss (%)	22.93	22.00	22.02	23.64	0.58	n.s.
Shear Force (kg)	1.49	1.49	1.39	1.37	0.05	n.s.

n.s.: not significant.

Table 39. **Proximate composition of breast meat (*n*=12 breasts/group) of broiler chickens fed a conventional soybean-based diet (CON) or diets with different dosages of single-cell proteins (2%, SCP2; 4%, SCP4; and 6%, SCP6) during grower and finisher phases.**

	CON	SCP2	SCP4	SCP6	SEM	*P*-value
Moisture (%)	76.13	76.23	75.94	76.27	0.17	n.s.
Crude protein (%)	22.14	21.53	21.79	21.89	0.12	n.s.
Total fat (%)	1.83	1.75	1.85	1.73	0.06	n.s.
Ash (%)	1.33	1.30	1.30	1.22	0.03	n.s.

n.s.: not significant.

As for plasma metabolome at 21 d (**Table 40**), 11 metabolites out of 54 identified showed significant variations in response to the dietary administration of SCP. At 42 d (**Table 41**), five molecules showed significant difference among the experimental groups.

Table 41. **Concentration (mmol/L) of plasma metabolites (identified through the ^{1}H-NMR analysis) showing significant differences in 21-d-old broilers fed a conventional soybean-based diet (CON) or diets with different dosages of single-cell proteins (2%, SCP2; 4%, SCP4; and 6%, SCP6) during grower and finisher phases.**

	CON		SCP2		SCP4		SCP6		SEM	*P*-value
Threonine	2.06E+00	A	1.81E+00	A	1.70E+00	AB	1.36E+00	B	6.44E-02	**<.001**
Betaine	1.37E+00	B	1.76E+00	A	1.40E+00	B	1.44E+00	B	4.67E-02	**<0.01**
Glycine	9.29E-01	b	1.15E+00	a	9.19E-01	b	1.01E+00	ab	2.76E-02	**<0.01**
Isoleucine	1.39E-01	B	1.49E-01	AB	1.77E-01	A	1.74E-01	A	4.89E-03	**<0.01**
Leucine	2.58E-01	b	2.48E-01	b	2.94E-01	ab	3.19E-01	a	8.94E-03	**0.01**
Glycerol	3.80E-01	ab	2.61E-01	b	2.99E-01	ab	5.49E-01	a	3.68E-02	**0.02**
2-Hydroxybutyrate	1.34E-01	b	4.06E-01	a	2.65E-01	ab	3.35E-01	ab	3.48E-02	**0.03**
Asparagine	5.74E-01	a	5.34E-01	ab	4.34E-01	b	4.69E-01	ab	1.86E-02	**0.03**
Ethanol	3.21E-02	b	5.35E-02	ab	3.70E-02	ab	6.12E-02	a	4.13E-03	**0.03**
Formate	1.21E-01	b	1.46E-01	ab	1.59E-01	a	1.52E-01	ab	5.01E-03	**0.04**
Uridine	2.80E-02	b	3.46E-02	ab	4.10E-02	ab	4.66E-02	a	2.56E-03	**0.05**

A,B: P<0.01; a,b: P>0.05.

Table 42. Concentration (mmol/L) of plasma metabolites (identified through the ^{1}H-NMR analysis) showing significant differences in 42-d-old broilers fed a conventional soybean-based diet (CON) or diets with different dosages of single-cell proteins (2%, SCP2; 4%, SCP4; and 6%, SCP6) during grower and finisher phases.

	CON		SCP2		SCP4		SCP6		SEM	*P*-value
Uridine	2.25E-02	C	3.09E-02	BC	3.49E-02	B	4.86E-02	A	1.74E-03	**<0.001**
Arginine	4.50E-01	A	4.41E-01	A	3.93E-01	A	2.43E-01	B	2.14E-02	**<0.001**
2-Aminobutyrate	4.60E-02	B	4.38E-02	B	3.92E-02	B	6.95E-02	A	3.02E-03	**<0.001**
Arabinose	1.05E-01	B	1.08E-01	B	1.23E-01	A	1.16E-01	AB	1.94E-03	**<0.01**
trans-4-Hydroxy-L-proline	1.87E-01	b	2.22E-01	ab	2.31E-01	ab	2.40E-01	a	6.98E-03	**0.03**

A, B: P<0.01; a, b: P<0.05.

