Development, optimization, and automation of diet preparation, administration and replacement

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Abstract:	*Tenebrio molitor* and *Acheta domesticus* have been recently approved as novel food by EFSA and are currently the leading edible insects due to their nutritional value and food conversion rate, and *Hermetia illucens* is considered as a potential future source of feed protein. To be able to produce insects of a high and consistent quality at a competitive rate, a continuous process flow and an optimal diet composition of diet and rearing conditions are needed. Within the European project CoRoSect, we aimed at formulating standardized diets for these insects by using by-products of agri-food sector and testing the diets with experiments in Italy and Finland. The by-products selected for *T. molitor* and *A. domesticus* diets included potato peels, tomato pomace, brewer's spent barley grain, wheat bran, and olive pomace in one experiment. Two experiments were carried out for *T. molitor*. In the first experiment, the by-products were tested separately. In the second experiment, combinations of by-products were tested based on the protein content. For *A. domesticus*, only mixed diet combinations were tested, reaching the protein content of the standard diet. For *T. molitor*, the best performance in the first experiment was shown by diets with tomato peels and brewer's spent grain. The second experiment appeared to yield the most promising results for diets containing 50% of wheat bran and different combinations of brewer's spent barley grain, tomato pomace and/or yeast. For *A. domesticus*, no statistically significant differences were found between the diets in terms of survival and development when compared to control. Hence, the results suggested that at least 60% of chicken feed often used in *A. domesticus* diets can be replaced with by-products of agriculture and food value chains, and without compromising crickets' production performance. Regarding *H. illucens,* the experiments sought to test the utilisation of different by-products and the level of two amino acids (tryptophan and methionine), which are often considered to be essential to insects, in the diet. The results suggest that the addition of higher amount of tryptophan (4.7–5.2%) can have a favourable effect on *H. illucens* growth and survival. By contrast, the high concentration of methionine reduced the performance of *H. illucens* larvae. A methionine content of g or 1.54% in the diet was found sufficient for an appropriate larval growth. The second-best performance result, after the chicken feed control diet, was achieved with a diet that included dried brewer's spent grain, feed yeast, potato protein, crushed tomato, breadcrumbs and fresh apple.

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19	CENTRO INTERNAZIONALE DI ALTISTUDI AGRONOMICI MEDITERRANEI http://www.iamb.it/	CIHEAM	IT

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ADF	Acid Detergent fibre
Ala	Alanine
Arg	Arginine
Asp	Asparagine
Ca	Calsium
Carb	Carbohydrates
CtP	Carbohydrates to protein-ratio
Cu	Copper
CyS	Cysteine
dm	Dry matter
Fe	Iron
Glu	Glutamic acid
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
K	Kalium
Met	Methionine
Mg	Magnesium
Mn	Manganese
N	Nitrogen
NDF	Neutral detergent fibre
Phe	Phenylalanine
Pro	Proline
Prot	Protein
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
Zn	Zinc

1 Introduction

World population is estimated to reach 9 billion people by year 2050 (FAO, 2009). To cope with the expected increase of food demand, alternative solutions are needed. Within the frame of the Farm to Fork strategy, introduced with the Green Deal recently approved by the European Community, the development of sustainable, environmentally friendly food systems becomes crucial.

Insects are globally an important food source, even if being rejected from the majority of people in developed countries (Yüksel, 2018). Insects are a source of vitamins, essential amino acids and fat acids (Bukkens, 1997). Yellow mealworm (*Tenebrio molitor*) and house cricket (*Acheta domesticus*) have been recently approved as novel foods by European Food Safety Authority and are among the leading edible insects due to their nutritional value and feed conversion rate. Rearing these insect species impacts the environment less than traditional livestock breeding does. For example, the relative greenhouse gases emitted by *T. molitor* breeding are substantially lower and feed conversion rate is better than that of traditional livestock.

In order to practice insect rearing successfully, information about the optimal rearing and nutritional needs of insects is needed. Although feeding and feeds can play a major role in determining environmental and economic sustainability of insect rearing as well as insect welfare, the nutritional requirements of insects are known poorly. Therefore, the main aim of CoRoSect task 3.2 was to develop standardized diets for insects intended to be used as food and feed derived from waste streams. Because the feed regulation does not permit the use of waste as feed in the food supply chain, "waste" here refers to residues and side streams that are permitted to be used as feed materials. Producing insect feed by recycling sidestream products obtained from food production chains would represent the closing of the circle of a fully sustainable industry, ensuring a low impact on the environment.

If the nutritional requirements (such as amino acid or minerals that are limiting insect growth) of insects are known, then mixtures of different side-streams can be used efficiently for the insect diets (Gold *et al.,*2018, Barragan-Fonseca *et al.,* 2017). This ensures that we able to produce insects of a high and consistent quality, and continuous process flow. To face this challenge, information is needed to help the utilisation of a wide variety of side-streams to formulate diets, which ensure consistent and high-quality production. On one hand, the diets should ensure good production performance and on the other hand, excessive provision of nutrients should be avoided to avoid environmental emissions.

Amino acid composition and digestibility of protein could be a key parameter driving larval development (Barragán-Fonseca, 2018). The comparative slaughter technique (comparison of the amino acid composition of the diet to the amino acid composition of the animal following slaughtering) previously used to determine energy requirements of livestock, can provide preliminary information on how well the amino acid requirements of insects are met by the diet (Cohen 2004, McDonald *et al.,* 2011; Gold *et al.,*2018). Carcass milling technique has used also for *H. illucens* (Woods *et al.*, 2018), but this comparative slaughter technique may be an oversimplification for *H. illucens* larvae treatment, as microbes in the biowaste and larval gut of these larvae can be additional consumers or producers of amino acids.

In most animal species, lysine is the first limiting amino acid. However, according to Koethe *et al.* (2022), the addition of lysine did not improve growth, development, or nutritional values of black soldier fly (*Hermetia illucens*) larvae. In contrast, the addition of lysine led to reduced larval size,

survival and prepupal rates, especially when high amounts (2.5% dry matter) of lysine were provided. The results of this study indicate that a lysine content of 0.3% in the diet is sufficient for optimal larval growth. The crude fat, crude protein, and lysine content of larvae did not differ significantly in relation to the supplementation of lysine.

From ecology point of view, it would be beneficial to use local side streams for feeding of insects. In these plant-based feed materials, that we used in our experiments, the first limiting essential amino acids for insects were arginine, tryptophan and methionine. Because synthetic amino acids are expensive, and because they are prohibited in organic farming, it is important to know, what are the sufficient levels of these amino acids and if there is any benefit from adding them to the insect diets?

This deliverable reports the results of CoRoSect task 3.2. Based on the findings of task 3.1, and related work conducted together with WP2, the performances (weight, total larval number, larval period and average larval weight, pupal period and pupal weight and total development time) of the mealworm (*T. molitor*), black soldier fly (*H. Illucens*) and of crickets (*A. domesticus*) were tested with diets including agricultural by-products such as wheat bran, brewer's yeast, brewer's spent grain, bread remains, in addition to residuals from the grapevine, potato, tomato, citrus and olive cultivation and transformation, where available. A novel method of diet administration, based on the inclusion of all the diet components in a single dry diet with a long shelf-life, recently developed by ENEA (Italian National Agency for New Technologies, Energy and Sustainable Economic Development), was also tested for the *T. molitor* diets yielding the best results in terms of insects' performance. The findings and data originating from this deliverable's experiments will be used as input to machine learning algorithms being developed in WP6 and WP7 aiming to automatise the processes of diet preparation, administration and replacement, as well as for the model of task 3.3. Hence, as automatization and optimisation task is partly conducted by forthcoming CoRoSect works, this deliverable does not yet report the automation part.

2 Materials and Methods

2.1. By-products selection

Each of three insect species has specificities in their diets. Hence the diets must be considered from the perspectives of each species. The principles of using sidestreams as feed materials were discussed and agreed by research teams in Italy an Finland before implementing the research. The views of CoRoSect industry partners enquired during the workshops and meetings carried out during the first year to the project under the leadership of WP2 were also taken into account. The experiments in *T. molitor* and *A. Domesticus* were conducted in Italy by CoRoSect partners CIHEAM and ICF, respectively, and the experiments in *H. Illucens* were conducted in Finland by partner Luke. One of the guiding principles was to examine feed materials that are available as side streams of food production and processing in the two countries. Hence, the by-products were selected by taking into account the availability of side streams both locally and in the two countries. Moreover, byproducts may have low protein content, which limits insects' growth, and they may contain harmful substances. Hence, these aspects deserved attention and affected the selection of byproducts and sidestreams for the experiments. This section and Table 2.1 provides an overview of feed materials selected for the experiments. Further details are provided in Section 2.2.

