Interreg North-West Europe

EUROPEAN UNION

ValuSect

European Regional Development Fund

Final report

Impact of substrate on the proximate composition of insects

2023

This report was written in context of the Interreg North-West Europe ValuSect project.

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1. Report background

This report is generated based on the results obtained after the research work carried out in the WPT2 (Quality improvement of insect processing, activity 1: Determining the impact of substrate on insect composition). This report is aimed to be a continuation of the report created within the research activities carried out in WPT1 (Quality improvement of the primary insect production process, activity 2: Testing side streams as substrate for insects). At early stages of the project it was clearly observed that the synergies and interaction between the two work packages were evident, therefore experimental trials in WPT1 were planned to understand the impact of substrate composition on the performance of the insects, but also to be able to answer how this is affecting the final composition of the animals intended to be used for food applications.

In a previous literature review[1] published by the Valusect team, it was highlighted how the diet is having a massive impact on the final insect composition. Very briefly, in this extensive document, it was detailed that in fact edible insects (*Tenbrio molitor, Acheta domesticus and Locusta migratoria*) are rich sources of proteins, fats, vitamins and minerals; however, it was also mentioned that the nutritional value of the insects varies significantly depending on key aspects such as insect species, life phase, climatic conditions, harvesting and processing technologies and specially diet.

In addition to this, one of the main focus of Valusect project was to explore the utilization of side streams as potential source of feed for edible insects[2], as published in this literature review. In this document it was reported that several agri-food co-products and by-products have the potential to be used as feeding material for insects, aiming to increase the re-circularity and to optimise the use of the natural resource to produce the massive amount of proteins we need to feed an increasing global population. In this sense and as reported in detail in WPT1 specific side streams were selected to be used as a substrate for insect growing. As part of the research work, it was investigate the availability and production scale of the selected side streams, as reported in the next table:

Table 1: Overview of the availability of the selected side streams (estimated, in tonnes/year) per country included in the ValuSect project.

	The UK	**The Netherlands**	**Belgium**	**Ireland**	**Switzerland**
Fruit & vegetables	unknown	unknown	12 528	-	unknown
Potato steam peels	80 350	196 000	148 000	5 768	5 850

[1] CHAPTER 1: EDIBLE INSECTS: NUTRITIONAL VALUE, BIOACTIVE FUNCTIONALITY, TOXICITY AND ALLERGIC POTENTIAL

[2] Van Peer, M.; Frooninckx, L.; Coudron, C.; Berrens, S.; Álvarez, C.; Deruytter, D.; Verheyen, G.; Van Miert, S. Valorisation Potential of Using Organic Side Streams as Feed for *Tenebrio molitor*, *Acheta domesticus* and *Locusta migratoria*. *Insects* **2021**, *12*, 796. https://doi.org/10.3390/insects12090796

Red blood cell fraction (porcine blood)	9187-13 780	13 368-20 053	9433 - 14150	100-200	2144 - 3216
Feathers	85 275	45 071	22 871	11.000-12.000	4 620
Grain middlings (wheat and spelt bran)	1 898 000	135 000	231 000	71 000	70 000
Foliage (Tomato, cucumber, chillies and pepper)	11 160 – 18 600	109 860 – 183 100	21 090 – 35 150	600 - 1000	8910 – 14 850
Forced chicory roots	-	45 900	36 000	-	-
Grassy biomass	657 000	800 000 – 1 200 000	639 000	-	73 000

Therefore, an experimental design was conducted to determine the viability, as feed source, of some of these side streams and also the impact on the insect performance and finally the impact on the insect composition. A summary of the feeding trial conducted is shown in the next Table 2 and Table 3. The reader is advised to refer to WP1.2 report to get more details about the diet composition, the level of inclusion of each side stream and the methodology implemented for growing, harvesting and processing the insects. Also, an explanation describing why two different types of diet were employed (wet and dry), can be found.

Table 1: overview of the experiments conducted when using dry feed

Side stream	**Insect species**	**Partner conducting the experiment**
Chicken feather meal	*Tenebrio molitor*	Thomas More
	Acheta domesticus	
Hydrolysed chicken feather meal	*Tenebrio molitor*	Inagro
	Acheta domesticus	
Red blood cells	*Tenebrio molitor*	Aberystwyth University
	Acheta domesticus	

Table 3 overview of the experiments conducted by each partner involved

Side stream	**Insect species**	**Partner conducting the experiment**
Oat bread ends	*Tenebrio molitor*	Aberystwyth University
	Acheta domesticus	
Oat husks	*Tenebrio molitor*	Aberystwyth University
	Acheta domesticus	

Horticultural foliage	*Tenebrio molitor*	Inagro
	Acheta domesticus	
Potato cuttings	*Tenebrio molitor*	Thomas More
	Acheta domesticus	
(Fermented) Forced chicory roots	*Tenebrio molitor*	Inagro
	Acheta domesticus	
Vegetables mix	*Tenebrio molitor*	Thomas More
	Acheta domesticus	

2. Methodology

With the objective of determining the impact of diet on insect composition the next analysis were conducted. Since the analysis were performed by different partners, the methodology can vary slightly between feeding trials. However, it was ensure that the results were harmonized and the methodologies employed, although different, were providing the same results in rode to be able to compare the data sets generated by each partner. Whether two different methods have been employed, both methods will be detailed in this section (as per Table 2) :

2.1. Dry matter content

Dry matter content was determined in an oven at 105°C for 24h until constant weight was achieved. The, the difference between fresh weight and dry weight was calculated.

2.2. Protein content

Crude protein content was determined by the nitrogen present in the samples by either the Kjeldahl (Thomas More) or the Duncan (Teagasc) method (LECO FP628 (LECO Corp., MI, USA) according to the Dumas method as described in the AOAC method 992.15) on the dried samples. A conversion factor of 5.33 was employed as agreed by the partners and in agreement with most recent scientific literature.

2.3. Fat content

Fat contents was determined either by total lipid extraction using petroleum ether (BP 40 – 60 °C) using Soxhlet equipment (Thomas More) or by means of Oracle NMR rapid Fat Analyser (CEM Corporation USA) according to AOAC method 2008.06.

2.4. Ash content

Ash content was determined according to AOAC method 920.153 (AOAC, 1920) using a muffle furnace (Carbolite, Sheffield, England) at 550°C.

2.5. Chitin/fiber content

Thomas More method:

Fibre content included the determination of NDF and CF, following the extraction protocols of Gerhardt (Gerhardt, Fibrebag system - manual), based on the Van Soest method (Van Soest et al., 1991). Neutral Detergent Fibre determination: A weighted sample of 1g was placed in a dedicated fibre bag (Gerhardt, ADF/NDF bag). The fibre bag filled with sample was defatted in petroleum ether (technical, boiling point 40 – 60 °C, VWR Chemicals) and dried in atmosphere. Defatted sample was extracted in a neutral detergent fibre solution with heat-stable alpha-amylase at boiling temperature for 1,5 hour. Subsequently, the sample was washed with boiling, demineralised water and dried overnight at 105°C. The weight of the dried fibre bag with residue was determined, followed by an ashing at 550°C for 4 hours in a muffle furnace. The weight of the ashes was determined and fibre content was calculated. Crude Fibre and chitin determination: A weighted sample of 1g was placed in a dedicated fibre bag (Gerhardt, CF bag). The fibre bag was filled with sample, defatted in petroleum ether and dried in atmosphere. Defatted sample was first extracted in acidic solution of 0,13M sulphuric acid for 30 minutes at boiling temperature. Extracted fibre bag with sample was washed in boiling, demineralised water and subsequently extracted in alkaline solution of 0,23 M potassium hydroxide. Extraction times differ from 30 minutes for CF-analysis in substrate and 2 hours for chitin determination in insects. Extracted fibre bag with sample was washed with boiling, demineralised water and dried overnight at 105°C. The dried bag with residue was weighted and ashed at 550°C for 4 hours in a muffle furnace. Ashes were weighted and fibre content was calculated. Total carbohydrate content is determined based on calculations. Non fibre-carbohydrates (NFC) is calculated using NDF (% NFC = 100% - (% CP + % NDF + % EE + % Ash)). Nitrogen-Free Extract (NFE) is calculated based on crude fibre determination (% NFE= % DM – (% EE + % CP + % Ash + % CF)).

Teagasc method:

The total dietary fibre (TDF), including insoluble dietary fibre (IDF) and soluble dietary fibre (SDF), was determined according to AOAC 991.43 [28] (enzymatic–gravimetric method) using Ankom automated dietary fibre analyser (Ankom, USA). Samples were dispersed in MES-TRIS buffer and were treated sequentially with a heat-stable α-amylase for 30 min at 100°C, while

being treated in sequence with a protease for 30 min at 60°C, and finally treated with an amyloglucosidase for 30 min at 60°C. Finally, the mixture was filtered in pre-weighed crucibles and the IDF was measured directly. From the filtrated portion, the SDF was precipitated with 95% ethanol at 4 × volume and then filtered. Both residues (insoluble and soluble) were washed with 78% ethanol, 95% ethanol, and acetone respectively, and then dried, weighed, and corrected by protein and ash content

2.6. Fatty Acid profile

Thomas More Method:

Fatty acids were determined with a Chrompack CP 9002 equipped with flame ionization detector and an SLB-IL111 (60 m × 0.25 mm i.d., Sigma-Aldrich) capillary column. Fatty acid methyl esters (FAMEs) were prepared from the extracted lipids by esterification in a methanolic KOH solution with the addition of a 20% BF3-MeOH solution according to Joseph and Ackman (1992). Fatty acids were identified by comparing their retention time with a 37 FAME-mix standard. (Chem-lab) or by comparing their MS-spectrum with online libraries such as NIST-database. A qualitative analysis was performed by calculation of the area-percentage of a peak relative to the total peak-area of the chromatogram.

Aberystwyth method:

Each freeze-dried sample (200 mg) was homogenised with 1 mL methanol, followed by the addition of 100 µL surrogate standard containing 100 µg heneicosanoic acid. Samples were vortexed for 1 min, supplemented with 2 mL chloroform, vortexed for an additional 2 min and subjected to centrifugation at 3000 rpm for 15 min. The supernatant was transferred to a separate tube and 3 mL 2:1 chloroform:methanol was added to the pellet, followed by vortex for 2 min and centrifugation at 3000 rpm for 15 min. The supernatants were then combined, followed by the addition of 2.25 mL 0.88% potassium chloride and mixing. The layers were allowed to settle, the upper layer was discarded and 2.25 mL 1:1 methanol: 0.88% potassium chloride was added, mixed and allowed to settle. The lower layer was passed through a sodium sulphate column. The cleaned sample was evaporated under nitrogen at 40 °C. Finally, 2 mL hexane was added to dissolve the lipid extract.

Basic methylation reagent was prepared containing 0.5 M sodium methoxide in methanol and methyl-acetate, followed by heating at 50 °C for 10 min. Extracted lipids in hexane were supplemented with 40 µL basic methylation reagent, incubated for 5 min and stopped with 60 µL 0.26 M oxalic acid in diethyl ether, followed by vortex for 20 sec. Samples were incubated for 3 min and subjected to centrifugation at 3000 rpm for 5 min. The supernatant was transferred to a clean tube containing 1 g anhydrous $CaCl_2$ and incubated for 30 min, followed by centrifugation at 3000 rpm for 5 min. The supernatant was then evaporated under nitrogen at 40 °C. Acid methylation reagent was prepared containing 1% (v/v) sulphuric acid in methanol. Dried samples were supplemented with 1.5 mL acid methylation reagent and incubated at 50 °C for 30 min. The solution was cooled, followed by the addition of 3 mL 5% (w/v) sodium chloride and

2 mL hexane, shaken for 1 min and subjected to centrifugation at 3000 rpm for 5 min. The upper organic phase was washed with 1.5 mL 2% (w/v) sodium bicarbonate and subjected to centrifugation at 3000 rpm for 5 min. The upper hexane soluble phase was added to 1 g sodium sulphate, followed by vortex for 20 sec and allowed to stand for 30 min. The sample was subjected to centrifugation at 3000 rpm for 5 min, then transferred to 200 µl glass vials with methyl-nonadecanoate as internal standard.

Fatty acid methyl ester samples were run on an Agilent 7890B GC with a Leco Pegasus BT time-of-flight mass spectrometer. The GC was equipped with a CP-Sil 88 capillary column (Agilent CP7489, 100m x 0.25mm x 0.2µm). The carrier gas was Helium at a flow of 0.7ml/min. The temperature gradient started at 100 'C for 4 minutes, ramped up to 200 'C at 25 'C/min with a hold at 200 'C for 8 minutes, followed by ramp up to final temp of 240 'C at 10 'C/min with a hold of 6 mins. The inlet and transfer line temperature were both set to 240°C. One µl sample was injection at a split ratio of 1:100.