In the ceca, only two metabolites showed variations at 21 d (**Table 43**). At 42 d, the number of metabolites whose concentration was significantly affected by the treatment increased to 10 (**Table 44**). The lower number of metabolites showing variations in response to SCP administration compared to previous studies on insect meal or microalgae meal corroborates the limited changes observed in growth performance of birds fed diets with SCP. However, some metabolites play important functions on bird metabolism and this can help us to better understand the effects of SCP administration in broiler chickens.

Table 43. Concentration (mmol/L) of cecal metabolites (identified through the ^{1}H-NMR analysis) showing significant differences in 21-d-old broilers fed a conventional soybean-based diet (CON) or diets with different dosages of single-cell proteins (2%, SCP2; 4%, SCP4; and 6%, SCP6) during grower and finisher phases.

	CON		SCP2		SCP4		SCP6		SEM	*P*-value
3-Phenylpropionate	3.76E-03	ab	3.56E-03	ab	3.27E-03	b	5.44E-03	a	1.62E-03	**0.03**
Glutamine	4.61E-03	ab	3.83E-03	b	6.01E-03	a	4.90E-03	ab	1.57E-03	**0.05**

a, b: P<0.05.

Table 44. Concentration (mmol/L) of cecal metabolites (identified through the ^{1}H-NMR analysis) showing significant differences in 42-d-old broilers fed a conventional soybean-based diet (CON) or diets with different dosages of single-cell proteins (2%, SCP2; 4%, SCP4; and 6%, SCP6) during grower and finisher phases.

Metabolite	CON		SCP2		SCP4		SCP6		SEM	*P*-value
Isovalerate	3.31E-03	A	2.73E-03	A	2.22E-03	AB	1.54E-03	B	1.12E-03	**<0.001**
2-Oxoisocaproate	2.96E-03	A	2.99E-03	A	2.57E-03	AB	2.04E-03	B	6.70E-04	**<0.001**
Glutamate	4.21E-02	AB	4.63E-02	A	3.62E-02	B	3.31E-02	B	9.37E-03	**<0.001**
Lysine	8.09E-03	AB	7.23E-03	B	6.15E-03	B	9.58E-03	A	2.38E-03	**<0.002**
Phenylacetate	1.30E-03	A	8.08E-04	B	1.01E-03	AB	7.78E-04	B	4.31E-04	**<0.01**
Aspartate	2.08E-02	ab	1.64E-02	b	1.75E-02	ab	2.27E-02	a	5.50E-03	**0.01**
Tyramine	1.68E-03	b	1.81E-03	b	2.75E-03	ab	3.16E-03	a	1.37E-03	**0.01**
Propionate	8.14E-02	a	5.72E-02	b	5.95E-02	b	7.02E-02	ab	2.14E-02	**0.01**
Betaine	1.27E-03	b	1.30E-03	ab	1.70E-03	ab	1.97E-03	a	7.06E-04	**0.03**
Serine	5.41E-03	ab	4.77E-03	b	6.90E-03	a	6.00E-03	ab	2.00E-03	**0.04**

A, B: P<0.01; a, b: P<0.05.

Regarding the cecal microbiota, at phylum level, the most abundant taxa was Firmicutes in both time points (**Table 45** and **Table 46**). At the end of the rearing cycle (**Table 46**), Firmicutes increased in groups SCP2 and SCP4 compared to CON while Bacteroidetes decreased in the same groups.

Table 45. Mean relative abundance (%) at phylum level in cecal content of 21-d-old broilers fed a conventional soybean-based diet (CON) or diets with different dosages of single-cell proteins (2%, SCP2; 4%, SCP4; and 6%, SCP6) during grower and finisher phases.

PHYLUM (%)	Experimental groups				*P*-value t-Test					
	CON	SCP2	SCP4	SCP6	CON vs SCP2	CON vs SCP4	CON vs SCP6	SCP2 vs SCP4	SCP2 vs SCP6	SCP4 vs SCP6
Firmicutes	97.5	97.4	92.1	95.3	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Bacteroidetes	0.00	0.00	1.60	0.00	n.s.	n.s.	.	n.s.	n.s.	n.s.
Tenericutes	1.60	1.50	1.50	1.60	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Proteobacteria	0.80	1.10	4.70	3.10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Actinobacteria	0.00	0.00	0.10	0.00	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

n.s.: not significant.