2.1.1. *H. illucens* diets

Poultry feed has very similar amino acid composition as *H. illucens* larvae (BSFL). In earlier studies at Luke, *H. illucens* larvae reared with poultry feed has had the fastest developmental time and it has produced the largest larvae compared to other experimental diets. Therefore, commercial store-bought layer mash (chicken feed) was used as a control diet and experimental diets were calculated to contain at least as much crude protein (6.2–6.3) and crude fat (1.3–1.6) as the control diet had. Experimental diets for each experiment were formulated to contain equal amount crude protein and fat. In the current study, the main focus of the *H. illucens* larvae experiment was in investigating 1) the suitability of agri-food sector byproducts in *H. illucens diets,* 2) investigating the amino acid composition of the diet as a key element for the performance of *H. Illucens* larvae (especially development time and growth) as well as 3) testing whether there is any benefit or harm for an extra dose of certain amino acids. More specifically, the aim of *H. illucens* studies was to evaluate, if the addition of extra amount of tryptophan or methionine to the plant-based side-streams containing diet will affect these larval performance parameters. For that purpose, 7-fold amount of these amino acids were added to a diet of *H. Illucens* larvae and they were analysed for performance characters.

The *H. illucens* experiment had two rounds of experiments. After the feed materials included in each diet were selected, the diets were prepared as explained in section 2.2.1. Several feed materials and by-products were included in the diets. There was a control diet and three experimental diets with four replicates per experiment. One of these replicates was used for monitoring the weight gain. One of these experimental diets had at least the same amounts of amino acids as control poultry feed and two of these experimental diets contained extra amount of tryptophan (first experiment) or methionine (second experiment).

Feed materials of the *H. Illucens* study were designed to model local side-streams (dried brewer's spent grain, vegetables, and breadcrumbs) and some of the feed materials were the same as used in the *T. molitor* experiments in Italy (i.e. potato and tomato). Domestic broad bean was added to the concentrate 3, because it contains methionine, cysteine, and arginine. Without broad bean the concentration of these amino acids would have been too low in diet 4.

2.1.2. *T. molitor* and *A. domesticus* diets

The selection of by-products suitable to formulate novel diets for *T. molitor* and *A. domesticus* was carried out by considering the following four aspects. Firstly, the availability of feed materials from the food industries in the study region was considered essential to ensure an easy retrieval of tested feeds in case they would be applied by commercial insect rearing companies. The availability of tested by-products in other European countries was also considered. Hence, food industries of typical of only one country only were discarded. Secondly, the percentage of water when by-products leave the factory or processing industry was considered. By-products with a low percentage of water were preferred, in sight of a higher storage time and logistic costs. Thirdly, the already existent recovery paths (biogas production, distilleries, animal feed) for the side streams was considered as a feature that was favouring the selection of a feed material to the experiment. Fourthly, the seasonality was considered as an important feature. This feature implied preferring by-products which were available all year round. Moreover, the nutritional content and the possibility of harmful substances in the diet required attention when selecting the feed materials for the experiments.

While the first *T. molitor* experiment tested diets consisting of single feed materials, the second experiment considered combinations of many feed materials in a diet. The comparison between single and multiple-material diets was included as a specific element in the *T. molitor* experiments because some byproducts, occasionally, have anti-nutritional factors and different combinations of them could be the solution for a diet that is sustainable and results in appropriate production performance.

In the final choice, it was not possible to respect the above-mentioned criteria for all selected side-streams for all the characteristics. The selected by-products and their provenance were:

- Potato peels: obtained from restaurants in Bari (Italy) by hand-peeling of raw potatoes. The peels included a thin layer of potato pulp.
- Tomato pomace: obtained from organic tomato sauce production industry located in Cerignola, Foggia (Italy). The pomace included peels, seeds and some pulp.
- Brewer's spent barley grain (SG): obtained from craft beer production industry in Bari (Italy).
- Wheat bran: obtained from organic durum wheat production of a mill located in Altamura, Bari (Italy).

Once in laboratory, each by-product was dried in a desiccator for 48 hours at 35°C to ensure a longer storage time and in the meanwhile preserving thermolabile elements such as vitamins. By-products were then ground in a kitchen blender to reach 4-6 mm of size. For house crickets, a further grinding was done with a knife grinder at 36.000 rpm, to obtain a size of 0.18-0.20 mm, more suitable for this species.

Table 2.1. An overview of feed materials included in different experimental diets of H. illucens, T. molitor and A. domesticus. The same feed materials included in different diets may vary in quality and nutritional composition. [1]

Diet	Chicken feed[1]	Wheat bran[1]	Brewers grain[1]	Kale	Spinach	Tomato[1]	Potato[1]	Yeast[1]	Breadcrumbs	Broad bean	Potato protein	Apple	L-Trp	L-met
H. Illucens														
C1, C2 (control)	✓													
S1			✓		✓		✓	✓					✓	
S2			✓	✓		✓		✓	✓				✓	
S3			✓	✓		✓		✓	✓					
S4			✓			✓	✓	✓	✓	✓	✓			✓
S5			✓			✓		✓	✓	✓	✓	✓		✓
S6			✓			✓		✓	✓	✓	✓	✓		
T. molitor														
A (Control)		✓												
B			✓											
C			✓											
D						✓								
E							✓							
F		✓	✓					✓						
G		✓					✓	✓						
H		✓				✓		✓						
I		✓	✓			✓								
A.domesticus														
A (Control)	✓													
B	✓	✓				✓		✓						
C	✓	✓					✓	✓						
D	✓	✓	✓					✓						
E	✓	✓	✓			✓	✓	✓						

[1] The potential feed materials include: two difference commercial chicken feed, three different brewer's grain (SG), tomato pomace or puree, potato or potato peels, different kinds of yeast, wheat bran, kale, spinach, breadcrumbs and synthetic amino acids.

2.2. Experiments' set-up and analyses of data

2.2.1. *H. illucens* experiment

2.2.1.1. Preparing of concentrates for H. illucens experiment

H. illucens diets were formulated so that each diet included a protein concentrate and other feed materials. First, the balanced protein concentrates of the side streams were formulated to make preparation of experimental diets, easier. Concentrates 1 and 2 were used in the first experiment. Concentrate 1 contained dried brewer's spent grain, feed yeast, and tryptophan as synthetic amino acid, so that all amino acid levels were at least at the same level as in poultry feed or alternatively 7-fold. Concentrate 2 did not include synthetic tryptophan. Concentrates 3 and 4 were used in the second experiment. Concentrate 3 contained broad bean, because it is widely available in Finland, and it is possible that we have also broad bean side streams, in the future. Potato protein is the side stream of potato industry. Feed materials of the concentrates (dry substances) were measured and moved to the bucket (20 litre) and blended with a concrete whisk.

Table 2.2 shows the used feed materials of these concentrates and their formulations (recipes) and calculated nutrient composition. Fresh brewer's spent grain (Brewery OlutMylly, Forssa, Finland, DM 19.1 %) was dried in a drying cabinet at 30 °C. After drying, the dried brewer's spent grain (DM 96.7 %) was grinded with the hammer mill. Feed yeast (Leiber Yeafi® BT) and Potato (variety 'Annabel') was purchased from a wholesaler. Spinach (frozen product chopped) and kale (Findus, pre-cooked chopped) was bought from a wholesaler. Tryptophan (Best Amino / L-Tryptophan, CJ BIO (CheilJedang Corp Korea, China), was bought from A-rehu feed company. Methionine (L-Met 100 Feed Grade, CJ-Bio, South Korea) was bought from Rehux feed company, Finland. Tomato (crushed) Eldorado, Italy was bought from a wholesaler. Breadcrumbs, MyllynParas (Hyvinkää, Finland) were bought from a wholesaler. Broad bean (variety 'Vire', Jokioinen, Finland) was grinded with a hammer mill. Potato protein was from Finnamyl Oy, Kokemäki, Finland. Apples (winter variety 'Jok 21') were picked from apple garden of Luke in Jokioinen, Finland.

2.2.1.2. Preparation of diets for H. illucens experiment

The experiments were designed to test *H. Illucens* larvae diets that consisted of a concentrate, rich in protein and utilizing local side streams (brewer's barley mash, feed yeast, potato protein), and some local (vegetables, breadcrumbs, apples) and European side streams (tomato, potato) to accompany the concentrate. The overall aim of the diet formulation was to use as much side streams as possible and that the protein and fat content of the different experimental diets would be at the same level. An additional specific aim of diet formulation for the first experiment was that the tryptophan content of the diet should be as high as, or higher than, the control diet (diets 1 and 2) or lower than the control diet (diet 3). Based on the results of the first experiment, the second experiment aimed at three diets with different methionine contents.