Data were analysed using the ChromaTOF software from Leco. Samples were compared to standard solutions run under the same conditions and peaks identified by both retention time and mass spectra. A dilution series of a Supelco 37 component FAME mix (Part #:CRM47885) was used for quantification.

2.7. Total amino acid profile

Thomas More Method:

Amino acid profiling was performed according to AOAC methods, described by Costa et al. (2020). 50mg of the dried, grinded mealworm sample was weighted into a glass tube. Protein hydrolysis was performed with 3 mL of 6M HCl with 0.1% phenol added, at 110 ◦C for 22 h under nitrogen atmosphere. The samples were filtrated through a 2-3 µm pore filter and 6 mL of 6 M NaOH was added. After adjusting the volume to 25 mL with ultrapure water, sample was taken for HPLC analysis with norvaline and sarcosine as internal standard. Total amino acids were separated by a high performance liquid chromatography (Agilent 1290 Infinity II HPLC with multisampler, Agilent) using an online, pre-column ortophthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) derivatization and an AdvanceBio AAA column (4.6 × 100 mm, 2.7 µm). A solvent gradient was used. Solvent A existed of a 10mM Na2HPO4 + 10mM Na2B4O7 solution at pH 8.2. Solvent B was a mixture of acetonitrile/methanol/water 45:45:10. Detection was performed in the UV region at 338 nm and 262 nm with a DAD detector.

Aberystwyth method:

Total nitrogen content, indicative of protein content, was measured using the standard Pig Swill or aspartic acid 2 methods on an Elementar rapid MAX N exceed using approximately 200 mg of each sample.

The free amino acid concentration of samples before protein digestion was measured using a modified ninhydrin assay. Dry samples were weighed to 100 mg and resuspended in 1 mL dH_2O. A TCA cleanup was performed by the addition of 1 mL 20% TCA, for a final TCA concentration of 10%. Samples were incubated with TCA on ice for

1 h followed by centrifugation at 3,000 × g for 10 min. Supernatants were neutralised with 1 M NaOH. Aliquots were diluted up to 200 µL in dH_2O. To each sample, 100 µL cyanide acetate buffer and 100 µL ninhydrin solution (2%) were added. Samples were immediately incubated at 100 °C for 15 min, then cooled on ice. Finally, 1 mL isopropanol:water (1:1, v/v) was added and absorbance was measured at 570 nm against an L-histidine standard curve.

2.8. Mineral and heavy metals profile

Thomas More Method:

Minerals and heavy metals were analyzed with graphite furnace atomic absorption (AAS) (Thermo Scientific, Thermo Scientific, ICE 3000 series with GFS Furnace Autosampler) after acid digestion in HNO3/HCl (1:3 V/V) solution with a microwave (CEM, MARS 5). Heavy metals that were determined are Cd, Cr and Pb.

Teagasc method:

All plastic material used in analysis, were dipped in 10% HNO3 for 24 hours, thoroughly washed with Milli-Q water and dried in an oven before use. A weight of 0.5 g of homogenized sample was added to the Teflon vessels, and then 5 mL of concentrated HNO3 was added and allowed to be pre-digested for 15 minutes. Then, samples were digested using the CEM-Xpress microwave accelerated unit at up to 200 °C for 20 min in closed vessels. Digested samples were diluted to 50 mL with ultra-pure deionized water. Certified reference samples, reagent blanks and spiked samples were included in each batch of digestion and treated the same manner. The calibration curve was prepared using Ca, Zn, Fe, Se, Co, K, Na, P, Mg and V stock solution (Sigma Aldrich) with appropriate dilution. After that, the sample was aspirated into the Agilent 7900 ICP-MS analyzer.

2.9. Disclaimer

All the results provided in this report were conducted following international standard methods or well stablished methods in the scientific literature; however, in any case, these results were generated in a certified lab and therefore these can't be use for any other purpose to the ones stated in this report. No legal claims can be made by using the contents of this report.

3. Main results

For more clarity the results have been divided in the impact of dry weight of all diets tested, followed by proximate composition, fatty acid profile, amino acid profile and mineral content.

3.1. Impact of diet on dry weight.

The results obtained for the dry matter for crickets and mealworms under different type of diets (protein rich and wet) are shown in Tables 3, 4, 5 and 6.

a) Protein rich diets

As it can be observed, no differences were found when crickets were reared using feather meal or red cells compared to the control diet, i.e. high protein diets. However, as described before, although he same rearing practices were followed, it could be seen that insects produced by Thomas More/Inagro were significantly higher on dry matter, meaning that small differences in the environment and manual operation could contribute, to some extend to these differences.

When analysing the results obtained for mealworms, it could be observed, in this case, that increasing the amount of feather meal had minimal impact on the final dry mass, although only significant when 15% of this protein source was included in the diet. When hydrolysed feather meal was employed, no differences were observed between inclusion levels o, control diet or feather meal.

More interestingly, when feeding mealworms with increased concentration of red cell, it could be clearly seen a tendency in where higher protein content in the diet was leading to a higher content of dry mass, being this value of 26% for control diets and 34% for the diets containing 20% of red cells as feeding. These results, in agreement with those reported in WP1.2 report; indicate that mealworms have a higher feed conversion ratio for this type of proteins. Similar results were observed for conversion ratios for red cells by crocket, although the results were not so evident due to huge standard variation in the samples.

These results can be explained due to differences between amino acid profile of the protein sources (feather and red cells), since the amino acid profile for red cells includes all the essential amino acids in higher quantities and therefore there is not expected to see amino acid limitations, considering the high amount lysine (3% of total amino acids). Feathers, on the contrary, is very poor source of lysine, histidine and other essential amino acids.

Table 3: Dry matter content of crickets reared on protein-rich side streams. Mean ± Standard Deviation. N = 3

Substrate	Dry Matter (g/100g)
Control Feather Meal	26,6 ± 1,7
Feather Meal 5%	27,4 ± 2,4
Feather Meal 10%	27,5 ± 1,6
Feather Meal 15%	26,0 ± 3,3
Feather Meal 20%	27,2 ± 0,9
Control Red Cells*	21,3 ± 0.1
Red blood cells 12.5%*	22,7 ± 0,1
Red blood cells 25%*	21,9 ± 0,1

Table 4: Dry matter content of mealworms reared on protein-rich side streams. Mean ± Standard deviation. N = 3

Substrate	Dry Matter (g/100g)
Control Feather Meal	29,3 ± 0,7
Feather Meal 5%	28,6 ± 1,0
Feather Meal 10%	30,3 ± 2,9
Feather Meal 15%	32,4 ± 2,7
Feather Meal 20%	30,0 ± 3,2
Control hydrolysed feather meal	30,0 ± 0,1
Hydrolysed Feather Meal 5%	30,7 ± 0,6
Hydrolysed Feather Meal 10%	30,9 ± 1,1
Hydrolysed Feather Meal 15%	31,0 ± 1,2
Hydrolysed Feather Meal 20%	31,5 ± 0,4
Control Red Blood Cells	25,9 ± 0,1
Red Blood Cells 5%*	28,7 ± 0,3
Red Blood Cells 10%*	32,8 ± 0,4
Red Blood Cells 15%*	30,4 ± 0,4
Red Blood Cells 20%*	34,2 ± 0,8

b) Wet diets

When determining the impact of the diet on dry weight if crickets after using wet diets, it was detected that vegetable mix and control diets reported similar lower values compared to the other diets. On the other hand, it was observed that those batches fed with fermented chicory roots and horticulture foliage had a significant higher dry weight to this found on the control diet or the other diets. In any case, the results observed, were always significantly lower to those reported for high protein diets. Indicating that proteins play a vital role when it comes to transform biomass into insect body weights.

When wet diets were provided to mealworms, it was determined that potato cuttings feeding was the one giving the highest dry weight, while the other diets tested were not statistically different to control diets.

Table 5: Dry matter content of crickets reared on wet streams. Mean ± Standard Deviation. N = 3

Substrate	Dry Matter (g/100g)
Control Wet Feed Thomas More	22,9 ± 0,8
Potato Cuttings	26,4 ± 0,3
Vegetables Mix	21,7 ± 0,3
Control Wet Feed Inagro	25,0 ± 0,7

Fermented Chicory Roots	27,7 ± 0,6
Horticultural Foliage	28,4 ± 0,2

Table 6: Dry matter content of mealworms reared on wet side streams. Mean ± Standard Deviation. N = 3

Substrate	Dry Matter (g/100g)
Control Wet Feed	28,3 ± 2,3
Fermented Chicory Roots	30,8 ± 1,6
Horticultural Foliage	28,9 ± 2,7
Potato Cuttings	35,3 ± 1,6
Vegetables Mix	29,4 ± 2,9

It could be concluded then, that when maximising dry weight on crickets, wet streams seems (but vegetable mix) to perform equally than high protein content diets when feathers are used. Meanwhile for mealworms, higher protein content (20% red cells) and potato cutting are the most promising diets.

These results may indicate that by modulating the amount of protein and fibre in the insects diet, is it possible to manipulate the dry mass, which could be beneficial to boost the insect biomass productions by providing to them those diets with higher conversion rates. Avoiding amino acid limitations and potentially developing ingredients blends able to provide all the essential nutrients for the insects requires more in depth investigation, since very little is known about the actual insect dietary requirements. However, it seems that providing enough protein is essential to a good insect development, more specially for mealworms, since crickets seems not to be that impacted by the diet, in terms of dry weight.

3.2. Impact of diet on macronutrients

After analysing the dry matter content, is it of relevance to determine the composition of such dry matter. As it was detailed, the main nutrients provided by insects are proteins, fat and fibre, therefore, the diets provided to the insects have to be able, not only to maximise conversion rate and dry weight, but also to generate a final product with the desired content of these three main ingredients, depending on the final application. For this reason, these three nutrients, togheter with ash content were analysed more in detail, as reported in Tables 7, 8, 9, and 10. In general terms, and depending on the diet, the more fat is detected in the dry body mass, less protein is detected, and vice versa.

a) Fat content

High protein diet

When describing the impact of rich protein diets on the fat content of mealworms and crickets different patterns were identified. In the case of crickets, higher amounts of feather meal was

highly correlated with a decrease in the fat content of the dry matter. In this sense, this value changed from 22% in the control diet, to 11% in the diet including the highest amount of feather meal. For the same species, no differences were observed in fat content, regardless the amount of red cell included in the diet.

On the other hand, mealworm fed with feather meal or hydrolysed feather meal, regardless the concentration, shown no differences in fat content, with values ranging from 18 to 20%. On the contrary. Mealworms fed with increasing concentration of red cell had increasing amounts of fat on their body composition (dry weight), moving from 13% using a control diet up to 22% when adding 20% of red cells to the substrate.

Wet diet

Similarly, the use of potato cuttings reported the highest fat content, dry basis, for crickets (compared to control diet) while no other differences were observed when comparing trial diets and controls. With the exception of vegetable mix, which yielded the dry biomass with the lowest value for this parameter (9.3%).

Wet diets fed to mealworms reported a similar results to these observed for crickets, where potato cuttings feeding led to the highest fat content of all the trials (35%), followed by a remarkable difference by chicory roots (20%).

b) Protein content

High protein diet

Increasing the protein content in the diet of crickets, had significant effect on the final protein content of the insect biomass, in detriment of fat content. When compared to the control diets, a higher protein content was found for both red cell and feather meal inclusion at any level.

In the case of the mealworms, no impact on protein content was observed for neither the feather nor the feather meal, with no significant differences when compared to the control. However, the increment of red cell proteins in the substrate, although minimal, had a negative impact on the protein content of the final biomass, being the highest one the one determined for the control diet (53,4%), in opposition to 20% red cells diet (50,0%), in favour of higher fat content.

Wet diet

As described, the diets providing crickets and mealworms with the highest fat content, were the same ones giving as a result insects with the lowest protein content. Based on the findings observed when using high protein diets, it can be hypothesised that wet substrates with richer protein content (and balanced amino acid profile) are the ones generating crickets with higher protein content and mealworms with higher fat content.

This findings strongly suggest that fat and protein content in the insect body composition can be modulated by modifying the diet, although the trends observed impacted in a different way for crickets and mealworms. For the first species, higher proteins seems to lead to higher protein and lower fat in the body. However, for mealworms it works in the opposite direction, since increasing protein content in the feeding has no impact on fat or protein content, or if any effect

is observed, is towards an increment of fat content in detriment of protein abundance. AS described in the previous section, these finding lead to think that insect body composition can be easily manipulated by designing specific diets or substrates, but not only considering the macronutrients, but also the composition on fatty acids and amino acids. It has been seen how protein sources with a complete amino acid profile (red cells) have different impact on these parameters when compared to a proteins source with deficiencies in essential amino acids. Also, requires special attention, that this impacts needs to be assessed individually for each insect species, since differences in metabolism, digestive systems and dietary preferences are playing a key role when determining the final body composition.