Table 46. Mean relative abundance (%) at phylum level in cecal content of 42-d-old broilers fed a conventional soybean-based diet (CON) or diets with different dosages of single-cell proteins (2%, SCP2; 4%, SCP4; and 6%, SCP6) during grower and finisher phases.

PHYLUM (%)	Experimental groups				*P*-value t-test					
	CON	SCP2	SCP4	SCP6	CON vs SCP2	CON vs SCP4	CON vs SCP6	SCP2 vs SCP4	SCP2 vs SCP6	SCP4 vs SCP6
Firmicutes	84.8	91.7	92.4	88.9	**<0.01**	**<0.01**	n.s.	n.s.	n.s.	n.s.
Bacteroidetes	13.2	6.30	5.80	9.30	**<0.01**	**<0.01**	n.s.	n.s.	n.s.	0.09
Tenericutes	1.10	1.00	1.20	1.40	n.s.	n.s.	n.s.	n.s.	**0.03**	n.s.
Proteobacteria	0.70	0.90	0.60	0.20	n.s.	n.s.	**0.03**	n.s.	n.s.	**0.03**
Actinobacteria	0.10	0.20	0.00	0.10	n.s.	**0.04**	n.s.	0.08	n.s.	0.09
Verrucomicrobia	0.10	0.00	0.00	0.00	n.s.	n.s.	n.s.	.	.	.

n.s.: not significant.

At the genus level, only few differences among experimental groups were appreciable at 21 days (**Table 47**). At 42 days, many genera were significantly modulated by the dietary treatment with SCP (**Table 48**). Specifically, large differences were observed between CON and SCP6 groups, while SPC2 and SPC4 were substantially similar.

Table 47. Mean relative abundance (%) at genus level in cecal content of 21-d-old broilers fed a conventional soybean-based diet (CON) or diets with different dosages of single-cell proteins (2%, SCP2; 4%, SCP4; and 6%, SCP6) during grower and finisher phases.

GENUS (%)	Experimental groups				*P*-value t-test					
	CON	SCP2	SCP4	SCP6	CON vs SCP2	CON vs SCP4	CON vs SCP6	SCP2 vs SCP4	SCP2 vs SCP6	SCP4 vs SCP6
Faecalibacterium	15.9	11.6	9.60	6.90	n.s.	0.07	**<0.01**	n.s.	0.10	n.s.
Ruminococcus	7.40	9.30	9.00	9.70	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Oscillospira	5.40	6.00	5.10	4.80	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Bacillus	1.70	2.00	1.40	1.10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Lactobacillus	1.30	3.20	3.60	2.50	n.s.	n.s.	0.08	n.s.	n.s.	n.s.
Coprococcus	1.20	2.30	1.50	1.40	**<0.01**	n.s.	n.s.	n.s.	**0.02**	n.s.
Blautia	1.20	0.60	0.80	0.60	0.10	n.s.	0.09	n.s.	n.s.	n.s.
Dorea	1.10	0.90	0.80	0.80	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Anaeroplasma	0.70	0.50	0.60	0.50	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Coprobacillus	0.60	0.60	0.80	0.50	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Clostridium	0.30	0.70	2.40	1.80	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Dehalobacterium	0.10	0.10	0.10	0.00	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Anaerostipes	0.00	0.20	0.40	0.60	**<0.01**	**<0.01**	**0.01**	**0.03**	**0.04**	n.s.
Lachnospira	0.00	0.00	0.10	0.10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Enterococcus	0.00	0.00	0.70	0.10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Bacteroides	0.00	0.00	1.60	0.00	.	n.s.	.	n.s.	.	n.s.
Candidatus Arthromitus	0.00	0.00	2.40	0.00	n.s.	n.s.	.	n.s.	n.s.	n.s.

n.s.: not significant.

<u>Table 48</u>. Mean relative abundance at genus level in cecal content of 42-d-old broilers fed a conventional soybean-based diet (CON) or diets with different dosages of single-cell proteins (2%, SCP2; 4%, SCP4; and 6%, SCP6) during grower and finisher phases.

