POULTRYNSECT

Diet chemical composition and report on *Hermetia illucens* larvae safety

Deliverables 3.1 and 3.2

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Abbreviations	
AA	Amino Acid
ASV	Amplicon Sequence Variant
BSF	Black Soldier Fly
BSFL	Black Soldier Fly Larvae
CP	Crude Protein
DM	Dry Matter
EAA	Essential Amino Acid
FA	Fatty Acid
FM	Fresh Matter
NEAA	Not Essential Amino Acid
RT PCR	Real Time Polymerase Chain Reaction
WP	Work Package

Content

Introduction

Introduction

The POULTRYNSECT Work Package 3 "**Laboratory and Sensorial Analyses**" aims to evaluate the impact of Black Soldier Fly live larvae (BSFL) inclusion as feed ingredient in chicken diet on chicken health and meat quality.

In order to characterize the nutritional quality and to verify the feed safety of the BSFL used for poultry trials, chemical and microbiological analysis should be performed. Concerning nutritional composition of the BSFL, the protein and fat compositions are impacted by what larvae consume. The natural variation among individuals and batches can be significant in fact, looking in literature, commercially available BSFL (sold as animal or pet feed) from the same company had values ranging from 31.7% to 47.6% crude protein and 11.8–34.3% fat in different studies (Bussler et al., 2016).

From a nutritional point of view, the amino acid (AA) spectrum is also an important parameter for the biological value of a protein source. The AAs like lysine, low in cereal proteins that are key staples in diets around the world, are very well represented in some insect species (Bukkens, 2005), and a same consideration could be done for the AAs tryptophan and threonine.

BSFL can process organic side streams into nutritious insect biomass, yielding a sustainable ingredient for animal feed. In processing such organic residues, the larvae impact the substrate and its microbiota. The microbiological safety of the insects and substrates remains a point of concern. Substrate-associated bacteria can dominate the larval gut microbiota, but the larvae can also alter the bacterial community in the substrate. However, their role relative to the feed substrate in shaping the bacterial community is unknown (Schreven et al. 2022).

The results of the assessment of chemical composition, amino acid (AA) composition, microbiological safety and microbiota composition of the BSFL produced in the Task 1.3, utilising the rearing substrate (diet) identified in Task 1.2 are summarized in this report.

The report, that joins the **results** obtained for deliverables 3.1 and 3.2, is focused on analysis carried out on different BSFL batches provided by the INAGRO partner that were delivered at the poultry facility centre of the UNITO partner and used to fed a medium growing chicken breed .

1. Material and Methods

All feeding trials were performed in accordance with the "Standard protocol for performing insect feeding trials" that was developed in the "Susinchain" project (European Union's Horizon 2020 research and innovation programme under grant agreement No. 861976). Feeding experiments start with five day old larvae of around 3 mg and results are compared to the larval performance on the Gainesville diet which consists of 67% water, 17% wheat bran, 6.6% maize flour and 9.9% alfalfa.

Larvae of the black soldier fly (*Hermetia illucens*) were grown on a mixture consisting of 30% Farm 1 Crumble (Hobby First) and 70% water. The first 5 days were placed in a propagator (polyethylene, 60 cm x 40 cm x 11 cm, Transoplast) 300 000 larvae grown on 5 kg of feed. The larvae were then distributed per 20,000 over different crates and were given 14 kg of feed per breeding box. The climatic conditions were kept constant at a temperature of 27 °C and a humidity of 70 %. The larvae were harvested after 14 days with an automatic vibrating sieve (Eco Separator, Russellfinex). The mesh width was 3 mm.

The larvae were frozen at –18°C immediately after harvest and then freeze-dried (Büchi, L200) for 48 hours. After the drying step, the samples were finely ground in a cutting mill (Moulinex, AR110830) until a fine powder was obtained.

Proximate composition

Dry matter content: Determination of the dry matter (DM) content was performed in a ventilated oven (Memmert,UF110) at 105°C for 17 hours. Each determination was performed in triplicate.

Fat content: The fat content was determined by a Soxhlet extraction. The solvent used was petroleum ether (boiling range 45 – 60 °C). Each determination was performed in triplicate.

Protein content: The method of Kjeldahl was used for the determination of the protein content. The digestion was carried out in a rendering furnace (Gerhardt, Kjeldatherm) and the distillation with a distillation apparatus (Gerhardt, Vapodest). 6.25 was used as the conversion factor. Each determination was performed in triplicate.

Chitin content: The chitin was isolated according to a procedure described by Liu et al. (2012) and then determined gravimetrically. Because the isolated chitin was almost colorless, the destaining with $KMnO_4$ was not included in the procedure. Every provision was made in performed in triplicate.