Only mealworm larvae fed with potato cuttings or fermented chicory roots had undergone considerable changes. A possible explanation for this outcome could be the fact that besides the chicory roots, also the potato cuttings were partly fermented (pH 4.7) making the starch/carbohydrates more digestible/available for the mealworm larvae. Meaning that pH may play a role in nutrients absorption, as also suggested for mineral availability.

Table 7: Proximate analysis of crickets reared on protein-rich side streams. Mean ± Standard Deviation. N = 3

Substrate	Ether extract (g/100g DM)		Crude protein (g/100g DM)		Crude ash (g/100g DM		Chitin (g/100g DM)		NFCH (g/100g DM)	
Control Feather Meal	21,8	±2,3	51,7	±2,8	4,8	±0,1	7,2	±0,6	14,5	±2,4
Feather Meal 5%	21,0	±0,9	51,2	±2,5	4,9	±0,2	6,9	±0,7	16,0	±2,1
Feather Meal 10%	21,1	±0,9	51,2	±2,5	4,9	±0,2	6,6	±0,7	16,1	±2,0
Feather Meal 15%	18,7	±3,6	57,0	±6,7	5,1	±0,4	7,8	±1,0	14,1	±3,4
Feather Meal 20%	10,7	±0,9	54,8	±2,6	5,0	±0,1	7,0	±0,5	14,5	±2,0
Control Red Cells*	10,6	±0,4	60,5	±0,7	5,3	±0,1	ND		23,4	±0,4
Red Cells 12.5%*	10,1	±1,8	62,6	±1,7	5,2	±0,2	ND		21,6	±0,7
Red Cells 25%*	10,1	±1,1	62,5	±1,4	5,2	±0,3	ND		22,1	±1,3

Table 8: Proximate analysis of mealworm reared on protein-rich streams. Mean ± Standard Deviation. N = 3

Substrate	Ether extract (g/100g DM)	Crude protein (g/100g DM)	Crude ash (g/100g DM)	Chitin (g/100g DM)	NFCH (g/100g DM)
Control Feather Meal	20,3 ± 1,1	51,9 ± 0,2	5,0 ± 0,5	7,2 ± 0,1	15,7 ± 1,5
Feather Meal 5%	18,0 ± 0,8	53,0 ± 1,1	4,8 ± 0,1	7,2 ± 0,4	17,1 ± 1,0
Feather Meal 10%	18,7 ± 1,1	51,3 ± 1,4	4,6 ± 0,2	7,0 ± 0,2	18,4 ± 1,8
Feather Meal 15%	20,2 ± 0,3	52,3 ± 0,7	4,3 ± 0,2	7,1 ± 0,5	16,2 ± 0,9
Feather Meal 20%	18,7 ± 0,3	52,4 ± 0,6	4,8 ± 0,4	7,2 ± 0,5	16,9 ± 1,4
Control Hydrolysed Feather Meal	18,7 ± 1,1	50,9 ± 0,2	4,6 ± 0,0	7,4 ± 0,6	18,4 ± 0,7
Hydrolysed Feather Meal 5%	19,1 ± 0,6	51,4 ± 0,7	4,5 ± 0,3	7,3 ± 0,1	17,8 ± 0,2
Hydrolysed Feather Meal 10%	19,3 ± 0,7	52,0 ± 0,5	4,1 ± 0,1	6,8 ± 0,1	17,9 ± 0,2
Hydrolysed Feather Meal 15%	18,9 ± 1,0	52,7 ± 0,7	4,2 ± 0,4	7,1 ± 0,1	17,0 ± 0,1
Hydrolysed Feather Meal 20%	18,6 ± 0,5	52,2 ± 0,0	4,2 ± 0,0	6,5 ± 0,7	18,6 ± 0,4
Control Red Blood Cells	12,9 ± 1,4	53,4 ± 0,8	5,6 ± 0,3	ND	28,2 ± 0,4
Red Blood Cells 5%	17,7 ± 1,7	50,6 ± 0,6	4,5 ± 0,1	ND	27,3 ± 1,6
Red Blood Cells 10%	17,7 ± 5,1	50,2 ± 3,4	4,2 ± 0,8	ND	27,9 ± 2,0
Red Blood Cells 15%	20,0 ± 0,9	49,7 ± 0,5	3,9 ± 0,1	ND	26,4 ± 1,3
Red Blood Cells 20%	22,1 ± 1,7	50,0 ± 0,5	3,5 ± 0,1	ND	24,4 ± 1,1

*analysed by Teagas

Table 9: Proximate analysis of crickets reared on wet streams. Mean ± Standard Deviation. N = 3

Substrate	Ether extract (g/100g DM)	Crude protein	Crude ash (g/100g DM)	Chitin (g/100g DM)	NFCH (g/100g DM)

	(g/100g DM)														
Control Wet Feed TM	11,8	±	0,5	63,4	±	0,4	6,8	±	0,8	8,7	±	0,2	9,4	±	1,3
Potato cuttings	16,9	±	0,8	61,3	±	0,7	5,3	±	0,0	7,5	±	0,2	9,0	±	1,3
Vegetables mix	9,3	±	0,6	66,1	±	0,7	6,5	±	0,2	8,2	±	0,3	9,9	±	1,3
Control Wet Feed Inagro	24,4	±	0,7	51,2	±	0,7	5,5	±	0,2	7,8	±	0,2	11,1	±	1,4
Forced Chicory Roots	23,0	±	0,5	49,5	±	0,4	5,5	±	0,2	6,4	±	0,1	15,6	±	1,1
Horticultural Foliage	23,5	±	1,7	50,7	±	0,5	5,2	±	0,3	6,7	±	0,1	13,9	±	1,6

Table10: : Proximate analysis of mealworms reared on wet side streams. Mean ± Standard Deviation. N = 3

Substrate	Ether extract (g/100g DM)			Crude protein (g/100g DM)			Crude ash (g/100g DM)			Chitin (g/100g DM)			NFCH (g/100g DM)		
Control Wet Feed	18,6	±	3,0	51,4	±	1,6	4,7	±	0,6	7,6	±	0,5	17,5	±	1,3
Fermented Chicory Roots	20,0	±	0,2	48,2	±	1,0	6,8	±	0,9	7,8	±	0,1	14,0	±	0,9
Horticultural Foliage	19,1	±	1,5	52,3	±	0,7	4,7	±	0,2	7,7	±	0,7	19,4	±	2,9
Potato Cuttings	35,5	±	1,6	40,7	±	0,5	3,7	±	0,3	6,1	±	0,2	17,2	±	1,0
Vegetables Mix	19,9	±	2,9	48,9	±	2,4	5,0	±	0,5	6,9	±	0,4	16,1	±	0,2

c) Impact on chitin and fibre content

The third main component of insects' biomass is fibre, mostly in the form of chitin. Fibre is present in insects most commonly in the form of chitin, which is an insoluble fibre and derives from the exoskeleton of the insects. Chitin is a modified polysaccharide (poly-beta-1,4-N-acetylglucosamine) containing nitrogen and it is much like the insoluble and indigestible polysaccharide cellulose found in plants.

For this component, no clear trends or differences were observed between the diets tested regardless the insect species. For crickets the chitin content varied between 6,4 and 8,7%, in dry basis; meanwhile for the mealworms this range was between 6,1 and 7,8% of dry weight.

Regarding the fiber content, which includes chitin, a similar lack of trend or correlation with the diet was observed for both insect species. Fiber content for mealworms varied between 23,3% and 27,9%; while for crickets this range was from 16.5 and 23,4%. Interestingly, it could be correlated, negatively, the fibre content of mealworms reared with an increasing concentration of red cells.

d) Impact on ash content

Ash content in the insects reared using high protein and wet diets will be discussed together. After analysing the data, no significant differences were observed from a technical point of view, since the ash content for crickets varied between 6,8% and 4,8%, regardless the type of diet. In the case of the mealworms, the ash ranged from 3,7 to 6,8 %. No clear patterns were identified when analysing the impact of feeding on ash content, with the exception of mealworms fed with increasing concentration of red cells. In this case a negative correlation between higher protein content in the substrate and lower ash content was identified. In any case, it will be more relevant to analyse the mineral profile obtained for the several feeding trial, rather than the total mineral quantities, since as described, these differences were not relevant from a nutritional point of view.

e) Correlation between macronutrients

In the next figures (Figure 1 and Figure 2), it was depicted the correlation between the main macronutrients analysed in this project. Figure 1 corresponds the crickets, and Figure 2 refers to mealworms. As previously described, for both species, the amount of fat is negatively correlated with the amount of protein found in the final body composition on dry basis. The results of the Pearse correlation test, for the significant ones, can be seen in Table 11.

Similar results were obtained for both species, in where the protein and fat content were negatively correlated, with R2 values of -0.87 and -0.89 for cricket and mealworms respectively ($p<0.01$). This supports the idea that when designing a production program for insects, it needs to be decided if the main goal would be to generate lipids or proteins, since different diets (as explained) will promote the accumulation of one or the other component.

Table 11: Pearse correlation values for protein vs fat, proteins vs dry weight and fat vs dry weight for crickets and mealworms

	Cricket	Mealworm
	Protein/Fat	
Pearse R2	-0.87	-0.89
p value	>0.01	>0.01
	Protein/Dry weight	
Pearse R2	-0.84	-0.60
p value	>0.01	>0.01
	Fat/Dry weight	
Pearse R2	0.77	0.73
p value	>0.01	>0.01

Very interestingly when analysing the correlation between protein content and dry weight, an strong negative correlation was found. It means that biomass generated from insects with high moisture content will be indicative of a final product with higher protein content. Reversely, a final product, which in origin had high levels of moisture, is indicative of low fat levels.

This is related on how the water molecules interact and accumulates in the insect bodies. Proteins have a good affinity for water, and therefore, insects with higher protein content in their bodies will be accumulating more water. On the other hand, insect reared using diets promoting fat accumulation, will have less protein in their bodies, and consequently, less moisture content. Moreover, the study revealed that using the by-products as wet feed had an effect on the nutritional profile of the T. molitor biomass. Specifically, providing the larvae with a high concentration of carbohydrates (i.e. potato cuttings) increased their fat content

These findings are capital to plan the diets and subsequent downstream scenario for protein or lipid extraction. If the goal is to generate large amounts of protein, drying process will be more costly from an energetic point of view (larger quantities of water need to be removed); while, on the contrary, the goal is to accumulate lipids in the insect bodies, the subsequent drying process could be more energetic efficient.

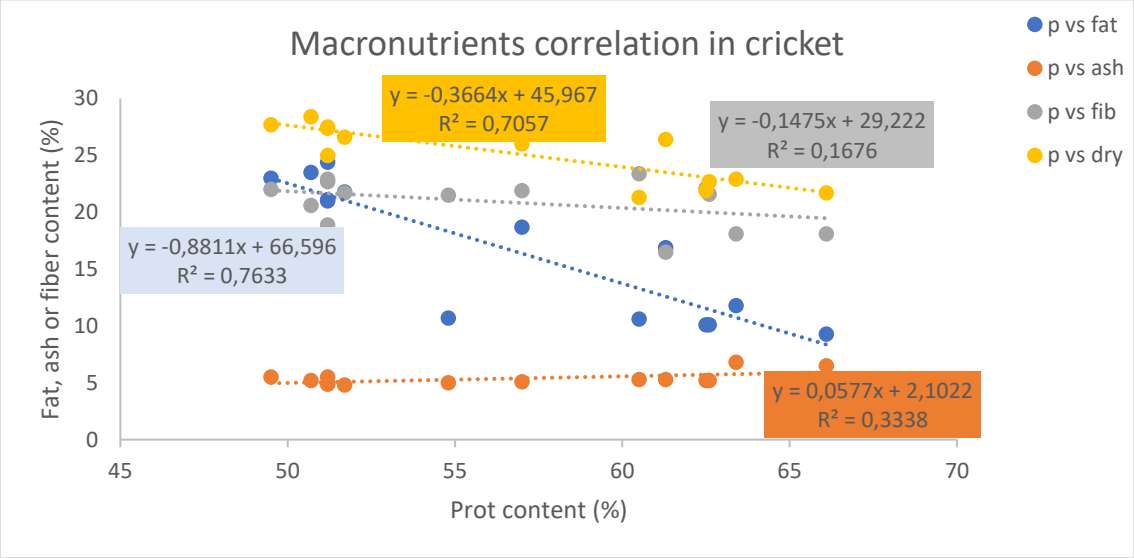

Figure 1: correlation between protein content and dry matter, fat, ash and fibre in crickets (n = 14 triplicates).