GENUS (%)	Experimental groups				*P*-value t-test					
	CON	SCP2	SCP4	SCP6	CON vs SCP2	CON vs SCP4	CON vs SCP6	SCP2 vs SCP4	SCP2 vs SCP6	SCP4 vs SCP6
Bacteroides	13.2	6.30	5.80	9.30	**<0.01**	**<0.01**	n.s.	n.s.	n.s.	0.09
Faecalibacterium	9.70	10.1	9.20	4.20	n.s.	n.s.	**<0.01**	n.s.	**<0.001**	**<0.01**
Ruminococcus	6.40	7.20	7.90	7.20	n.s.	**0.02**	n.s.	n.s.	n.s.	n.s.
Oscillospira	3.00	3.40	2.90	1.90	n.s.	n.s.	**<0.001**	n.s.	**<0.001**	**<0.01**
Coprococcus	1.60	1.50	1.40	1.20	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Lactobacillus	1.50	2.60	2.10	2.50	n.s.	n.s.	**0.04**	n.s.	n.s.	n.s.
Bacillus	1.00	0.80	0.70	0.40	n.s.	n.s.	**0.01**	n.s.	0.09	n.s.
Dorea	0.80	0.80	0.60	0.50	n.s.	n.s.	0.06	n.s.	**0.02**	n.s.
Blautia	0.40	0.60	0.50	0.20	n.s.	n.s.	**0.02**	n.s.	**<0.01**	**0.03**
Dehalobacterium	0.40	0.30	0.30	0.20	n.s.	n.s.	**<0.001**	n.s.	**<0.01**	**0.02**
Clostridium	0.40	0.20	0.20	0.30	n.s.	0.06	n.s.	n.s.	n.s.	n.s.
Coprobacillus	0.20	0.30	0.40	0.20	n.s.	**0.02**	n.s.	n.s.	n.s.	**0.03**
Anaeroplasma	0.10	0.00	0.00	0.00	**<0.05**	0.09	n.s.	n.s.	n.s.	n.s.
Akkermansia	0.10	0.00	0.00	0.00	n.s.	n.s.	n.s.	.	.	.
Turicibacter	0.10	0.20	0.30	0.30	n.s.	**<0.01**	**<0.05**	n.s.	n.s.	n.s.
Anaerostipes	0.10	0.20	0.40	0.20	**<0.05**	**<0.01**	**0.05**	0.07	n.s.	n.s.
Eubacterium	0.00	0.00	0.50	0.20	n.s.	n.s.	0.08	n.s.	0.08	n.s.

n.s.: not significant.

7. In-field validation

According to the outcomes of the different lab-scale trials presented in this deliverable, a field trial on broiler chickens, involving approximately 8,000 birds, is currently ongoing to validate the effects of the dietary substitution of soybean with the most promising alternative protein source according to the aim of the project (i.e. to find alternative to soybean in poultry diets), namely insect meal at a dosage of 9%. In this study, the growth performance as well as slaughter yields will be monitored. At the same time, insights into product quality and safety will be carried out.

8. References

AOAC (1990). Association of Official Analytical Chemists. Official methods of analysis. 15th ed. Washington, DC, USA.

Beski, S. S., Swick, R. A., and Iji, P. A. (2015). Specialized protein products in broiler chicken nutrition: A review. Animal Nutrition, 1(2), 47-53.

Ekstrand, C., Carpenter, T. E., Andersson, I., and Algers, B. (1998). Prevalence and control of foot-pad dermatitis in broilers in Sweden. British Poultry Science, 39(3), 318-324.

FEFAC (2022). Feed and Food. Published February 2023. Available at https://fefac.eu/wp-content/uploads/2023/03/FF_2022_final.pdf

GLEAM (2017). Global Livestock Environmental Assessment Model. Available at http://www.fao.org/gleam/en/- http://www.fao.org/gleam/en.

Kim, S. W., Less, J. F., Wang, L., Yan, T., Kiron, V., Kaushik, S. J., and Lei, X. G. (2019). Meeting global feed protein demand: challenge, opportunity, and strategy. Annual review of animal biosciences, 7, 221-243.

Kluth, H., and Rodehutscord, M. (2006). Comparison of amino acid digestibility in broiler chickens, turkeys, and Pekin ducks. Poultry science, 85(11), 1953-1960.

Mottet, A., and Tempio, G. (2017). Global poultry production: current state and future outlook and challenges. World's Poultry Science Journal, 73(2), 245-256.

Ritchie, H., and Roser, M. (2021). Forests and Deforestation. Published online at OurWorldInData.org. Retrieved from: https://ourworldindata.org/forests-and-deforestation

Zampiga, M., Calini, F., and Sirri, F. (2021). Importance of feed efficiency for sustainable intensification of chicken meat production: implications and role for amino acids, feed enzymes and organic trace minerals. World's Poultry Science Journal, 77(3), 639-659.