To standardise the nutrient contents of the diets (substrates), they were designed by WinOpti-software (AgroVision, the Netherlands). The information on the chemical composition of the feed materials was collected from the official Finnish feed tables using data from Natural Resources Institute Finland (Luke) and the Eva-Pig program and myfooddata.com website. The main wet volume consisted of local vegetables (spinach, kale, potato and apple) or tomato or potato-based materials, which were used also in two other experiments. Vegetables obtained from the stores were used to mimic equivalent plant-based side-streams from food industry. In addition, breadcrumbs, which were used also in *T. molitor* experiments, were used as dry side streams in *H. illucens* diets.

Before the diet formulation, dry matter content of the feed materials (dried brewer's spent grain, spinach, kale, crushed tomato, potato, breadcrumbs) were determined on a sample of the

fresh/defrost sample by a dry matter analyzer and the diet composition/formulation was adjusted based on analysed dry matter content. The feed materials, formulas and calculated composition of concentrates and diets are presented in Tables 2.2, 2.3 and in Annex 1 Table A4.

The poultry feed was used as a control diet, because in the previous studies at Luke insect lab, poultry feed has been good diet for growing *H. Illucens* larvae. Treatments in experiment 1 (diets 1–3) and 2 (diets 4–6) were prepared according to Table 2.2 and Table A4. Experimental diets were formulated to contain equal nutrient and amino acid profile as control diet (one diet) and otherwise equal nutrient and amino acid profile, except for 7-fold content of tryptophan (diets 1 and 2 in the first experiment) or methionine (diets 4 and 5 in the second experiment).

Larvae of the control group did not receive any additional tryptophan/methionine but only the basic diet (chicken feed), which tryptophan content was analysed in our previous experiment (4.29%). The diets of the first experiment were formulated based on the information from this analysis. In the first, experiment tryptophan was added to the basic plant-based diets (1 and 2) to obtain a tryptophan content of 5.79, 5.79 respectively. Diet 3 did not contain any synthetic tryptophan and its tryptophan content was calculated to be 0.94%. The analysis after the experiment however showed that the tryptophan content of the chicken feed was much lower than expected (0.82%). Because of this misinformation, the second experiment included two diets, where methionine content was calculated 7-fold compared (9.87 and 9.87%) to the control diet (1.41%) and one diet, where methionine content was calculated to be at the same level (1.50%) as in the control diet.

Frozen feed materials (spinach and kale) were transferred to the refrigerator day before the experiment to allow them to melt down before use. Spinach and kale were pre-chopped products. Raw potatoes were sliced with a mandolin into slices of 3 mm and chopped with a kitchen blender (Wilfa, BL1B-P1200, Norway). Fresh apples were chopped with a kitchen blender. The concentrates and the other feed materials of the diets were weighed according to the formula (Table 2.3) and mixed with each other with a ladle. After the control and experimental diets were prepared and mixed properly, a sample of 500 g of prepared diet was taken for chemical analysis. The final batch size was 5.0 kg. Before the larvae were added to the diet, it was warmed to the ambient temperature of rearing chamber (27 °C).

Table 2.2. Ingredients and calculated chemical composition of formulated concentrates used in H. illucens diets.

	Concentrate 1	Concentrate 2	Concentrate 3	Concentrate 4
Ingredients (%)				
Brewer grains (dried brewer's spent grain)	80.65	82.0	45.0	66.92
Feed yeast	17.83	18.0	6.0	15.0
L-Tryptophan	1.53	–	–	–
Broad bean (*Vicia faba*, Vire)	–	–	33.05	–
Potato protein	–	–	15.95	18.08
Calculated chemical value (%)				
Dry matter	92.0	92.0	89.81	92.30
Moisture	8.0	8.00	10.19	7.70
Crude protein	23.77	22.84	31.92	33.27
Crude fat	7.81	7.95	4.86	6.98
Amino acid composition[1)]				
Alanine	12.91	13.12	15.78	17.75
Arginine	10.06	10.22	18.45	15.94
Aspartic acid	15.55	15.80	32.40	31.1
Cysteine	2.54	2.58	4.0	4.0
Glutamic acid	41.82	42.51	48.52	50.3
Glycine	9.39	9.54	14.17	14.65
Histidine	4.04	4.10	6.87	6.57
Isoleucine	11.19	11.38	16.17	17.61
Leucine	19.37	19.70	28.53	30.37
Lysine	9.13	9.27	19.37	18.65
Methionine	3.78	3.84	5.25	6.35
Phenylalanine	10.42	10.59	16.17	17.67
Proline	21.02	21.37	20.55	24.97
Serine	9.95	10.11	15.31	15.7
Threonine	7.63	7.75	13.67	14.49
Tryptophan	17.37	2.46	3.46	3.76
Tyrosine	4.89	4.96	11.55	11.61
Valine	11.58	11.77	18.01	19.24

[1)]The essential amino acids for the rearing of insects are in italic (essential amino acids according to Cohen 2004).

Table 2.3. Ingredient and calculated chemical composition of formulated H. illucens diets (Control C1, C2 and experimental diets S1 through S6).

	C 1[2)]	C 2	S 1[1)]	S 2	S 3	S 4	S 5	S 6
Ingredients (% of diet)								
Laying hen feed	39.19	39.19	–	–	–	–	–	–
Water	60.81	60.81						
Concentrate 1	–	–	32.47	32.55	–	–	–	–
Concentrate 2	–	–	–	–	32.55	–	–	–
Concentrate 3	–	–	–	–	–	24.33		
Concentrate 4	–	–	–	–	–	–	22.0	19.74
Spinach (frozen product)	–	–	60.53	–	–	–	–	–
Potato (fresh)	–	–	7.0	–	–	29.00	–	–
Kale (frozen product)	–	–	–	62.00	62.00	–	–	–
Tomato (crushed)	–	–	–	5.15	5.15	41.83	42.0	40.31
Breadcrumbs	–	–	–	0.30	0.30	4.0	6.76	9.95
L-methionine	–	–	–	–	–	0.85	0.84	–
Apple (fresh)	–	–	–	–	–	–	28.4	30.0
Calculated chemical composition (%)								
Dry matter	35.0	35.0	35.45	35.5	35.5	35.0	35.0	35.14
Moisture	65.0	65.0	64.55	64.5	64.5	65.0	65.0	64.86
Crude protein	6.31	6.17	8.81	8.83	8.53	9.82	9.31	8.47
Crude fat	1.61	1.31	2.72	2.99	3.03	1.32	1.79	1.70
Amino acid composition[3)]								
Alanine	2.45	2.46	4.76	4.78	4.84	4.37	4.43	4.18
Arginine	4.09	3.45	3.91	3.90	3.96	5.02	4.05	3.87
Aspartic acid	5.41	4.67	6.14	6.14	6.23	9.81	8.35	7.84
Cysteine	0.99	1.13	0.97	0.98	0.99	1.21	1.13	1.13
Glutamic acid	12.73	13.56	15.04	15.27	15.5	16.81	16.44	16.42
Glycine	2.68	2.62	3.58	3.60	3.65	3.92	3.73	3.56
Histidine	1.43	1.41	1.57	1.96	1.98	1.96	1.75	1.69
Isoleucine	2.57	2.29	4.22	4.31	4.37	4.38	4.31	4.06
Leucine	4.48	4.33	7.18	7.10	7.21	7.68	7.45	7.04
Lysine	3.30	2.84	3.67	3.64	3.69	5.25	4.55	4.24
Methionine	1.33	1.41	1.44	1.35	1.37	9.87	9.87	1.50
Phenylalanine	2.98	2.92	3.91	3.98	4.03	4.64	4.47	4.25
Proline	4.03	4.63	7.28	7.53	7.64	5.77	6.51	6.37
Serine	2.86	2.86	3.66	3.73	3.78	4.30	4.06	3.90
Threonine	2.44	2.36	2.97	3.00	3.03	3.82	3.64	3.43
Tryptophan	4.29	0.82	5.79	5.79	0.94	0.98	0.96	0.92
Tyrosine	1.87	1.86	2.03	1.99	2.01	3.23	2.89	2.47
Valine	3.17	2.91	4.42	4.38	4.45	4.96	4.78	4.53

[1)]See Table 2 for a description of the treatments. [2)] Control 1 diet amino acid compositions are from previous experiment. [3)]The essential amino acids for the rearing of insects are in *italic* (essential amino acids according to Cohen 2004).

2.2.1.3. Larvae

Little *H. Illucens* larvae were obtained from a laboratory colony of Luke. *H. Illucens* is continuously propagated by Luke Insect Lab (Jokioinen, Finland). Facilities are in place to rear and process insects at laboratory and pilot level. For these experiments in this study, *H. Illucens* newly hatched larvae were

grown at the plastic container (20 cm x 20 cm x 12 cm) on a mixture of 0,5 g chicken feed (chicken feed, metabolizable energy content 11.2 MJ kg^{-1}) and tap water (the dry matter content of complete feed was 30%). During the pre-experimental period larvae were maintained in a controlled growth chamber at 27 ^{o}C and 50% RH.