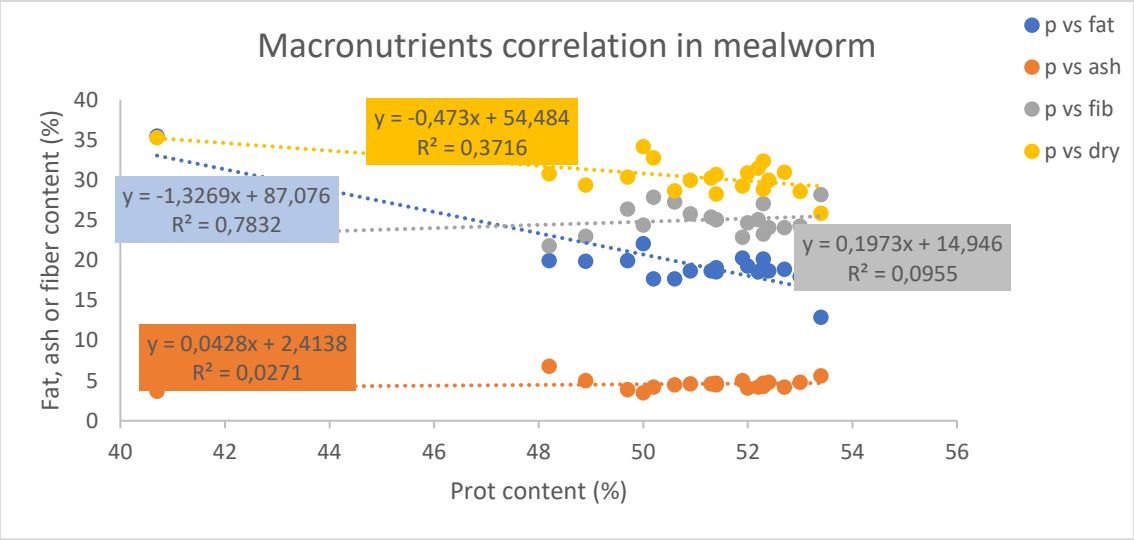

Figure 2: correlation between protein content and dry matter, fat, ash and fibre in mealworms (n = 20 triplicates).

3.3. Impact on fatty acid profile

Once the main macronutrients have been discussed, was part of the objectives of this work to investigate how the diets could affect, more in detail, the profile of specific compounds that are of relevance when assessing the nutritional potential of using insect proteins and fats as part of our diets. In this regard, it was determined the fatty acid profile and the amino acid profile, following the methodology described in Section 2.6.

As it has been previously reported, diet can affect the fat content, protein content and dry mass content. However, it needs to be determined if those changes are having also a reflection on the proportion of the fatty acids and how the balance between SFA (saturated fatty acid), MUFA (monounsaturated fatty acids) and PUFA (polyunsaturated fatty acids).

a) Impact on mealworms fatty acid profile

As described, mealworms were fed on two types of substrates. As previously described by several authors[3] the main faaty acids profile found in insects, regardless the species are palmitic acid (C16:0), oleic acid (C18:1) and linolenic acid (C18:2), which in general compromises more than the 80-86% of total fatty acids.

These results were confirmed in our trial, in where was clearly observed the predominance of these fatty acids, compared to the remaining ones. However, it was very interesting to observe that while, in mealworms, the amount of C16:0 remained practically constant was a very clear trend on an increased concentration of C18:1 as more proteins was added to the diet, in detriment to C18:2, which levels decreased as more protein was added to the substrate. It indicates that higher protein content in the feeding formulation is leading to fatty acid balance with higher content of MUFA and reduced amounts of PUFAs. For instance, the MUFA/PUFA ratio using standard diet, or a vegetable mix diet was of 0.61 and 0.66, respectively. However, when high protein content was employed (feather meal 20%) this ratio was of 0.95. When looking at the results coming from mono streams, a similar trend was observed, where potato peel yielded the fatty acid profile with a MUFA/PUFA ratio 1.63. In this case a lower value ratio is desirable, since is evidence of higher PUFA content in the insects dry product. In general, there was a high variation in this ratio, ranging from 0.60 to 1.63.

From a nutritional point of view, is relevant to increase the intake of unsaturated fatty acids, especially those classified as polyunsaturated. In this case, it seems that including high levels of proteins is detrimental for the fatty acid profile, since more C18:1 is accumulated in the insects tissues.

[3] Barroso, F. G., C. de Haro, M.-J. Sánchez-Muros, E. Venegas, A. Martínez-Sánchez and C. Pérez-Bañón (2014). "The potential of various insect species for use as food for fish." Aquaculture **422-423**: 193-201.
Zielińska, E., B. Baraniak, M. Karaś, K. Rybczyńska and A. Jakubczyk (2015). "Selected species of edible insects as a source of nutrient composition." Food Research International **77**: 460-466.

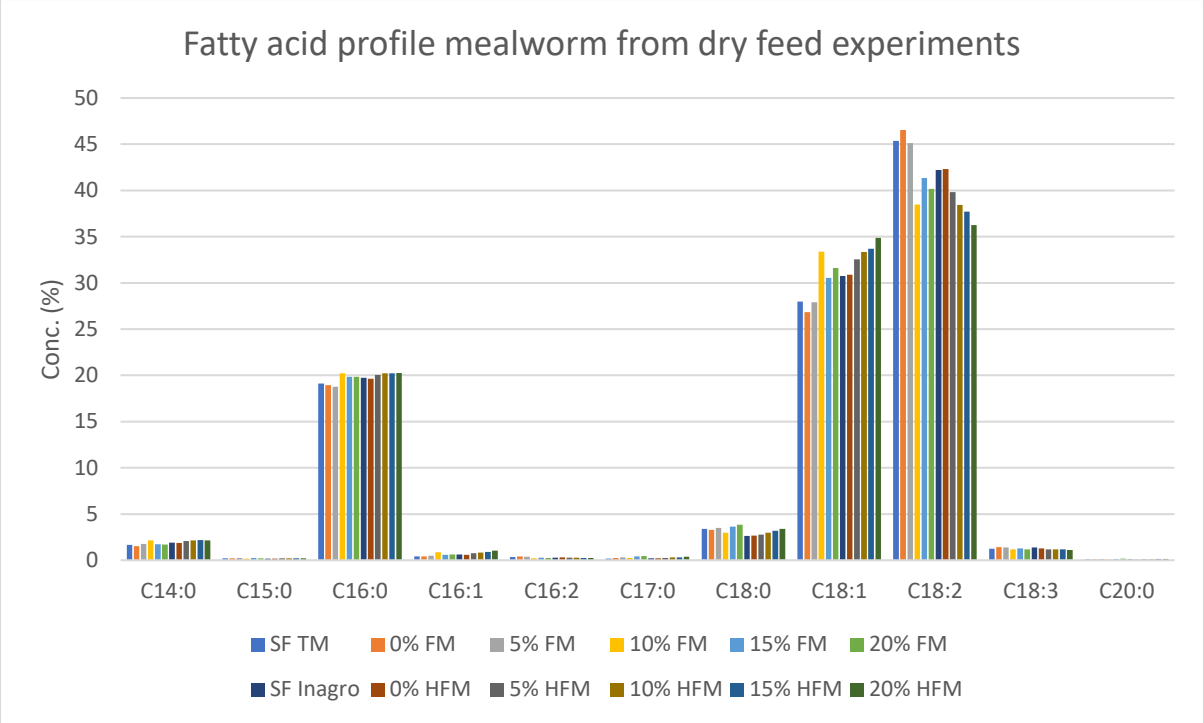

Figure 3: fatty acid profile of mealworms fed different protein rich side streams

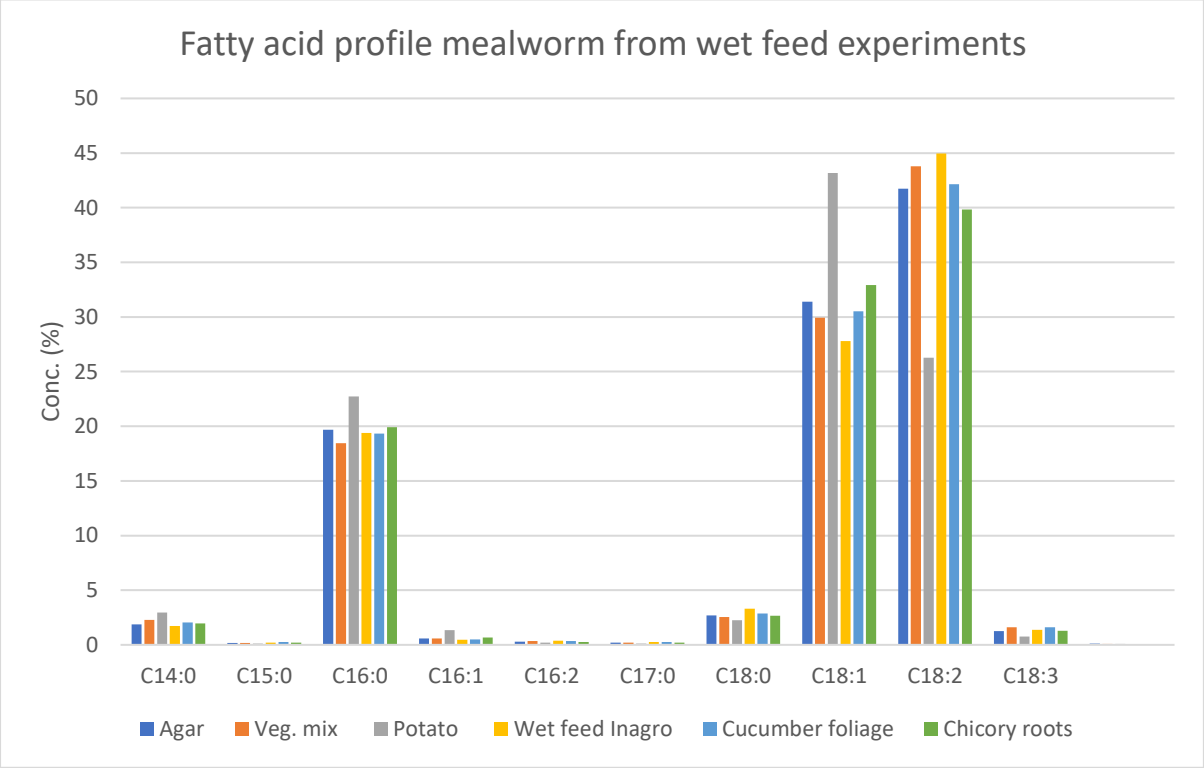

Figure 4:: fatty acid profile of mealworms fed mono side streams (wet feed)

b) Impact on crickets fatty acid profile

A similar analysis was performed for the crickets reared during Valusect project. AS expected, the main fatty acids, were again palmitic acid (C16:0), oleic acid (C18:1) and linolenic acid (C18:2); however, for this specie C18:0 was more abundant when compared to mealworms.

Although for this insect, not so clear trend was identified, it seems, again, that as higher protein levels are included as part of the diet the levels of C18:2 decreased consistently; but in this occasion (when specifically looking at the insect fed with red cells) it was not an increase (significantly) in the levels of C18:1. The mono side streams diets, yielded profiles in where C16:0, and C18: didn't vary significantly, while once again, the ratio between C18:1 and C18:2 is highly dependent on the type of diet. The MUFA/PUFA ratios found in this type of insect ranged from 0.41 to 0.91; which are significantly lower (and, therefore, more beneficial) to those calculated for mealworms.