Ash content: The determination of ash content was performed in a muffle furnace (Nabertherm, B 180) at 550 °C. It was dried until a constant mass was obtained. Every provision was made in performed in triplicate.

Carbohydrate content: The carbohydrate content was calculated according to:

Carbohydrate content (%) = (1 – (ωfat + protein + ash + ωchitin)) ×100%

Amino acid composition

AA composition of insect feed and BSFL was estimated after hydrolysis in 6 M HCl for 22 h at 110 ◦C and analysed using HPLC and fluorescence detection (Cohen and Michaud, 1993). Values are expressed as g/100g of crude protein (CP)

Microbiological analysis

The potential presence of three pathogens species (*Campylobacter* spp., *Listeria monocytogenes* and *Salmonella* spp.) were investigated using microbiological and real time PCR (RT-PCR) assays.
Campylobacter spp*, L. monocytogenes* and *Salmonella* spp detection was evaluated according to ISO 10272-1:2006, ISO 11290–1, 2017 and ISO 6579, 2002 reference methods, respectively.
Moreover*, L. monocytogenes* and *Campylobacter* spp. were searched in feed and larvae samples by SureFast® PLUS RT-PCR assay (R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions.
The RT-PCR assay was also applied to identify the presumptive *L. monocytogenes* and *Campylobacter* spp. colonies grown in the culture plates.
The DNA of feed and larvae samples and colonies was extracted by the SureFast PREP bacteria kit (R-Biopharm) and all the RT-PCR amplification reactions were performed on an Eco Real-Time PCR System (Illumina, San Diego, CA, USA).

Microbiota composition

Total DNA from feed and BSFL samples was extracted using the RNeasy Power Microbiome KIT (Qiagen. Milan. Italy) following the manufacturer's instructions. RNase (Illumina Inc. San Diego. CA) was used to digest RNA in the DNA samples with an incubation of 1 h at 37 °C. DNA was quantified using the Qubit ds assay and standardized at 5 ng/µL. The extracted DNA was used to amplify the V3-V4 region of the 16S rRNA gene (F: 5'- CAGCCTACGGGNGGCWGCAG-3'; R: 5'-GAGACAGGACTACHVGGGTATCTAATCC-3'). PCR products were purified and tagged according to the Illumina metagenomic standard procedure (Illumina Inc., San Diego, CA, USA). Sequencing was performed with a MiSeq Illumina instrument with V3 chemistry and generated 250 bp paired-end reads in accordance with the manufacturer's instructions.

Bioinformatics and Statistical Analysis

After sequencing, .fastq files were imported in QIIME v.2 (Bolyen et al., 2019). Sequence adapters and primers were trimmed by using cut adapter and DADA2 algorithm was used to trim low quality reads, remove chimeric sequences, and to obtain amplicon sequence variants (ASVs). Taxonomic assignment was performed by QIIME feature-classifier plugin against the Greengenes 16S rRNA gene database. Taxonomy assignment for 16S was double checked on BLAST (Basic Local Alignment Search Tool) suite tools; ASVs tables display the highest taxonomic resolution reached.

2. Results & discussion

The proximate composition of BSFL fed with poultry feed (Farm 1 Crumble) is showed in **Table** . Values are expressed as % of the fresh matter (FM) for the DM while all the other parameters are expressed as % of DM. The highest content of nutrient is the CP (41.9%, calculated utilising a 6.25 nitrogen conversion factor), followed by carbohydrates (26.1%), crude fat (14.5%) and ash (11.7%). The most interesting parameter is related to the CP content that is very similar to the CP of soybean (usually around 42%), one of the protein source most utilised in poultry nutrition. Moreover, BSFL showed a very high content of fat that according to the fatty acid (FA) analysis reported in the Deliverables of the WP1 are characterized by a high percentage of saturated FAs mainly dominated by lauric acid (C12:0), which have demonstrated prebiotic effects on the microbiota of livestock and antibiotic effects on gastrointestinal disease-causing bacteria.