The size of the little larvae in the beginning of the experiment was determined by calculating the number of larvae per gram. Larvae were assigned to three treatment groups/experiment: control group, and tryptophan/methionine supplementation groups diet 1 and diet 2, (first experiment) and diet 4and diet 5 (second experiment) and groups 3 (first experiment) and 6 (second experiment), which did not have any synthetic amino acid addition.

2.2.1.4. Rearing of larvae during the experiments

The larvae were reared in grey plastic boxes (30 cm x 40 cm x 15 cm) placed inside a Eurobox (40 cm x 60 cm x 20 cm) as illustrated in Figure 2.1. Temperature during the experiment was adjusted to 27 °C and humidity to 50 % (RH). Pro loggers (screw tag) were screwed to the bottom of the box (centre location) for temperature measurements. The screw hole under the box was secured with duct tape. The process of monitoring of rearing conditions with the sensors was prepared in collaboration with the partner OAMK.

The control feed was dissolved in water and feed materials of experimental diets were mixed with each other's according to formulation to achieve the dry matter content of 35–36% (64–65 RH %). For each treatment group, there were four replicates. Diet amount was 5 kg per box (single batch feeding) and there were about 5,500 larvae per 5 kg diet. The size of the box was 40 x 30 x 15 cm and the thickness of the substrate in the box was about 10 cm (12 000 cm^{2}), so the larval density was about 2.2 larvae cm^{-2}. Prior to adding the larvae to the diet, the temperature of the diet was adjusted to the 27 °C . The boxes were without the lids. Larvae were reared in these boxes until in most boxes 10 prepupae (dark larvae) were visible. The treatment box place in the rack level was contributed by randomized design (Table 2.4). Samples were numbered with the code series_row (Table 2.5). The growth of the larvae was measured from the larvae of the boxes at row 2. Temperature loggers were placed at the rows 1 and 4 in every box.

Figure 2.1. Rearing devices used in the H. illucens experiment (box-in-box arrangement and a rearing box with larvae (upper photos), a logger attached in the box (bottom left photo) and sieving device (bottom right).

Table 2.4. Placement of treatment boxes in the level of the rack.

Box	Row	Column	Treatment
1	1	1	2
2	1	2	3
3	1	3	4
4	1	4	1
5	2	1	3
6	2	2	4
7	2	3	1
8	2	4	2
9	3	1	1
10	3	2	2
11	3	3	3
12	3	4	4
13	4	1	4
14	4	2	1
15	4	3	2
16	4	4	3

Table 2.5. Samples were numbered with the code series_row in both H. Illucens larvae rearing experiments.

Code series_row			
2_1	3_1	4_1	1_1
3_2	4_2	1_2	2_2
1_3	2_3	3_3	4_3
4_4	1_4	2_4	3_4

The total weight of the diet boxes at different time points were measured on days 1, 4, 6 and 8 (first experiment) and on days 1, 3, 6, 8, 10 and 11 (second experiment). On experiment day 3 water was added (0 to 60 g) to the diet boxes according to the weight of the chicken feed diet (control) box (4900 g) (first experiment). On day 6 and 8 all the diets were mixed with a ladle. The mean larval weight (sample 30 larvae) at different time points was measured from the rearing boxes on the row two. To obtain a representative estimate of the larval size, the larvae sub-samples were collected from different locations of the box to the cup and the larvae were sampled from it. Before the weighing the larvae were cleaned from residual diet by washing them on the sieve and afterwards dried with paper towels. After weighing the larvae were placed back on the diet. The pH (Eutech Elite Ph Spear, Thermo Scientific) of the diet was analysed also from the rearing boxes on row 2 on days 1, 4 and 8 (first) and on days 1, 4, 7 and 11 (second experiment). The different stages of the experiment were documented by taking photos.

At the end of the experiment, the larvae, prepupae and frass were separated from each diet by sieving (3 mm; Figure 2.1). The sieve was washed up after each diet box. After the sieving the larvae, prepupae and frass were weighed and sampled (about 400 g, 2-liter bag) for the chemical analyses.

All diets were weighed when applied, as well as the total remaining material (larvae and prepupae and frass) at the end of the experiment before and after sieving. Survival and the number of the dark larvae were estimated for each experimental group. The dead larvae were not taken into account when

calculating the diet-to-biomass conversion ratio (BCR). After the experiment temperature sensors were measuring the temperature at the bottom of these empty experimental boxes for two more days (4.6-5.6.).

2.2.1.5. Chemical analyses of the diet, larvae and frass

The sample of diets (500 g) in the beginning of the experiment was analysed for chemical composition. Representative sample for analysis was collected as the combined sample from all the treatment boxes after sieving. Diet (before the experiment), larvae and prepupae and frass (after the experiment) were analysed in analytical triplicates per treatment group for dry matter, crude fat, crude fiber, crude protein (N), ash, ADF and NDF. Diets were also analysed for amino acid content. All four replicates were analysed for dry matter, ash and crude protein (N).

Chemical analyses of combined sample of diets, larvae and frass involves calculating the amount of moisture, dry matter, crude protein (N), ash, crude fat, crude fibre, ADF, NDF and amino acids. The composition of mineral substances was also analysed. Chemical analyses were conducted at Luke laboratory (Jokioinen), except amino acids, which were analysed at Eurofins lab. Analytical methods for chemical composition analyses used in these experiments are presented in Table 2.6.

Sample pre-treatment: Sample oven drying before analysis: The common procedure is drying at 60°C for 18 h (overnight). If amino acid determination is needed from the sample then the procedure is freeze drying. Freeze drying: Christ gamma 2-20 with controller LMC-2, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany. Drying period 3-4 days beginning with -25 °C, 0.370 mbar. Grinding: After drying (60 °C or freeze drying) the samples are grinded using sample mill (Sakomylly KT-120, Koneteollisuus Oy, Helsinki, Suomi) and 1 mm sieve.

Table 2.6. Analytical methods used for chemical composition analyses.

Analysed parameters	Description of used method
Dry matter	Dry matter content was determined by drying at 105°C for 20 h (2 h at +50°C at first).
Crude ash	Part of the sample that remains after incineration at 510°C 16 h. Official method AOAC-942.05 Ash of Animal Feed (Official Methods of Analysis of AOAC International (2019) 21st Ed., AOAC International, Rockville, MD, USA, ISBN 0-935584-89-7).
Total N	Total nitrogen content (Kjeldahl) based on Official Method AOAC 20001.11: Protein (Crude) in Animal Feed, Forage (Plant Tissue), Grain, and Oilseeds (Official Methods of Analysis of AOAC International (2019) 21st Ed., AOAC International, Rockville, MD, USA, ISBN 0-935584-89-7) using Cu as a digestion catalyst and Foss Kjeltec 2400 Analyzer Unit (FOSS, Hillerød, Denmark).
Crude protein	Crude Protein content is calculated by multiplying total nitrogen content by an appropriate conversion factor.
Amino acids	Amino acids were made by Eurofins Scientific Finland (Eurofins Vitamin Testing Denmark - DS EN ISO/IEC 17025 DANAK 581), by the method: Amino acids with acid hydrolysis accredited method ISO 13903:2005; EU 152/2009 IC-UV.
Crude fat	AOAC Official Method 920.39 Fat (Crude) or Ether Extract in animal Feed (Official Methods of Analysis of AOAC International (2019) 21st Ed., AOAC International, Rockville, MD, USA, ISBN 0-935584-89-7) and acid hydrolysis method (ISO 6492: Animal feeding stuffs - Determination of fat content. The equipment used was Foss Soxtec/Hydrotec 8000™ System for total fat analysis, consisting of Soxtec™ 8000 extraction unit and Hydrotec™ hydrolysis unit, (FOSS, Hillerød, Denmark).
Crude fibre	With Fibertec 2023 FiberCap system (FOSS, Hillerød, Denmark, FOSS Application note 3437). European commission (1992).
NDF	Neutral detergent fibre (NDF): According to Application Note 3445/Rev. 6. which is based on methods of: AOAC 2002:04: Amylase-treated Neutral Detergent Fiber in Feeds, Official Methods of Analysis of AOAC International (2019) 21st Ed., AOAC International, Rockville, MD, USA, ISBN 0-935584-89-7 and Goering and van Soest (1970) and also: Van Soest *et al.,*(1991). The Fibertec apparatus is Fibertec™ 8000 (Hot Extraction Unit) (FOSS Analytical A7S, Hillerød, Denmark). Sodium sulphite was used in NDF-detergent solution and heat-stable alpha amylase is used in samples containing starch and the result is ash-free (aNDFom).
ADF	Acid Detergent fibre (ADF): According to Application Note 3444/Rev. 6. which is based on methods AOAC 973.18 Fiber (Acid Detergent) and Lignin (H_2SO_4) in animal feed, Official Methods of Analysis of AOAC International (2019) 21st Ed., AOAC International, Rockville, MD, USA, ISBN 0-935584-89-7 and Method EN ISO 13906:2008. ADF result is on basic of organic matter (ADFom). The Fibertec apparatus is Fibertec™ 8000 (Hot Extraction Unit) (FOSS Analytical, Hillerød, Denmark). NDF- treatment was made prior ADF-treatment. Detergent solution was made according to Robertson and Van Soest (1981).
Minerals and trace elements	(Ca, P, K, Na, Mg, Mn, Fe, Cu, Zn, S): Determination of total nutrients: Samples for the total nutrients were digested by the closed wet HNO3-H2O2 digestion method in a microwave oven (CEM MDS 2000, CEM Corporation, Matthews, Canada) and the extract was analysed by a iCAP 6500 DUO ICP-emission spectrometer (Thermo Scientific, United Kingdom) (Kalra 1998).