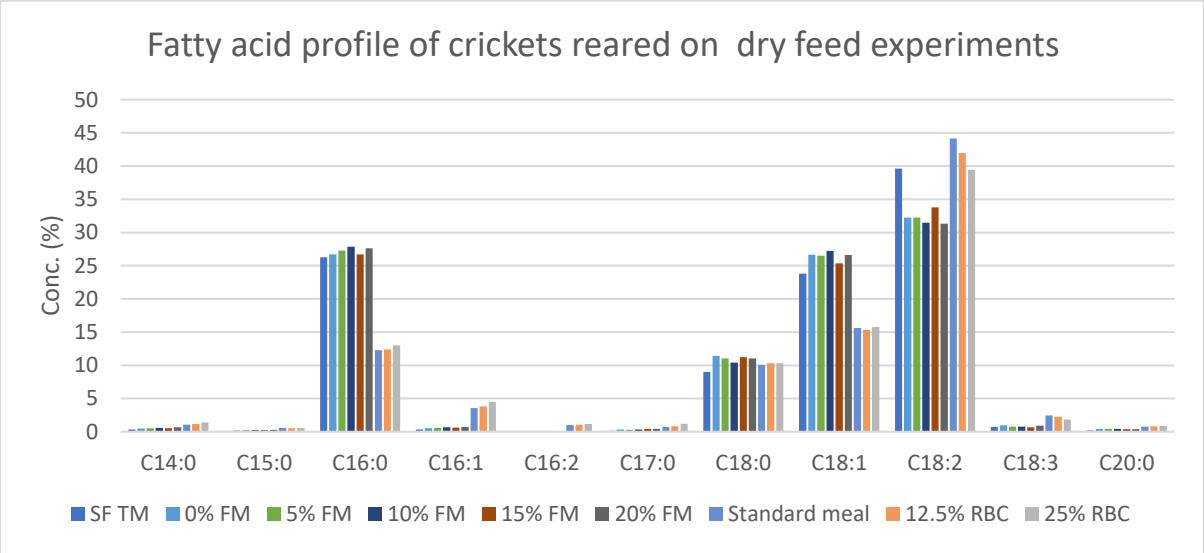

Figure 5: fatty acid profile of crickets fed different protein rich side stream

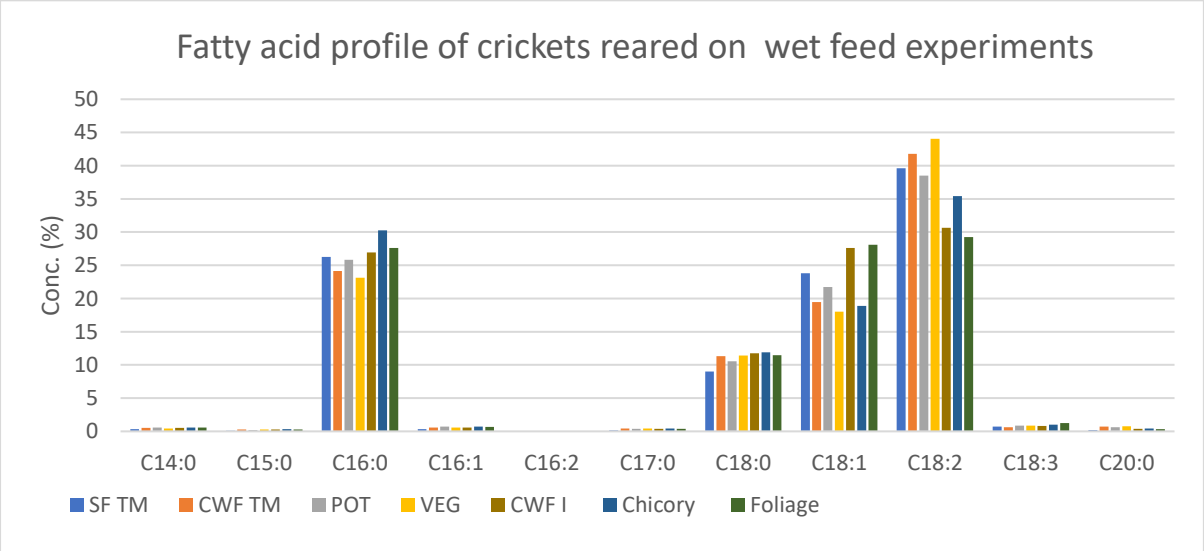

Figure 6: fatty acid profile of crickets fed mono side streams (wet feed)

As a general remark, for this section, I t can be concluded that diet has a very remarkable impact on the fatty acid profile of insects fat, but these interactions are only observed for two main fatty acids (C18:1 and C18:2) which seems to be interconnected. Insects have the capability of desaturate and elongate some fatty acids, as for instance C18:1, and since both of them play vital role in their metabolism. However, this capacity is not present in all insect species according to the literature[4], for instance *T. molitor* is unable to synthetize linoleic acid, while *A. domesticus* has this capacity. It means, that mealworms depend on the diet to obtain and accumulate this fatty acid, while cricket are not so dependant, since it can be created from C18:1 and C18:0. It may indicate, that mealworms reared on diets with low C18:2 content, will have less abundance on their bodies, as it happens with rich protein diets. This impact, thus, was not evident in cricket, as observed on the constant C18:2 levels observed after incrementing the amount of feather meal or feather hydrolysate.

3.4. Impact of diet on amino acid profile

Similarly to what happened to fat content, where the profile of the same was analyse, a similar approach was taken to understand of the diet is having an impact on the amino acid profile of the insects. It could be observed how the diet had an impact on the protein content, of the dry mass, but to understand the potential of these proteins for human nutrition, the quality of the protein needs to be determined. An initial stage for assessing protein quality is to investigate the amino acid profile, which indicates the quantities of essential and non-essential amino acids, as well as the absences of essential amino acids that could be limiting, and therefore, affecting negatively the nutritional quality of the protein under assay. Nevertheless, amino acid profile is just the first step, and more research is required regarding the digestibility, absorption and bioavailability of insect derived proteins, to fully understand their potential.

The results reported for amino acids, are given using the same units than used for protein content, i.e. g/100g Dry Matter. As expected, the values obtained differ from the total amino acid sum and total protein content for several reasons: some amino acids are destroyed during analysis, and some nitrogen is present in other forms other than proteins and considered as proteins following protein determination.

a) Impact on mealworms amino acid profile

The result obtained for this insect species are depicted in Figures 7 and 8. Also a summary of the average percentage of each on of the amino acids, considering all the trial performed with mealwroms is shown in Table 12.

As it can be seen, there is no significant differences regarding the amino acid percentage among any of the diets here employed (both wet and dry). However, as demonstrated in Figure 9, there is a very strong correlation between the amounts of protein provided in the diet and the amount of total amino acids found in the dry mass. It means, that for mealworms, higher protein diets led to more abundance in amino acids. A similar response is observed for the wet diets, where the protein content in the feed is indicative of the total amino acids determined in the dry mass.

[4] Malcicka, Miriama. "Evolution of linoleic acid biosynthesis in Collembola and different species of arthropods." (2018).

This trend observed for a higher amino acid content as the protein intake is increased, is in contradiction with the observations done for total protein content., where no real impact was observed. Main reason for this disagreement, as explained, is the different type of methodology employed for both determinations. From a nutritional point of view, amino acid profile is more relevant that protein content, and in this scenario, it could be assessed that this parameter is not being affected by the diet.

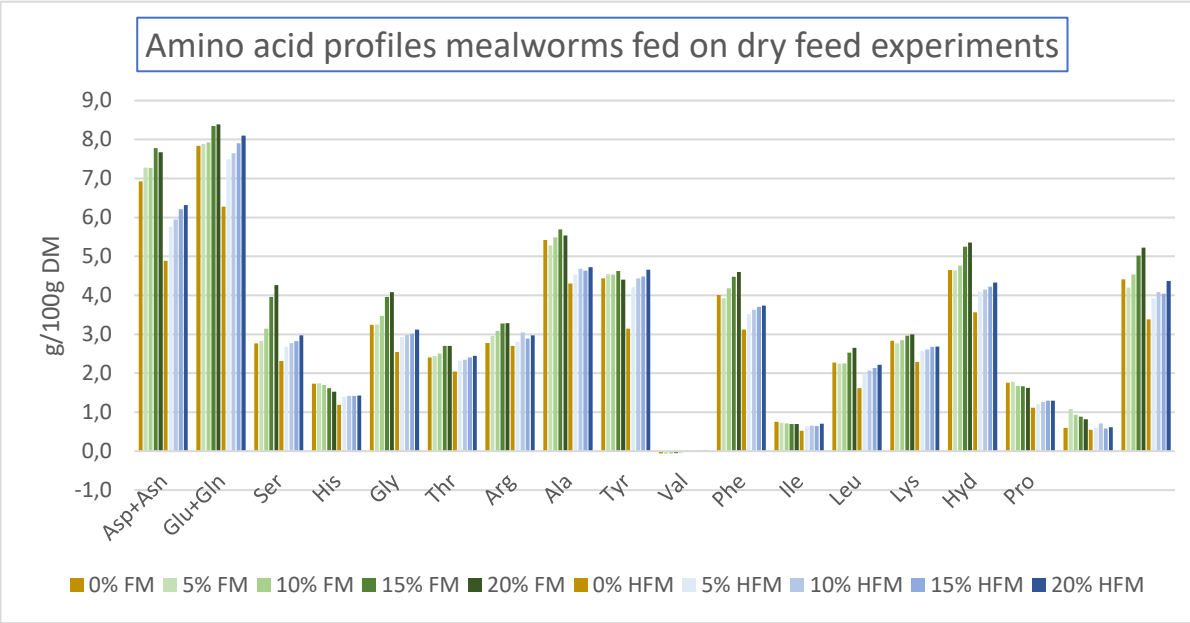

Figure 7: amino acid profile of mealworms fed with different protein rich side stream

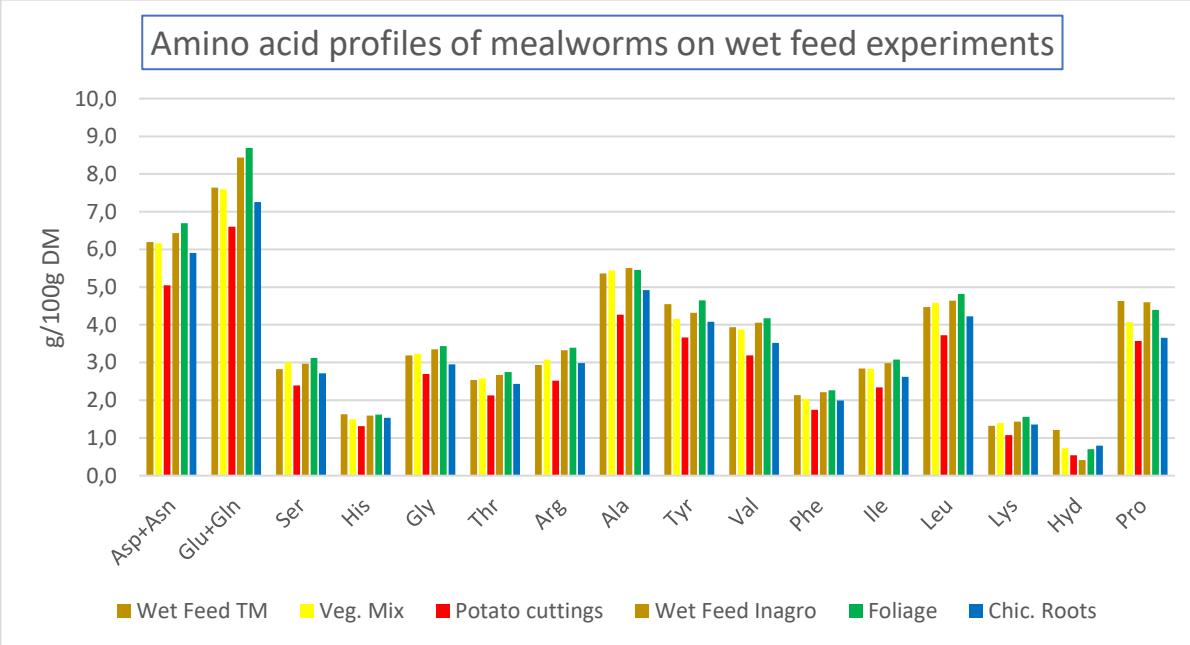

Figure 8: amino acid profile of mealwroms fed with wet side streams

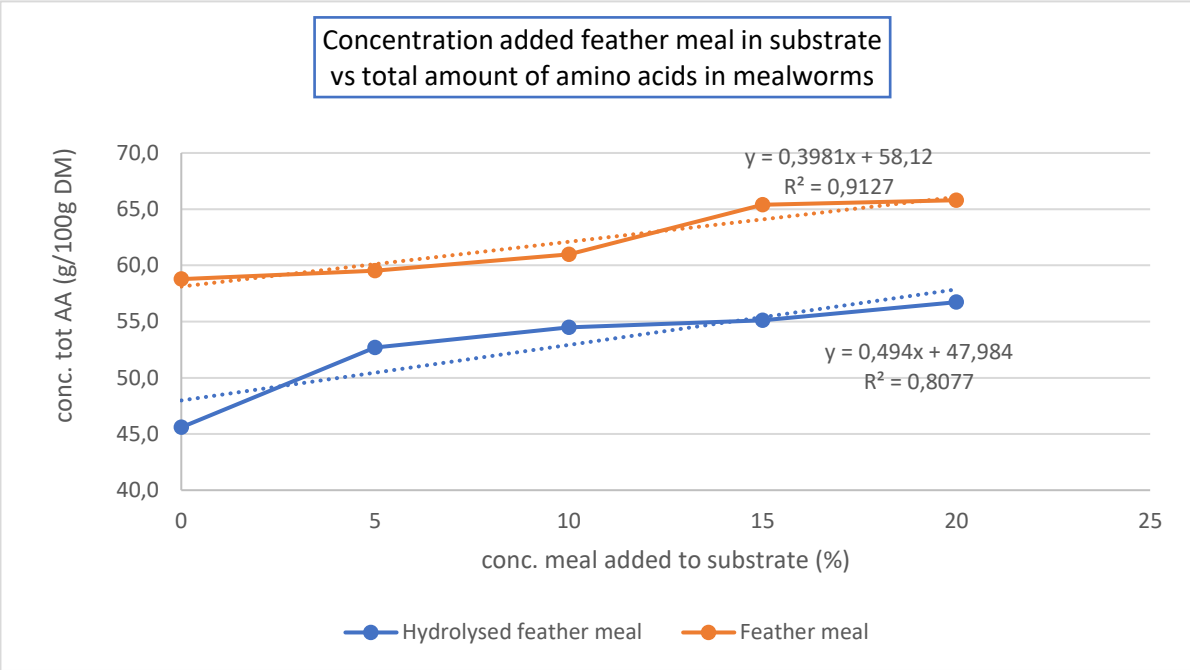

Figure 9: correlation between protein content in the diet and amino acid content in the dry mass for mealworms.