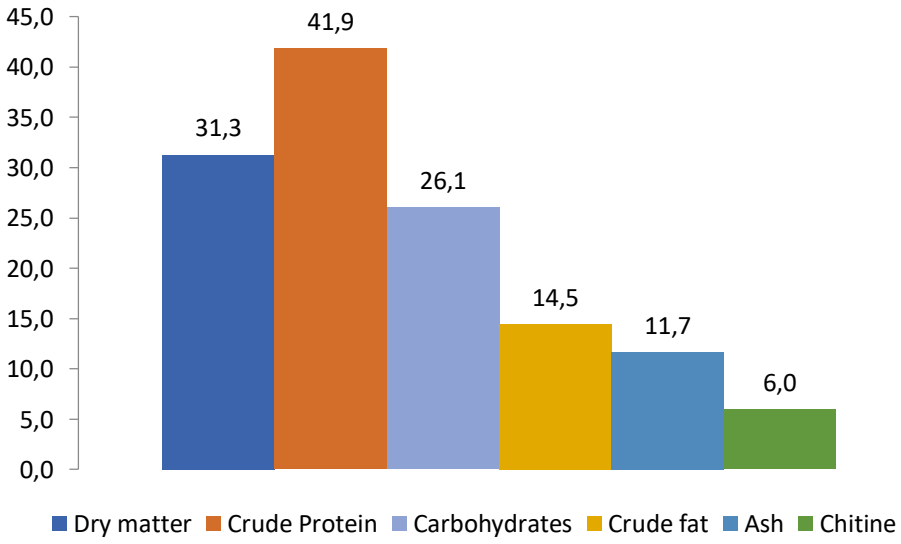


Figure 1: Proximate composition of BSFL fed with poultry feed (Farm 1 Crumble)

The amino acid (AA) composition of poultry feed (Farm 1 Crumble) and BSFL reared on this substrate are reported in **Table** . Values are expressed as g/100g of crude protein. In the same table a comparison within common protein sources (fish meal and soybean meal) utilised in animal feeds is showed. Concerning the AA profile the BSFL showed an interesting spectrum with values for the main essential AA (EAA) comparable, and in some cases (i.e. histidine, valine) higher, to those of common protein sources (fish meal and soybean meal) utilised in poultry nutrition. As far as non EAA (NEAA), the BSFL profile showed for the most part of the NEAA higher values in particular if compared to fish meal.

Table 1: Amino acid composition of the larvae substrate (poultry feed), BSF larvae and classical feed protein sources. Data are expressed as g/100 g crude protein

Amino acid (AA)	Poultry feed	BSFL	FM*	SBM*
Arginine	5.8	5.4	5.8	7.3

Histidine	2.5	3.3	2.2	2.7
Isoleucine	3.9	4.6	4.3	4.6
Leucine	8.2	7.1	7.0	7.7
Lysine	5.6	7.1	7.5	6.2
Methionine	1.8	2.0	2.8	1.4
Phenylalanine	5.0	4.3	3.8	5.1
Threonine	3.8	4.1	4.1	3.8
Tryptophane	1.5	1.6	1.1	1.4
Valine	4.8	6.1	4.9	4.8
Essential Amino Acid (EAA)	**42.9**	**45.7**	**43.5**	**45.0**
Alanine	5.3	7.6	6.1	4.3
Aspartic Acid	9.4	9.7	8.7	11.3
Cystine	2.3	1.3	0.8	1.6
Glutamic Acid	20.6	12.5	12.6	17.9
Glycine	4.6	5.9	5.9	4.2
Proline	7.0	5.9	3.8	5.0
Serine	5.0	4.8	4.0	4.6
Tyrosine	2.9	6.6	2.9	3.5
Non Essential Amino Acid (NEAA)	**57.1**	**54.3**	**44.8**	**52.4**

Abbreviations: BSFL; Black Soldier Fly Meal; FM; Fish Meal; SBM; Soybean Meal

***From Feedipedia https://www.feedipedia.org/**

The results of the microbial analysis of BSFL and poultry feed (Farm 1 Crumble) are reported in Table 2. From a feed safety point of view, results showed that none of the tested pathogens were found neither in the feed nor in the larvae. A far as the *Listeria* spp, therefore including different species such as *L. innocua*, is concerned its presence has been detected in all samples but successive RT PCR analysis did not detect the presence of the pathogen species *L. monocytogenes*.