2.2.1.6. Calculations

Performance parameters were calculated as described by Gold *et al.,*(2020). However, instead of direct calculation of initial and final larva numbers, the count estimates were done as total larval mass divided by the average size (based on sample of 30 larvae, sampled with good randomization). Larval counts, and residue and larvae dry weights, were used to calculate five *H. Illucens* larvae performance parameters. First, larval survival rates were calculated using Eq. (1) as the ratio of larvae at the end ($larvae_{end}$) and the beginning ($larvae_{beg}$) of the experiments (Van Der Fels-Klerx *et al.,*2017).

$$\text{Survival rate } (\%) = \frac{\text{larvae}_{\text{end}}}{\text{larvae}_{\text{beg}}} \times 100 \qquad (1)$$

Waste reduction was calculated using Eq. (2) as the ratio of residue dry mass ($residue_{mass}$) to the dry mass of total feed ($feed_{mass}$) provided (Diener *et al.,*2009):

$$\text{Waste reduction } (\%\ \text{DM}) = \left(1 - \frac{residue_{mass}(g)}{feed_{mass}(g)}\right) \times 100 \qquad (2)$$

The bioconversion rate was calculated using Eq. (3), for which the larval dry weight gain ($larval_{gain}$) was calculated as the difference between the final larval dry weight and the initial larval dry weight multiplied by the number of larvae at the end of the experiment:

$$\text{Bioconversion rate } (\%\ \text{DM}) = \frac{larvae_{gain}(g)}{feed_{mass}(g)} \times 100 \qquad (3)$$

Waste conversion efficiency (Liu *et al.,*2018), also called efficiency of conversion of ingested/digested food (Diener *et al.,*2009, Oonincx *et al.,*2015), was calculated using Eq. (4):

$$\text{Waste conversion efficiency } (\%\ \text{DM}) = \frac{larvae_{gain}(g)}{feed_{mass}(g) - residue_{mass}(g)} \times 100 \qquad (4)$$

Finally, the protein conversion efficiency was calculated using Eq. (5) as the ratio of the amount of larval protein accumulated ($protein_{gain}$) to feed provided ($feed_{mass}$). Larval protein accumulated was calculated as the difference between the amount of final larval protein and the initial larval protein multiplied by $larvae_{end}$. The amount of larval protein was calculated by multiplying the larval protein content with the larval weight:

$$\text{Protein conversion efficiency } (\%\ \text{DM}) = \frac{protein_{gain}(g)}{feed_{mass}(g)} \qquad (5)$$

Neonate protein, fat and ash content were taken as 6 dol (matches our larval stage) from the study of Liu *et al.,*(2017).

2.2.1.7. Rearing Experiment

The size of the little larvae in the beginning of the experiment was determined by calculating the number of larvae per gram. In the first experiment the results (120, 125, 139 and 142 pieces/g) indicated that the gram of little larvae contained an average of 131 pcs, so it was estimated that 42 g contained 5500 larvae. First experiment started on 25 May 2022 at 4 pm (day 1) and it lasted for 10 days. In the second experiment, the gram of little larvae contained an average of 184 pieces. Hence, that weight of 5 500 larvae batch was 30 g. Second experiment started on 28 October 2022 at 4 pm (day 1) and it lasted for 11 days.

Little larvae (average size 7.63 mg first and 5.45 mg second experiment) were placed in diet at the first day of experiment. Larvae were assigned to three treatment groups/experiment: control group, and tryptophan/methionine supplementation groups diets 1, 2 and 3 (first experiment) and diets 4, 5 and 6 (second experiment).

Larvae were transferred into a plastic container without lids with 5.0 kg control or experimental substates. Batch feeding was used to match the industry practice. The experiments were conducted in four replicas. The three replicas were for exploring the performance of *H. Illucens* larvae in the feeding experiment and fourth replicate was for monitoring of the weight gain of the larvae. It has concluded in previous studies that weighting disturbs the growth of the larvae. Growth of the larvae was monitored by weighing 30 larvae for one sample (fourth replica, always the same container) on

days 0, 3, 6, 8 and 10 (second experiment also on day 11). The experiment was finished when there were 10 visible larvae, which had transformed into prepupae. The survival rate was also estimated from this sample.

At the end of the experiment, on day 10 (first experiment) and day 11 (second experiment), larvae were separated from the remaining residue by sieving. After harvesting the larvae, total mass of the larvae and residues (frass) was determined.

2.2.2. *T. molitor* experiment

2.2.2.1. Insects and experimental facilities

Two experiments were carried out for *T. molitor* at IAMB facilities (Valenzano, Bari, Italy). In the first experiment, the diets composed of each by-product were tested separately (oligidic diets). In the second experiment, combinations of by-products were tested based on the protein content of diets (meridic diets).

In both experiments, 40-days old larvae collected from lab rearing were placed inside plastic transparent cups, after being weighed. For each diet, 10 replicas with 20 larvae were performed. Wheat bran was used as negative control. 0.25 g of fresh pumpkin were provided as source of water and replaced every 4 days. The single replica was concluded at the first pupa appearance.

Temperature and relative humidity were maintained at 27±1 and 60±5% in a dark environment.

2.2.2.2. Experiment with single by-products

In the first experiment, each cup was filled with 5 g of diet, that acted as diet, and further diet was added in case of shortage (for an illustration, please see Figure 2.2). Only in this part of the experiment, also an additional by-product was tested. This was dried and ground olive pomace, coming from local olive oil mill. Unfortunately, no chemical analysis is available for this compound.

Figure 2.2. Photos from the first experiment with T. molitor

2.2.2.3. Experiment with mixed by-products

In the second experiment, diets were combined to achieve approximately the same level of proteins contained in wheat bran (16.68%). To reach this level, brewer's yeast powder for livestock feed was added. Since the brewer's spent grain retrieved for this experiment (SG1) showed a low protein content compared to what we expected (12.9%), in this part of the experiment also a spent grain coming from another beer industry was included (SG2). SG2 contained 24.85% of proteins.

All diets combinations were formulated on the protein content basis because proteins are crucial for yellow mealworm growth (John *et al*., 1979). The composition diets tested in the second experiment are presented in Table 2.7.

To prevent mealworms from selecting the most palatable components of the diets, the by-products were merged to form a biscuit according to the method conceived by Juan A. Morales Ramos (personal communication; for an illustration, please see Figure 2.3). Also single by-products were tested again following the same procedure. Overall, nine diets, including the control diet, were tested.

All the by-products used in the *T. molitor* and *A. domesticus* experiments were sent to a chemical analysis to a laboratory (*EuroQuality lab* srl, Gioia del Colle, Bari – Italy). The macronutrients were determined, and the following relative analysis methods were applied:

1. Humidity/ volatile substances (according to procedures reported in Baldini et al. (1996), *Rapporti ISTISAN 1996/34, page 7*).
2. Total amount of the non fibercarbohydrates on the dry matter (calculated by subtracting the amounts of proteins, ashes, fats and fibers from 100)
3. Total ashes (according to procedures reported in Baldini et al. (1996), *Rapporti ISTISAN 1996/34, p. 77*)
4. Total nitrogen (according to procedures reported in Baldini et al. (1996), *Rapporti ISTISAN 1996/34, p. 124*)
5. Crude Protein content (calculated by multiplying nitrogen content by 6.25)
6. Fats (according to procedures reported in Baldini et al. (1996), *Rapporti ISTISAN 1996/34 p. 41*)
7. Crude fibers (according to procedures reported in *AOAC 985.29 1986*)

Once in laboratory, each by-product was dried in a desiccator for 48 hours at 35°C to ensure a longer storage time and in the meanwhile preserving thermolabile elements such as vitamins. By-products were then ground in a kitchen blender to reach 4-6 mm of size. For house crickets, a further grinding was done with a knife grinder at 36.000 rpm, to obtain a size of 0.18-0.20 mm, more suitable for this species. Finally, each by-product was sent to a chemical analysis laboratory to determine the composition in macronutrients. The chemical composition of the feed materials is presented in Table 2.8).