b) Impact on crickets amino acid profile

The amino acid profiles obtained for crickets were very similar among all the feeding trials, with The abundance of the different amino acids can be observed in figures 10 and 11, for both type of diets. However, as it also happened with mealworms, the total amount of essential amino acids was impacted by the type of diet, more significantly for those diets rich in protein content, both feather and red cells. In this case, the results are also in disagreement with the results obtained for total protein content, where it was observed that higher protein content was leading to lower protein content in the dry mass. Once again, this can be attributed to the role of non-nitrogen-protein compounds, and the different methodologies applied to calculate amino acid profile and total protein content. Similarly, a good correlation was found between the protein content of the diet, and the final amino acid content in the dry insects' bodies, as observed in Figure 12.

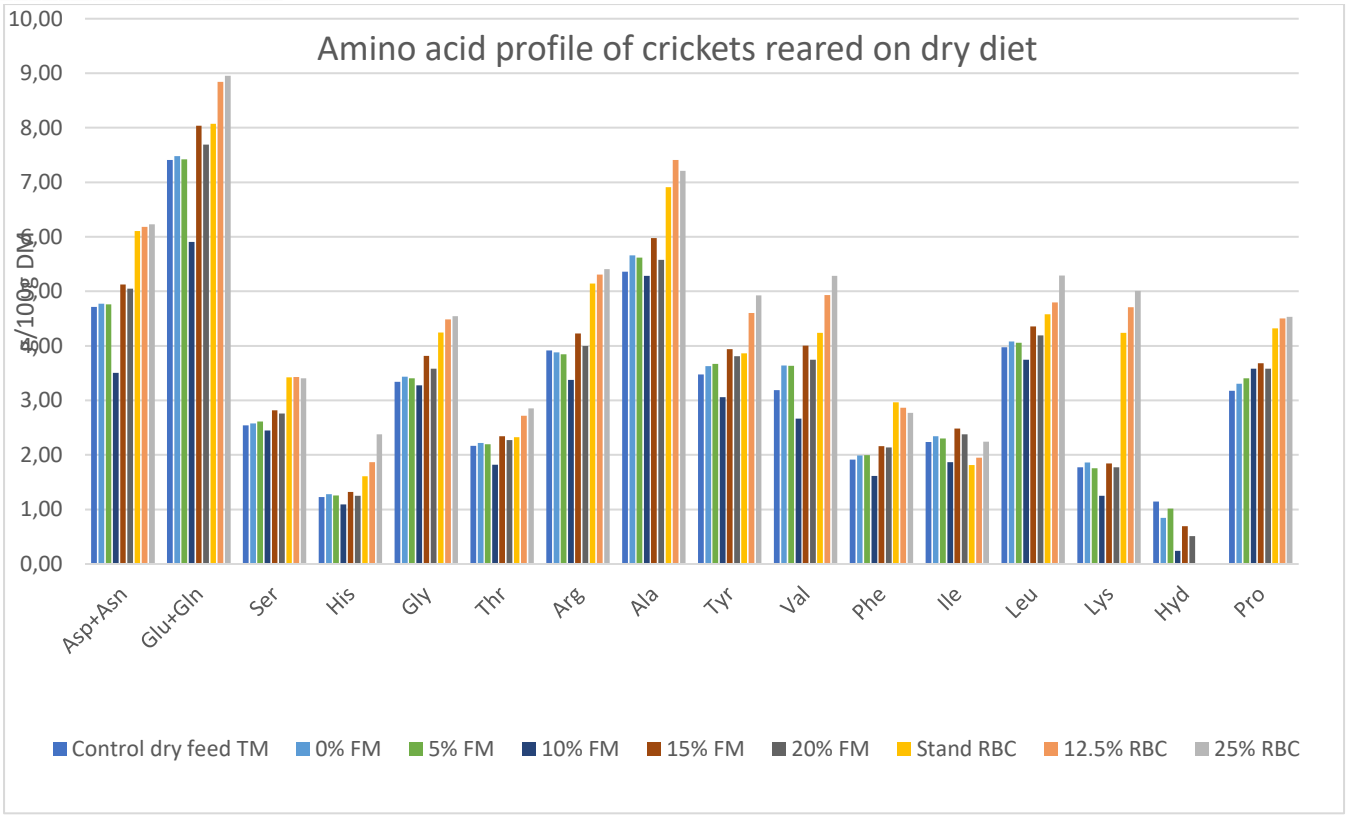

Figure 10: amino acid profile of mealworms fed different protein rich side streams

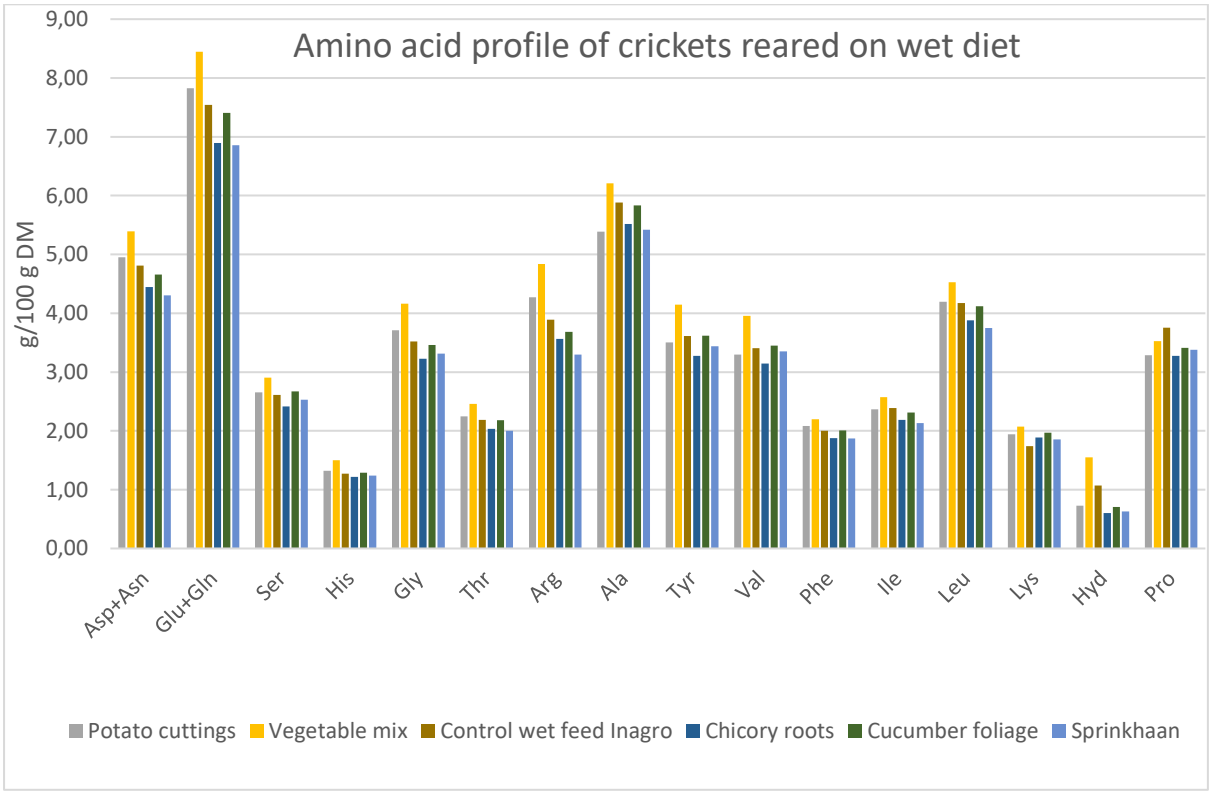

Figure 11: amino acid profile of crickets fed on different wet side streams

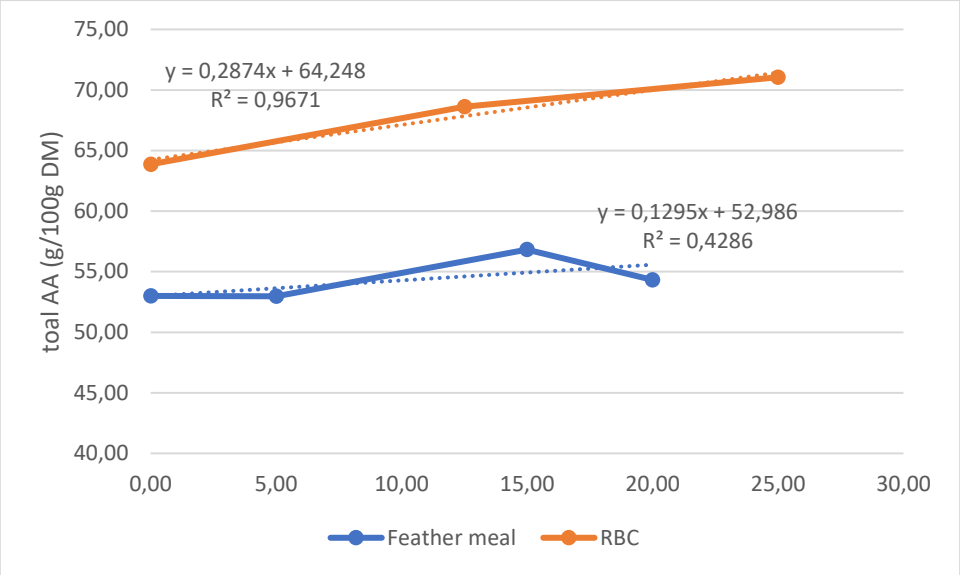

Figure 12: correlation between protein content in the diet and amino acid content in the dry mass for crickets.

c) *Overview of amino acid profile of both insect species*

Minimal differences regarding the percentage of each one of them to the total amino acid content were observed for both species, regardless the diet implemented. This can be observed in Table 12. The main implication of these finding is that amino acid profile, within the scope of the trials here performed, are nit subjective to be modified by means of dietary interventions, conversely to what was observed for fatty acid profiles. On the other hand, it seems possible to increase the amount of amino acids in the final body mass by adding extra protein to the insects' diets, for both insects' species. However, the results obtained from total crude protein, were in contradiction with those obtained for total amino acid content in the dry mass. This issues has been previously reported, since the protein to nitrogen conversion ratio is not well defined and also, that both method have their own limitations for accurate determination of total protein or amino acid content.

When comparing the amino acid profile of both insect species (crickets and mealworms). Not actual differences were observed regarding the relative abundance of each one of the amino acids determined. Implying that both insects could have the same potential nutritional value when intended for human consumption. As described by other authors, insect proteins are having low concentration in essential amino acids, mainly lysine and methionine, or threonine. As reported in Table 12, the average value of essential amino acids was of 31.2% and 30.3% for mealworms and crickets respectively, which are considerable lower than other protein sources such as poultry (41%), pork (37%) or beef (37%); but higher than those values found in lentils (21%), peas (22%) or chickpeas (20%).