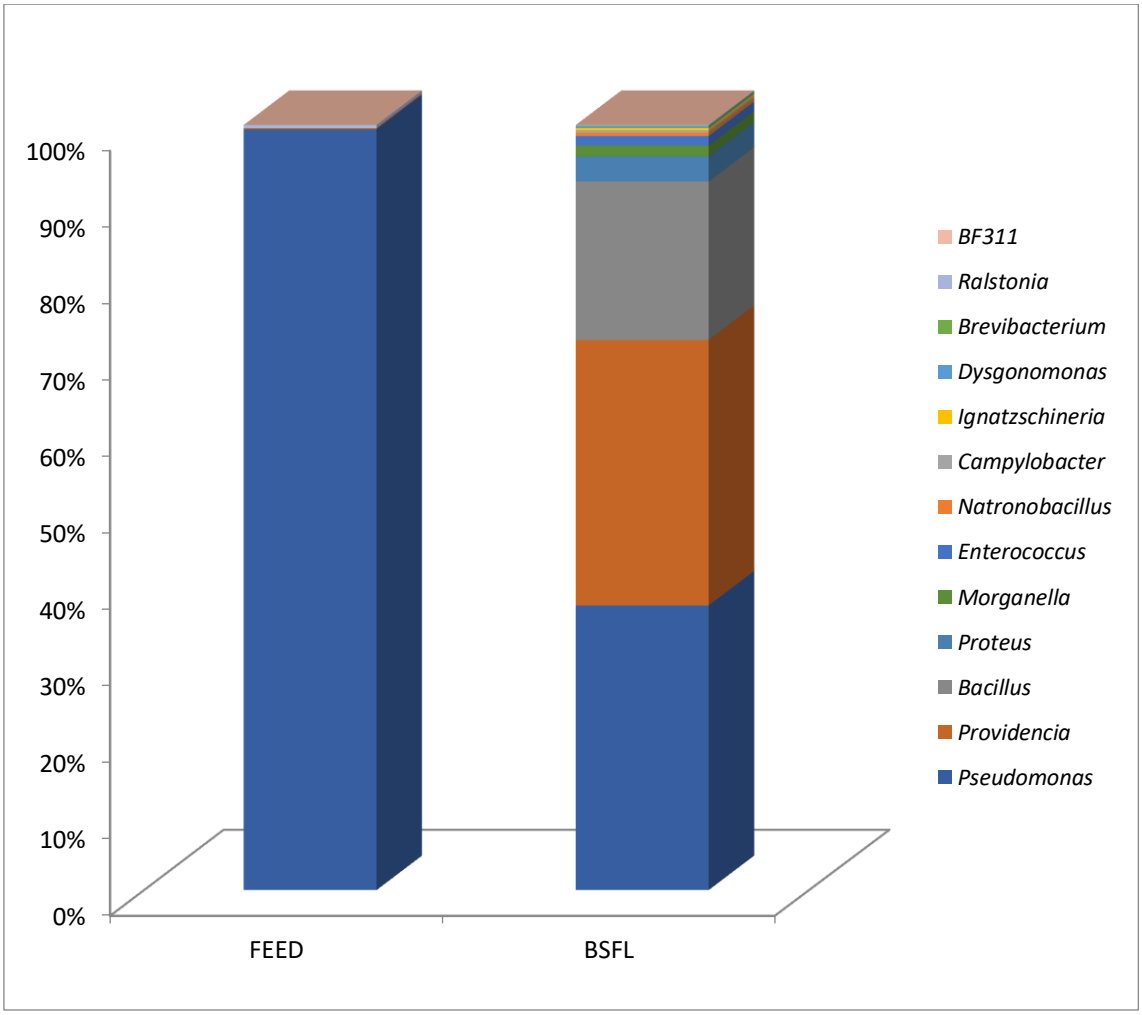
Table 2: Microbial analysis of the selected pathogens in the larvae substrate and BSFL.

Bacterial strain	Official Method	Sample	Quantity (g)	Result
Campylobacter spp.	ISO 10272_1	BSFL I batch	10.0	Absent
		BSFL II batch	5.2	Absent
		Feed Farm 1 Crumble	8.0	Absent
Listeria monocytogenes	ISO 11290_1	BSFL I batch		
		BSFL II batch	6.0	Absent*
		Feed Farm 1	6.6	Absent*
		Crumble	8.0	Absent*
Salmonella spp.	ISO 6579	BSFL I batch	9.4	Absent
		BSFL II batch	7.7	Absent
		Feed Farm 1 Crumble	8.2	Absent

Abbreviations: BSFL; Black Soldier Fly Larvae; * Presence of *Listeria spp*

The microbiota of feed and BSF larvae reported as ASV(%) is reported in **Figure 2**. It appears that the BSF larvae showed a bacterial richness evidenced by the presence of 13 bacterial strains with the most abundant genus represented by *Pseudomonas* (34 %), *Providencia* (32 %) and *Bacillus* (19 %) while the poultry feed seems exclusively dominated by the genus *Pseudomonas* (98 %).

Figure 2: Amplicon Sequence Variant (%) of poultry feed (Farm 1 Crumble) and Black Soldier Fly Larvae



3. Conclusion

Based on the nutritional and microbiological analysis performed in these Tasks, we can affirm that the Black soldier fly larvae batches produced in the WP1 by the INAGRO partner could be surely defined as nutritious and safety for the feed purposes.

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