Table 2.7. Composition of the second experiment diets with T. molitor.

Diet	Wheat bran (%)	Brewer's spent grain (SG1, %)	Brewer's spent grain (SG2, %)	Tomato pomace (%)	Potato peels (%)	Yeast (%)	Protein content (%)
A (Control)	100						16.68
B		100					12.85
C			100				24.85
D				100			9.5
E					100		11.49
F	50	45				5	16.50
G	50				43	7	16.61
H	50			41		9	16.52
I	50		23	27			16.60

Table 2.8. Chemical composition in macronutrients of the selected by-products.

	Wheat bran	Potato	Tomato	Brewer's spent grain (SG1)	Chicken feed
Humidity (g/100g)	8.8	5.2	7.9	7.5	12
Non fiber carbohydrates (g/100g DM)	30.2	60.6	8.9	50.3	-
Total ashes (g/100g)	4.2	6.5	3.8	2.2	5.5
Total nitrogen (%w/w)	2.7	1.8	1.5	2	-
Crude protein (g/100g)	16.7	11.5	9.5	12.9	19
Fat (g/100g)	6.5	0.6	3.2	3.9	4
Crude fiber (g/100g)	36.1	18.9	67	27	5.5

Figure 2.3. Photos illustrating the biscuits used in the second T. molitor experiment.

2.2.3. *A. domesticus* experiment

2.2.3.1. Insects and experimental facilities

The experiments with the house crickets were performed at Italian Cricket Farm (Torino, Italy). Four diets and a control diet were formulated considering the amount of protein of chicken feed for free-range chickens commonly used for house cricket rearing (19%; Table 2.9). For this purpose, chicken feed, wheat bran and brewer's yeast were present in all diets to increase the protein amount. The diet was composed by adding each by-product in the determined proportions and mixing by hand.

2.2.3.2. Rearing experiment

Thirty-days old crickets were collected from ICF rearing population and placed inside the breeding crates (26 cm x 37 cm x 14 cm) made of polypropylene. These crates contained a rudimentary cricket drinking trough also made of polypropylene and consisting of a sheet of pure cellulose blotting paper rolled up and soaked in water, and two overlapping cellulose egg cups to increase the breeding surface. The crates were stacked on top of each other and covered with a fine-mesh grid to prevent the crickets from escaping. Figure 2.4 illustrates the setup of crates.

Forty crickets were weighed and placed in each tank, together with 20 g of diet. Water was replaced every seven days and 20 g of feed was administered every ten days. 5 replicas were performed for each diet. Temperature and Relative Humidity were maintained at 28±2 °C and 55±5%.

The experiment was interrupted after 21 days because almost all the survived individuals had reached the adulthood and because cannibalistic behaviour often becomes more pronounced at the onset of the adult phase (Stefano Magnaghi, personal communication).

Table 2.9. Composition of diets tested with A. domesticus.

	Chicken feed (%)	Yeast (%)	Wheat bran (%)	Tomato pomace (%)	Potato peels (%)	Brewer's spent barley grain (SG1, %)	Protein content (%)
A (Control)	100	0	0	0	0	0	19
B	50	10	10	30	0	0	18.78
C	50	10	10	0	30	0	19.38
D	50	10	10	0	0	30	19.78
E	40	10	10	10	10	20	18.70

Figure 2.4. Photos illustrating the rearing crates used in the A. domesticus experiment.

2.2.4. Statistical analysis

The growth rates of *H. illucens* were analysed with Petzoldt's (2022) growthrates package. Maximum growth rates were estimated from log-linear part of the growth curve by using smoothing splines. Row-column type experiments were analysed using mixed models using rows and columns as random factors and diets or diet characteristics as fixed factors using lme4 – R package (v. 1.1.30) (Bates *et al.,*2015). The multiple testing correction was done test wise using Tukey's method. Standard linear regression analyses were done using lm – function in R-package stats (v. 4.2.1) (R Core Team, 2022) and predictors were chosen using exhaustive search in R-package leaps (v. 3.1) (Miller TlboFcbA, 2020) with maximum adjusted r2 criterion. Co-inertia analysis (CIA) is a multivariate method that identifies

trends or co-relationships in multiple datasets which contain the same samples. CIA can be applied to datasets where the number of variables far exceeds the number of samples (Dray *et al*., 2007). COI was performed using ade4 (v. 1.7-20) (Dray and Dufour, 2007). CoIA was done for Principal Components Analysis with scaled and centered values. In *H. illucens* experiment 2, monotone Hermite splines were calculated for interpolation according to the method of Fritsch and Carlson (1980) by using splinefun in stats -R package (v. 4.2.1) (R Core Team (2022).

Regarding *T. molitor* and *A. domesticus* experiments, the data were analysed and summarised by CIHEAM. To compare the performances of diets on insect growth, the data were analyzed first with a Kruskal–Wallis test. Thereafter, Mann-Whitney pairwise test with Bonferroni corrected p-values (α=0.05) was carried out. The statistical analyses were performed using the software Past (Hammer *et al*., 2001).

3 Results and discussion

3.1. *H. illucens experiment*

3.1.1. Chemical composition of diets, larvae and frass

Formulation of diets was quite successful except for the first control diet, which had an incorrect tryptophan concentration (analysed in previous experiment). The plan of testing amino acid levels was adjusted so that a 7-fold amino acid content was tested in parts of the experiment. Analysed chemical composition of used diets was near the calculated values. The analyzed chemical compositions of the control and experimental diets are presented in Table A1 of Annex 1. Table A2 in Annex 1 shows analysed chemical composition of *H. Illucens* (pooled sample) in control and experimental diets (fresh weight). Analysed chemical composition of *H. Illucens* larvae frass (pooled sample) of control and experimental diets (fresh weight) is presented in Table A3 in Annex 1. Correlations between diet characteristics are illustrated in Figures 3.1 (first experiment) and 3.2 (second experiment).

The protein content is usually calculated from total nitrogen using the nitrogen-to-protein conversion factor (Kp) of 6.25. This factor overestimates the protein content, due to the presence of nonprotein nitrogen in insects. In the studies of Janssen *et al.,*(2017), a specific Kp of 4.76 ± 0.09 was calculated for larvae from *T. molitor*, *Alphitobius diaperinus*, and *H. illucens*, using amino acid analysis. The protein content of the larvae was adjusted according to Janssen *et al.,*(2017) to use multiplier 4.76 instead of 6.25.