Table 12: Average value of amino acid percentages, regarding total amino acids, for all mealworms feeding trials (n = 19 triplicates) and all cricket trials (n = 16 triplicates)

	Mealworm		Cricket	
	% of total AA	SD	% of total AA	SD
Aspartic acid	11.20	0.54	8.94	0.35
Glutamic acid	13.73	0.75	13.79	0.59
Serine	5.19	0.43	4.99	0.19
Histidine	2.70	0.19	2.50	0.24
Glycine	5.66	0.27	6.67	0.24
Threonine	4.37	0.24	4.08	0.13
Arginine	5.29	0.35	7.49	0.37
Alanine	9.02	0.49	10.68	0.44
Tyrosine	7.54	0.42	6.78	0.23
Valine	6.73	0.13	6.63	0.40
Methionine	1.18	0.07	Nd	Nd
Phenylalanine	3.74	0.13	3.85	0.24
Isoleucine	4.86	0.22	4.09	0.55
Leucine	7.89	0.31	7.64	0.29
Lysine	2.50	0.24	4.07	1.36
Hydroxyproline	1.36	0.37	1.28	0.80
Proline	7.48	0.31	6.52	0.49
Total essential %	31.27		30.35	

3.5. Impact of diet on mineral profile

After considering the main macronutrients of relevance obtained from edible insects, it is also very relevant to understand the mineral profile of the insects, to understand if these can be a potential source of minerals relevant for human nutrition, and if the diet is having an impact on the concentration of such components. In this section, we will pay attention to next relevant minerals: phosphorus, magnesium, potassium, calcium, sodium, zinc, copper, iron and manganese. According the EFSA 2017 report [5], and as a guidance, the recommendation for daily intakes for the most relevant minerals are as follows, for adults older than 18 years of age (although some differences are proposed in this report based in sex and other demographic conditions):

- Calcium: 860 mg/day
- Iron: 8 mg/day
- Zinc: 7.5 mg/day
- Manganese: 3.0 mg/day
- Phosphorus: 550 mg/day
- Potassium: 3500 mg/day
- Copper: 1.6 mg/day
- Magnesium: 350 mg/day

a) Impact of diet on mealworms mineral profile

The data obtained during this project are not providing significant evidences that the mineral profile of the mealworms biomass has been affected by the diet provided to the insects when it comes to Mg, Na, Zn, Cu, or Mn[6]. All the above mentioned minerals are well represented in the insect composition and for the most of them an intake of 100g of dry matter will be enough to cover the 100% of the needed amount (Zn, Cu) or more than 80% of this requirement (Mg or Mn).

On the other hand, minerals such as P, K, Ca, or Fe were significantly affected by the diet. In the case of phosphorus, a significant decrease of the value was observed when high protein diets were provided to the insects, being these values around 15% lower than those on diets based on wet side streams. All the samples analysed were providing more than 100% of the daily requirement. Potassium was seen to be very consistent throughout all the diets, with the exception of the red cells, in this case, an increment in the content of RBC was indicative or a decrease for K detected. Regarding dietary requirement, 100 gr of mealworms are enough to cover the 30% of the recommended daily intake. Calcium was served to be the most variable mineral in this insect species. The diet allowing more calcium accumulation was that based on fermented chicory roots, but even in this case the amount of

[5] https://www.efsa.europa.eu/sites/default/files/2017_09_DRVs_summary_report.pdf
[6] Noyens, I., Schoeters, F., Van Peer, M., Berrens, S., Goossens, S., & Van Miert, S. (2023). The nutritional profile, mineral content and heavy metal uptake of yellow mealworm reared with supplementation of agricultural sidestreams. Scientific Reports, 13(1), 11604.

calcium is less than 10% of the daily requirement per 100 g of dry mass. Finally, iron was observed to vary significantly with the diet when increasing concentration of red cell were provided, increasing its concertation from 7.27 up to 11.99 mg/100 g as the protein content (and thus the iron content in the feed) was increased. More relevant result were observed when chicory roots were fed to the insects, giving a final iron content of 45 mg/100g. In both cases, 100g of dry mass will be sufficient to cover daily intake requirement. This can be explained because of the very high levels of iron found on this substrate (521 mg/100g)

b) Impact of diet on crickets mineral profile

When the mineral profile of crickets was determined, it was found that the diet had no significant impact on the content of K, Na, Zn, Cu or Mn. For these minerals, the amount provided , in average, per 100 g of dry mass is sufficient to cover 30% of K needs, 100% of Zinc, 100% of Cu and 100% of Mn.

Likewise to what happened to mealworms, phosphorus content was decreased at higher levels of protein inclusion in the diet, giving results in the same range that those found for the mealworms; meaning that P intake will be cover at 100% with 100g of crickets dry biomass.

Interestingly, Mg levels in crickets were much lower than those found in mealworms, with a value arounf ten times lower. Similarly, high protein diets were detrimental in the amount of magnesium content. It means, that cricket are a poor source of this mineral, and only a 10% of the daily intake can be covered with 100G of dry crickets. Potassium levels were similar between the two insect species, in the case of the crickets higher values were observed (in general) when control meals were used to rise the insects.

Conversely to mealworms, calcium levels in crickets were found to be remarkably higher, although not enough to cover the recommended intake of 850 mg/day. Calcium content was favoured by foliage and chicory based diets, where maximum levels of 200 mg/100 g were observed.

Finally, iron content was very consistent among the different trial, but it was observed, as it happened with the mealworms, that increasing amount so red cell led to an increase accumulation of this mineral in the insects body. Specifically, the iron content increased from 9.81 to 16.12 mg/100g. Such values indicate that these particular insects are an excellent source of iron, although it needs to be determined how available this iron is once consumed.

c) Overview of mineral profile of both insect species

Based on the information here provided, it seems that insects are good source of essential minerals such as P, Zn, Cu, Fe and Mn. While, conversely, minerals such as Ca, K or Mg are found in limited concentrations. Main differences between insects were found in the levels of Mg and Ca, being the first more abundant in mealworms, and the second one in crickets.

Clear trends of mineral accumulation were found for Fe when red blood cells were fed to both species and more remarkably when chicory roots were provided to mealworms. Such results indicate that insects could be a potential vehicle to alleviate the actual issue of iron deficiency, which has been observed in extensive cohorts of populations. More research is required to determine whether

the iron accumulated by insects is bioavailable and how it compares to other iron sources as plants or red meat.

The results observed in mealworms indicate that The study also found that it selectively accumulated Ca, Fe and Mn from acidic mineral rich side streams.

Table 12: mineral profile of mealworms under different substrates.

Substrate	P		Mg		K		Ca		Na		Zn		Cu		Fe		Mn	
	mg/100g	*SD*	mg/100g	*SD*	mg/100g	*SD*	mg/100g	*SD*	mg/100g	*SD*	mg/100g	*SD*	mg/100g	*SD*	mg/100g	*SD*	mg/100g	*SD*
Standard feed TM	1079.68	71.36	256.07	10.32	1249.04	35.39	24.84	2.00	86.83	11.91	12.53	0.37	1.76	0.08	5.00	0.56	1.08	0.01
Control wet feed TM + Inagro	*1094.76*	*111.54*	*273.52*	*32.49*	*1216.22*	*130.95*	*23.73*	*3.83*	*114.57*	*42.47*	*13.60*	*1.20*	*2.23*	*0.16*	*5.20*	*2.50*	*1.37*	*0.39*
Control wet feed	1085.77	39.75	248.03	12.74	1255.36	47.94	25.44	2.72	76.95	6.09	12.70	0.62	2.19	0.51	6.42	1.78	1.02	0.05
Vegetables mix	938.69	38.25	297.26	13.89	1128.00	27.17	27.55	3.12	104.53	6.48	15.78	3.18	2.01	0.07	5.73	0.68	1.24	0.05
Potato cuttings	690.14	30.06	165.47	12.66	890.64	45.30	1.66	0.36	95.82	10.81	12.30	1.44	2.11	0.27	4.58	0.38	1.58	0.07
Control feather meal	989.20	36.21	219.98	9.73	1190.09	55.28	3.02	3.44	27.11	3.26	13.03	1.16	2.03	0.08	4.58	0.48	1.46	0.07
Feather meal 5%	1020.24	33.20	249.84	14.39	1179.97	39.08	6.65	3.43	61.98	4.55	12.95	0.36	2.07	0.05	6.82	3.49	1.57	0.09
Feather meal 10%	980.56	19.62	249.46	12.46	1142.22	31.09	4.02	3.69	80.06	4.38	13.73	1.21	2.18	0.04	7.41	3.49	1.62	0.07
Feather meal 15%	977.36	46.80	262.77	14.86	1101.57	33.29	10.18	1.25	108.59	6.83	13.74	2.33	1.96	0.05	5.32	0.31	1.58	0.04
Feather meal 20%	911.60	18.10	265.35	11.42	1070.01	13.90	9.31	8.74	124.86	3.13	11.97	0.39	1.92	0.04	5.54	0.45	1.79	0.05
Standard feed Inagro	1043.04	18.88	264.99	5.21	1202.75	13.66	0.00	0.00	141.38	2.89	13.41	0.56	2.45	0.06	4.41	0.27	1.91	0.06
Control Feather meal	933.90	10.93	239.84	10.40	1103.36	38.14	1.82	3.15	133.43	4.32	13.72	1.72	2.33	0.05	4.40	0.25	1.77	0.09
Hydrolysed feather meal 5%	889.85	27.31	263.30	14.41	1107.92	50.96	2.84	2.98	136.40	9.94	13.55	1.54	2.21	0.06	4.59	0.31	1.86	0.08
Hydrolysed feather meal 10%	863.99	23.68	267.43	11.65	1118.50	17.64	0.70	1.22	132.45	7.01	13.08	0.65	2.09	0.06	4.30	0.19	1.98	0.10
Hydrolysed feather meal 15%	862.91	19.38	278.32	11.27	1115.47	11.07	3.89	3.81	136.25	5.37	12.95	0.58	2.02	0.04	4.59	0.23	1.97	0.03
Hydrolysed feather meal 20%	892.66	17.68	274.37	8.25	1016.93	18.22	21.63	2.46	144.66	9.90	13.24	0.45	1.62	0.05	4.05	0.29	1.76	0.08
Standard RBC	1220.71	*ND*	310.52	*ND*	1572.33	*ND*	54.63	*ND*	111.36	*ND*	14.75	*ND*	*ND*	*ND*	7.27	*ND*	*ND*	*ND*
5% RBC	1011.46	*ND*	299.26	*ND*	1265.89	*ND*	47.45	*ND*	162.02	*ND*	13.16	*ND*	*ND*	*ND*	8.73	*ND*	*ND*	*ND*
10% RBC	826.19	*ND*	261.29	*ND*	1042.23	*ND*	38.99	*ND*	155.35	*ND*	11.64	*ND*	*ND*	*ND*	10.32	*ND*	*ND*	*ND*
15% RBC	853.73	*ND*	268.50	*ND*	1041.08	*ND*	42.05	*ND*	169.24	*ND*	12.34	*ND*	*ND*	*ND*	11.99	*ND*	*ND*	*ND*
20% RBC	739.26	*ND*	229.36	*ND*	922.48	*ND*	35.44	*ND*	139.52	*ND*	11.87	*ND*	*ND*	*ND*	11.18	*ND*	*ND*	*ND*
Control wet feed Inagro	1103.74	33.73	299.02	16.08	1177.09	27.23	22.02	3.61	152.18	5.64	14.49	0.34	2.27	0.06	3.97	0.24	1.71	0.05
Cucumber foliage	1065.50	14.18	222.30	9.03	1206.37	20.27	65.84	6.89	63.02	1.55	13.82	0.30	1.76	0.07	4.56	0.20	1.58	0.03
Fermented chicory roots	970.70	31.83	243.94	14.00	1219.15	32.68	79.09	4.69	175.42	4.58	12.80	0.43	2.37	0.06	45.19	3.06	2.31	0.07

This policy brief aims at providing the essential information. A complete document can also be found here.

Table 13: mineral profile of crickets under different substrates

Sample	P		Mg		K		Ca		Na		Zn		Cu		Fe		Mn	
	mg/100g	SD	mg/100g	SD	mg/100g	SD	mg/100g	SD	mg/100g	SD	mg/100g	SD	mg/100g	SD	mg/100g	SD	mg/100g	SD
Standard feed	1033.99	*18.45*	1.47	*2.55*	1263.16	*27.50*	478.92	*18.12*	108.65	*19.42*	30.38	*0.40*	2.66	*0.13*	8.59	*1.50*	6.52	*0.15*
Control wet feed	1074.90	*43.35*	1.72	*2.98*	1387.42	*63.36*	111.69	*19.12*	531.25	*16.63*	30.64	*1.31*	1.96	*0.16*	6.51	*0.45*	4.67	*0.35*
Potato cuttings	1015.82	*69.93*	0.00	*0.00*	1262.80	*81.84*	67.07	*1.59*	400.28	*30.48*	28.07	*2.12*	1.79	*0.07*	5.51	*0.47*	4.16	*0.13*
Vegetables mix	1077.24	*30.85*	7.60	*13.16*	1367.05	*32.08*	114.67	*14.64*	549.04	*0.91*	30.89	*0.93*	2.02	*0.05*	7.30	*1.30*	4.51	*0.24*
0% Feather meal	931.87	*44.65*	20.24	*7.98*	1115.95	*90.39*	199.54	*15.99*	754.09	*415.13*	25.77	*1.92*	3.16	*0.26*	9.09	*0.44*	5.98	*0.01*
5% Feather meal	874.09	*28.44*	20.83	*12.84*	1070.39	*21.97*	195.79	*27.54*	458.83	*31.64*	23.69	*1.05*	2.71	*0.20*	9.77	*0.72*	5.94	*0.27*
10% Feather meal	916.62	*17.87*	19.19	*5.31*	1065.40	*38.78*	185.63	*11.99*	488.86	*90.10*	23.96	*0.62*	2.79	*0.38*	7.68	*0.06*	5.88	*0.14*
15% Feather meal	958.22	*69.90*	19.87	*9.58*	1139.35	*95.23*	193.77	*32.57*	510.01	*77.34*	26.67	*3.20*	3.25	*0.87*	8.78	*1.03*	5.95	*0.49*
20% Feather meal	910.64	*31.48*	11.98	*6.96*	1091.00	*47.08*	175.87	*7.80*	453.96	*33.45*	24.80	*1.51*	2.63	*0.21*	7.45	*0.05*	5.51	*0.35*
Control RBC	1123.23	*ND*	108.91	*ND*	1500.11	*ND*	134.20	*ND*	620.81	*ND*	34.73	*ND*	ND	*ND*	9.81	*ND*	ND	*ND*
12.5% RBC	742.10	*ND*	66.91	*ND*	1015.81	*ND*	86.52	*ND*	430.74	*ND*	21.22	*ND*	ND	*ND*	7.64	*ND*	*ND*	*ND*
25% RBC	779.5	*ND*	73.08	*ND*	1088.31	*ND*	98.2	*ND*	497.93	*ND*	23.62	*ND*	ND	*ND*	16.12	*ND*	*ND*	*ND*
Control wet feed I	953.22	*23.30*	14.80	*8.98*	1073.41	*43.02*	193.10	*6.47*	536.29	*17.80*	26.13	*0.83*	4.05	*0.53*	9.93	*6.94*	6.79	*0.16*
Chicory roots	956.16	*16.25*	24.83	*6.97*	1040.02	*6.32*	202.71	*3.64*	436.97	*9.08*	24.04	*0.99*	2.96	*0.18*	9.85	*1.07*	6.68	*0.14*
Foliage	921.04	*8.17*	28.25	*10.74*	1011.66	*11.05*	231.53	*17.67*	422.44	*7.31*	24.31	*0.19*	3.05	*0.03*	8.68	*0.47*	6.77	*0.30*

3.6. Heavy metals profile

The current EU legislation describing the maximum limits of heavy metals in food products[7] states that for cadmium and lead these limits are of 0.10 mg/kg (10ug/100g) for meat products, which we are using as a guideline. While for chromium there is no maximum limit stablished, Cr is commonly present in the environment, food and food supplements and is known to be an essential nutrient for the human body, in small amounts.

As reported, no cadmium was found in any of the samples analysed, or at least the values were below the limit of detection. Regarding lead, the amount determined is slightly higher than the EU legal limit for fresh meat, but it needs to be considered that this is determined in dry weight and not in fresh wait as it happens with the meat. Taking into account that moisture content, in average, for the insects was, around 70%, it means that the heavy metals were concentrated (after drying) by a factor of 3. When this is applied to the calculation, the level of lead in fresh weight are below of established limits.

These analyses show the possibility of accumulating heavy metals in the insects' bodies, when present in the diet, as it was observed for iron, for instance. However, the heavy metal levels found in the mealworm and crickets biomass in this study are considered safe for human consumption. Regular close monitoring is advised when using side streams that can contain heavy metals as feed source for insects, since the main source of heavy metal accumulating in the insect tissues is the diet. This was particularly evident when looking at the insect fed with chicory roots.

Table 14: heavy metals concentration in crickets and mealworms reared in different substrates

Diet	*Cd*		*Cr*		*Pb*	
	ug/100g DM	SD	*ug/100g DM*	SD	*ug/100g DM*	SD
Crickets						
Standard feed	*0.00*	*0.00*	*40.25*	*7.79*	*0.00*	*0.00*
Control wet feed	*0.00*	*0.00*	*72.19*	*33.94*	*28.74*	*9.01*
Potato cuttings	*0.00*	*0.00*	*79.46*	*16.83*	*22.62*	*2.54*
Vegetables mix	*0.00*	*0.00*	*62.53*	*35.40*	*28.40*	*2.88*
0% Feather meal	*0.00*	*0.00*	*43.39*	*11.85*	*26.71*	*7.75*
5% Feather meal	*0.00*	*0.00*	*70.30*	*37.63*	*44.51*	*1.42*
10% Feather meal	*0.00*	*0.00*	*43.16*	*3.05*	*27.22*	*19.58*
15% Feather meal	*0.00*	*0.00*	*196.25*	*26.87*	*35.27*	*1.17*
20% Feather meal	*0.00*	*0.00*	*46.75*	*15.98*	*35.32*	*1.18*
Control wet feed I	*0.00*	*0.00*	*50.37*	*5.38*	*47.96*	*3.63*
Chicory roots	*0.00*	*0.00*	*116.77*	*63.24*	*35.40*	*4.99*
Foliage	*0.00*	*0.00*	*61.21*	*18.74*	*40.82*	*5.14*
Mealworms						
Standard feed TM	*0.00*	*0.00*	*9.50*	*2.72*	*11.92*	*3.28*
Control wet feed TM + Inagro	*0.00*	*0.00*	*39.51*	*24.99*	*40.53*	*23.88*
Control wet feed	*0.00*	*0.00*	*15.02*	*2.68*	*8.96*	*2.41*
Vegetables mix	*0.00*	*0.00*	*57.84*	*10.78*	*9.80*	*1.45*

[7] Regulation (EU) 2023/915

Potato cuttings	*0.00*	*0.00*	*56.14*	*10.69*	*17.44*	*8.08*
Control feather meal	*0.00*	*0.00*	*54.64*	*4.45*	*29.38*	*9.21*
Feather meal 5%	*0.00*	*0.00*	*62.79*	*1.70*	*36.27*	*2.77*
Feather meal 10%	*0.00*	*0.00*	*58.01*	*14.38*	*46.97*	*4.80*
Feather meal 15%	*0.00*	*0.00*	*66.04*	*7.41*	*24.56*	*6.00*
Feather meal 20%	*0.00*	*0.00*	*68.33*	*1.67*	*37.37*	*6.17*
Standard feed Inagro	*0.00*	*0.00*	*36.78*	*3.47*	*36.86*	*2.92*
Control Feather meal	*0.00*	*0.00*	*40.81*	*0.42*	*38.61*	*3.43*
Hydrolysed feather meal 5%	*0.00*	*0.00*	*48.98*	*4.76*	*37.35*	*1.94*
Hydrolysed feather meal 10%	*0.00*	*0.00*	*38.37*	*0.47*	*36.31*	*4.70*
Hydrolysed feather meal 15%	*0.00*	*0.00*	*42.75*	*1.83*	*38.80*	*2.30*
Hydrolysed feather meal 20%	*0.00*	*0.00*	*29.95*	*1.86*	*42.67*	*11.63*
Control wet feed Inagro	*0.00*	*0.00*	*53.90*	*7.47*	*44.78*	*3.11*
Cucumber foliage	*0.00*	*0.00*	*40.88*	*0.57*	*49.56*	*6.85*
Fermented chicory roots	*0.00*	*0.00*	*153.52*	*38.38*	*108.65*	*13.52*

4. Overall conclusion

In this extensive trial two edible insect species (mealworm and crickets) were reared on different substrates, which were selected based on their composition, availability and status of co- or by-products of the food industry. An extensive number of combinations of substrate and insect species were tested, in triplicate, to determine the impact of such substrates in the insect performance (Report WP1.2) and the nutritional composition of the bio mass generated.

The results obtained indicate that the nutritional profile of both species, and the amount of dry weight obtained, can be manipulated, to some extent, by modifying the composition of their diet, the format and the pH. The proportion of carbohydrates and proteins provided to the insects are capital in order to anticipate the amount of macronutrients, especially fat and protein, in the final body composition.

Results clearly indicated that increasing the protein content in the feeding is leading to an increase of the fat content in mealworms and protein content in the crickets, when using dry substrates. On the other hand, when wet diets are used, the more carbohydrate is included more fat the mealworms and crickets are accumulating, as observed for potato peel diets. It was also observed, not surpsiginly, that fat and protein content are closely correlated, and when one of this components increase is in detriment of the other one. Regarding the other macronutrients such as ash, fibre and chitin, the impact of diets was not celery correlated with the diet and the variability of these parameters was less evident than that found for fat and protein.

When analysing the fatty acid profile of the insects, it was observed that for dry diets, the higher protein content included, the less abundance of C18:2 was observed, which was correlated negatively with an accumulation of C18:1. This impact is much more evident in mealworms, than in crickets who can biosynthesize C18:2 and it procurement is not so much dependant on the diet. Between both species, the main difference observed was the higher values of C18:0 found in crickets.

The diet had no actual impact on the amino acid profile of any of the species under any of the diets tested in this trial. However, a strong correlation of total amino acids in dry mass and protein content in the diet was determined; such trend was observed for both species. This observation contradicts

the crude protein finding, but this may be due to fundamental differences between both analytical methods. Both insects are able to provide all the essential amino acids (30% of total amino acids), although some of them are found in very low concentration and could be limiting, such as lysine or methionine.

Insect have the capability of accumulating some mineral, that are present in the diet as iron, phosphorus or magnesium. Considering the daily intake recommendations, the two insects here tested are able to provide the required amounts of iron, copper, phosphorus, and manganese (only for crickets). While are poor sources of calcium (especially mealworms), potassium or magnesium (especially crickets).

Finally, the capacity of accumulating minerals and metals is also associated with heavy metals accumulation coming for the diet. Although all the results where below the legal limits for cadmium and lead, in wet weight, it is recommended to quantify the substrate to minimise heavy metal accumulation in insects bodies.

5. Future research work and recommendations

Based on the work and analysis conducted for this report, the next recommendations are suggested for future research work:

1. Optimizing Insect Diets: Further research can focus on optimizing the composition of insect diets to achieve desired nutritional profiles. This could involve experimenting with different combinations of carbohydrates, proteins, and fats in the diet to determine the most efficient and cost-effective way to produce insects with the desired macronutrient content.

2. Diet Format and pH: Investigate the influence of diet format (dry or wet) and pH on the nutritional composition of insects. Understanding how these factors affect insect growth and nutrient content can help refine rearing practices.

3. Fatty Acid Profiles: Continue research on the fatty acid profiles of insects and how they are influenced by diet. Understanding the impact of different fatty acid compositions on the quality of insect biomass is crucial, especially for their use as a food source or feed for livestock.

4. Mineral Accumulation: Investigate the mineral accumulation capacity of insects, particularly for minerals like iron, copper, phosphorus, and manganese. Research can focus on ways to enhance the accumulation of essential minerals while minimizing the accumulation of minerals that insects are poor sources of, such as calcium, potassium, and magnesium.

5. Heavy Metal Accumulation: Continue to study heavy metal accumulation in insects, with a particular focus on quantifying the impact of the substrate. Research can also explore methods to minimize heavy metal accumulation in insects when reared on certain substrates.

6. Nutrient Bioavailability Studies: Investigate the bioavailability of nutrients from insects. This involves determining how well the human digestive system can extract and utilize the nutrients present in insects. It is essential to assess whether the nutrients are readily absorbed and whether any factors in insects may enhance or inhibit nutrient absorption.

7. Comparative Studies: Conduct comparative studies to assess how the bioavailability of nutrients in insects compares to that of traditional protein sources, such as meat, fish, or plant-based proteins. This can provide insights into the potential nutritional advantages of including insects in the human diet.

8. Digestibility: Research the digestibility of insect-based products, including factors that may affect digestibility, such as cooking methods or food processing. Understanding how cooking or processing techniques impact nutrient availability is crucial.

9. Nutrient Interaction: Investigate the interactions between nutrients in insect-based foods and how these interactions may affect overall nutrient absorption. For example, how the combination of proteins, fats, and other components in insects may influence nutrient uptake.

10. Mineral and Micronutrient Absorption: Examine the absorption of essential minerals and micronutrients from insects. Determine whether insects contribute to the daily recommended intake of minerals and how effectively these nutrients are absorbed by the human body.