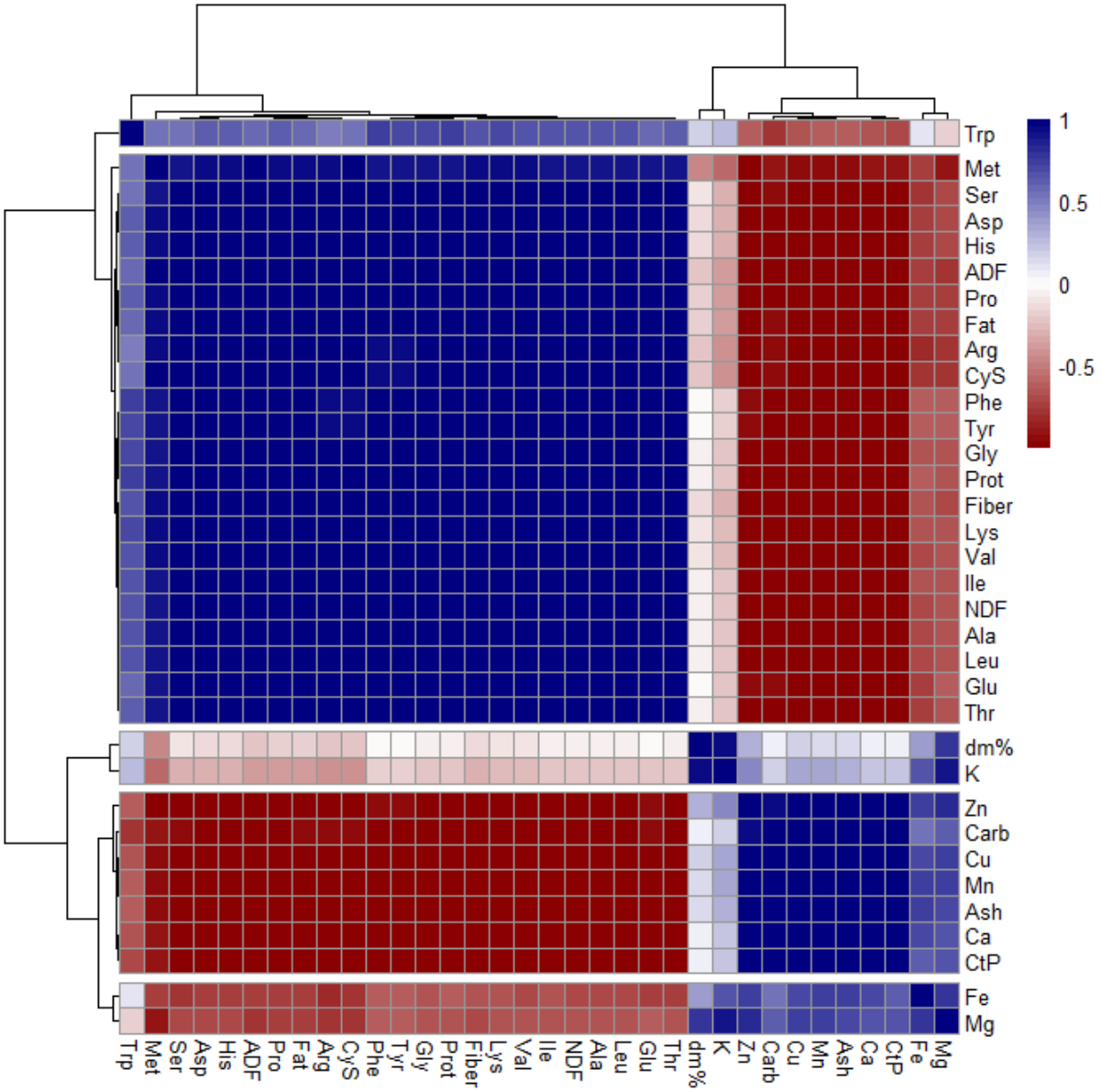

Figure 3.1. Correlation between diet characteristics (first experiment).

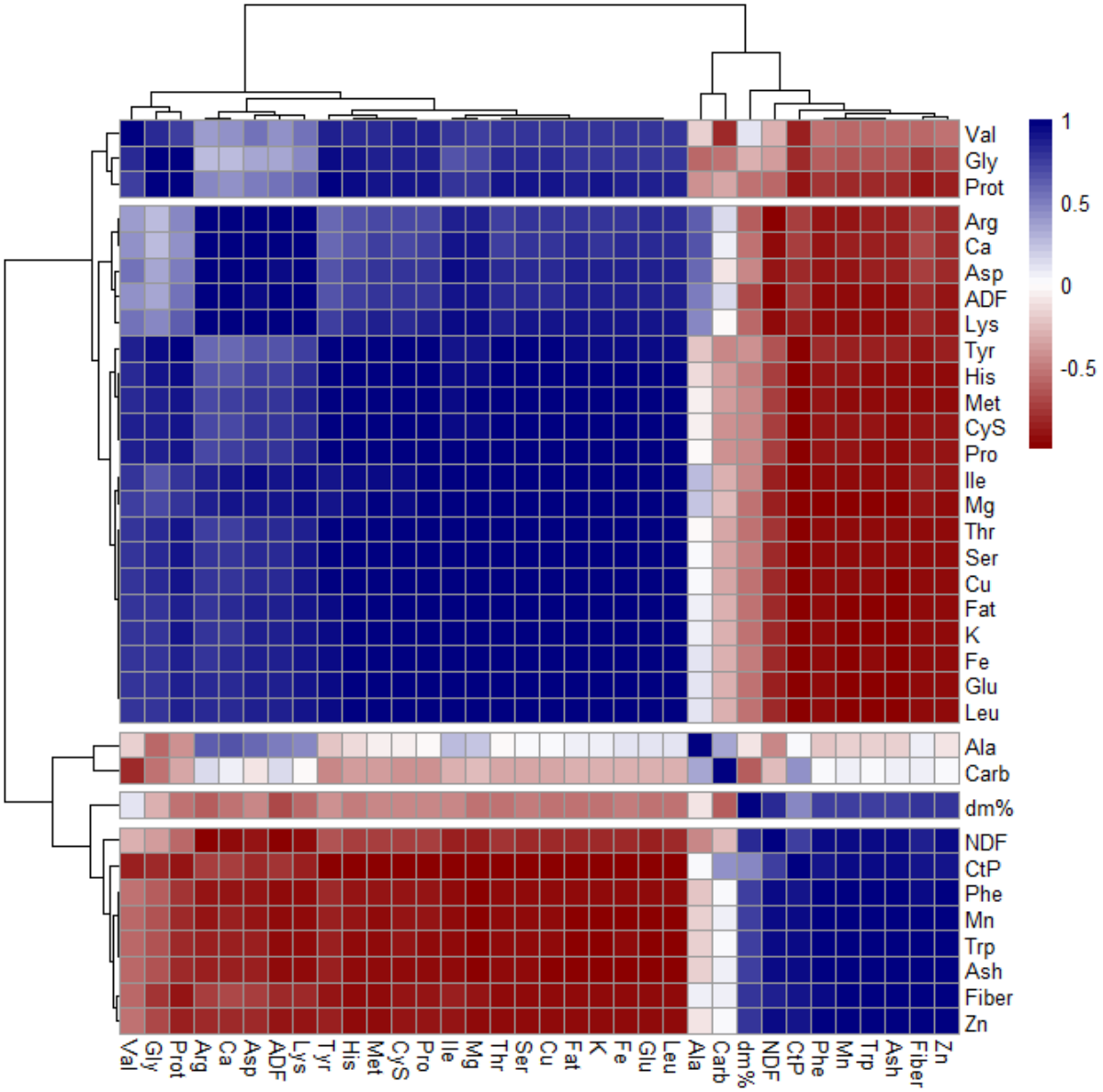

Figure 3.2. Correlation between diet characteristics (second experiment).

3.1.2. Temperature sensor data

Data from temperature sensors of the first (Figure 3.3) and the second (Figure 3.4) experiments show that the maximum temperature of the diet on the bottom of the rearing box was very high (over 45 °C). It has been observed also in previous studies, that during the feeding process of the larvae, the diet temperature can reach as much as 45 °C, which is the optimum temperature for proteolytic activity at a pH of about 8 in the posterior midgut of *H. Illucens* larvae (Bonelli *et al.*, 2019; Kim *et al.*, 2011).

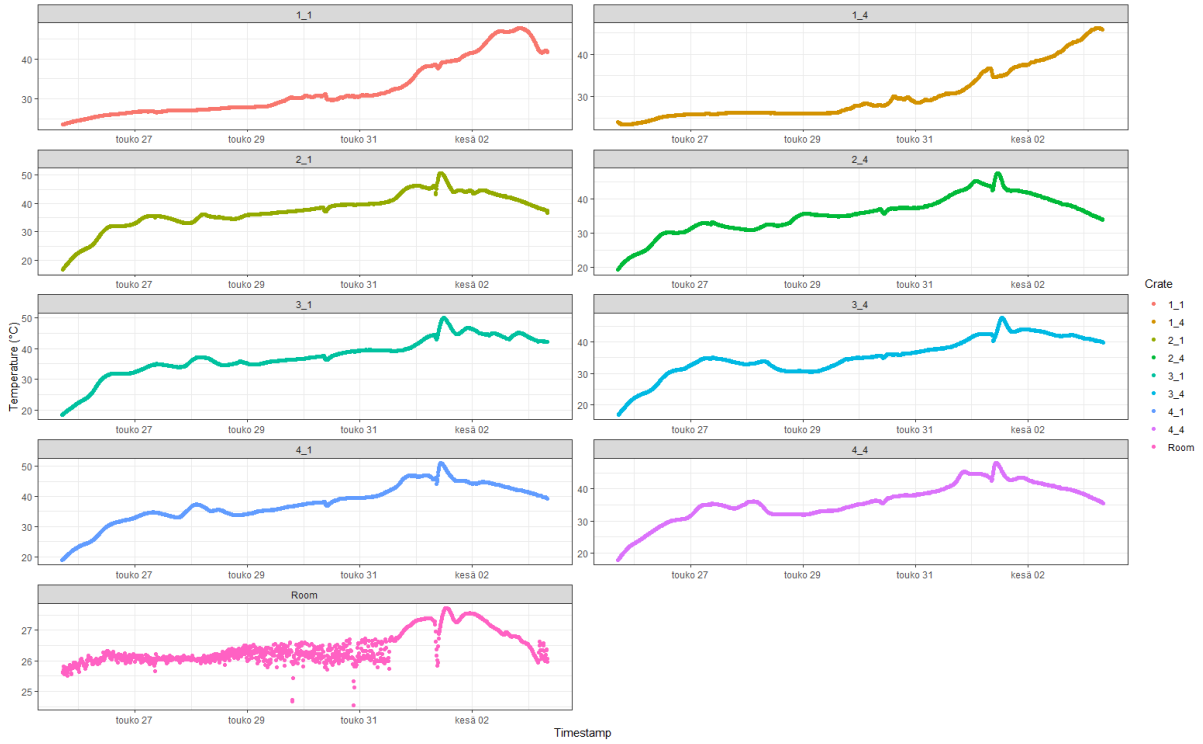

Figure 3.3. Illustration of measurement data from the temperature sensors used during the first experiment (two measurements per treatment in each row and the room temperature measurement).

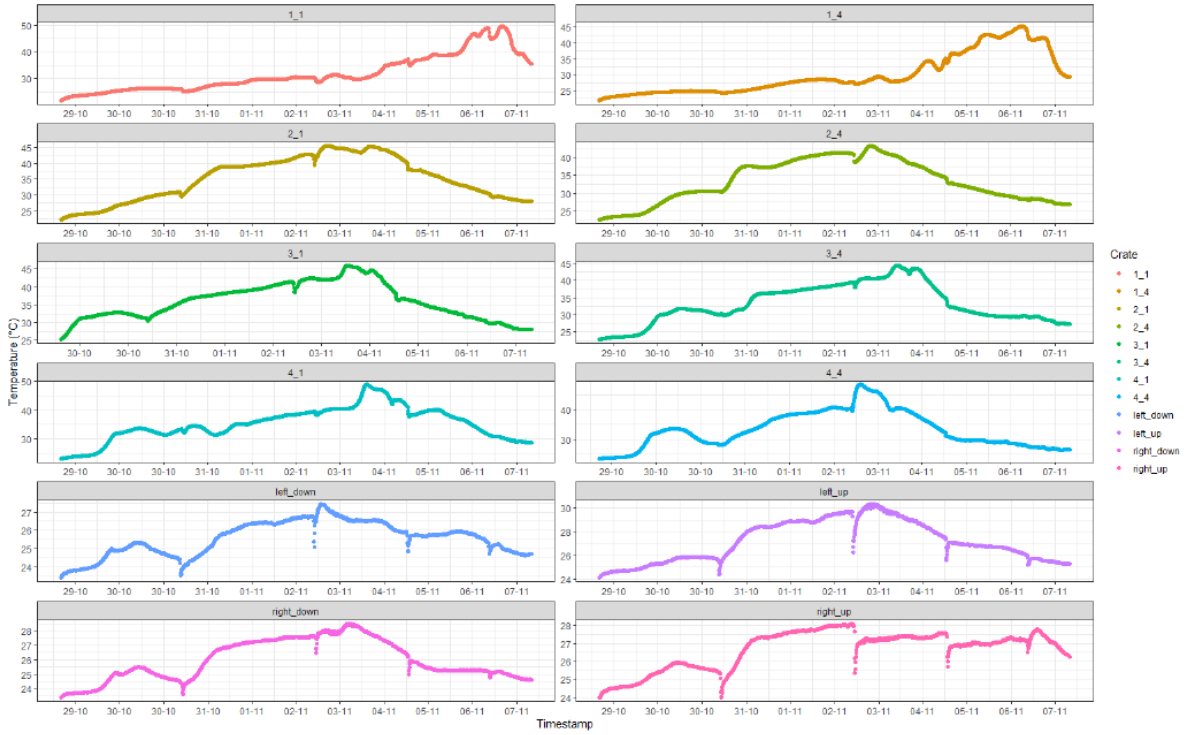

Figure 3.4. Illustration of measurement data from the temperature sensors used during the second experiment. Possible interruptions in the measurement data were fixed by interpolating the missing observations.

3.1.3. Performance of larvae in the first experiment

3.1.3.1. Performance indicators

In the first experiment, the observed and modelled total weight of the batch and weight of one larva was the highest for the control diet on day 6 of the experiment, and thereafter (Table 3.1, Figure 3.5, Table A5 in Annex 2). With the control diet, the larvae weight peaked around day 7.0-8.5 of rearing and the modelled growth curve illustrates. Growth analysis results demonstrated a faster growth and a smaller final size of larvae for the experimental diets compared to the control diet. However, the experimental diets resulted in quite similar growth rates. (Table 3.2, Figure 3.5). The control had a lower observed pH than three other diets (Table A5 in Annex 2).

Table 3.1. Table of modelled growth with initial size (y0), maximum exponential growth rate (mumax), maximum weight (y_max), time when larval size is within 95% of the maximum (time_for_max) and the final larva weight (FinalWeight) in the first experiment.

Diet[1)]	y0	mumax	y_max	time_for_max	Final weight
Control 1	7.6	0.51	221.6	[7,8.5]	196.9
Diet 1	7.6	0.80	171.6	[5.3,9]	163.8
Diet 2	7.6	0.79	168.4	[5.5,9]	163.2
Diet 3	7.6	0.77	179.9	[5.8,8.4]	165.3

[1)]See Section 2.2.1. for a description of the treatments.

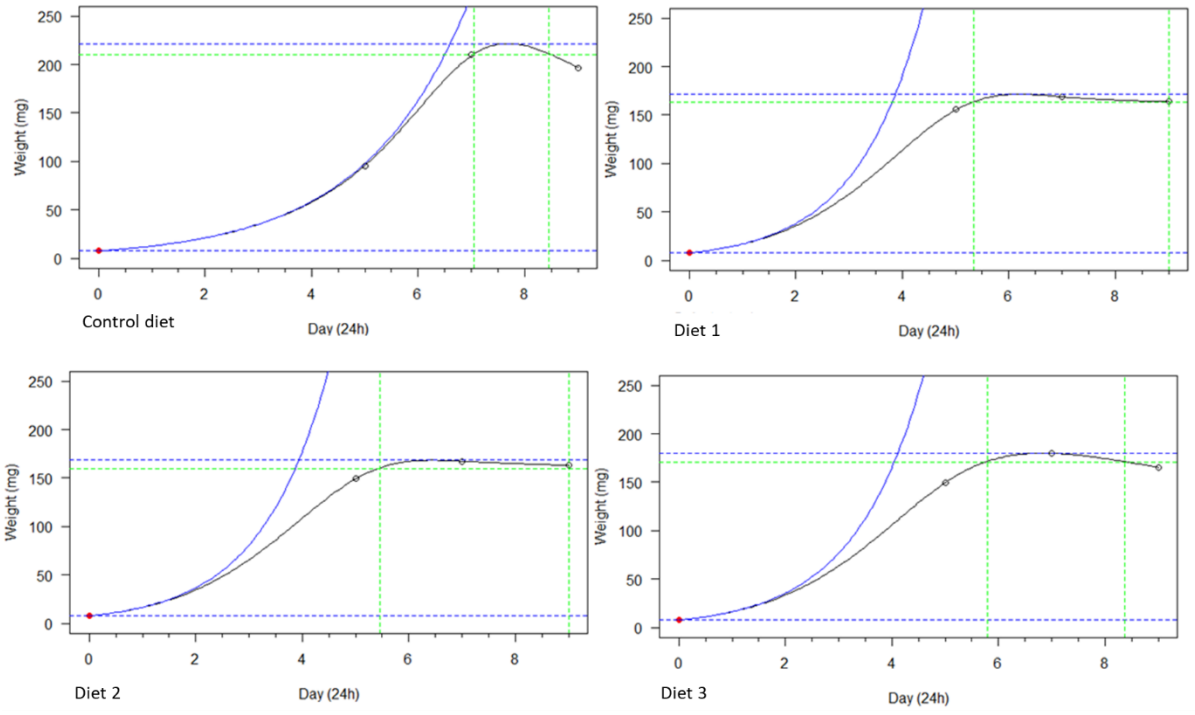

Figure 3.5. Plot of estimated growth based on subsamples. Plot indicates the maximum growth rate curve (blue solid) and maximum estimated size (blue hatched) and the region when larvae are estimated to be within the 95% of the maximum size (green hatched lines).

The diets differed e.g. regards to total produced larval matter and protein, bio conversion and dry and wet reduction (Table 3.2, Figure 3.6 below) and there were clear differences in the survival rates of larvae. Mixed model test indicated a very significant impact for the diet ($p < 0.001$). Random factors did not have significant impact, though top and lowest rows grew slightly better than the middle rows. Columns had no clear pattern.

The estimated impacts for the experimental diets compared to the control were Diet 1: -258 g; Diet 2: -206 g; Diet 3: -228 g. In other words, experimental diets are predicted to provide approximately (600-225)/600 ≈ 60% of the dry larval material compared to the control. Diet 1 had a lower bioconversion rate than diets 2 and 3. (Figure 3.7).

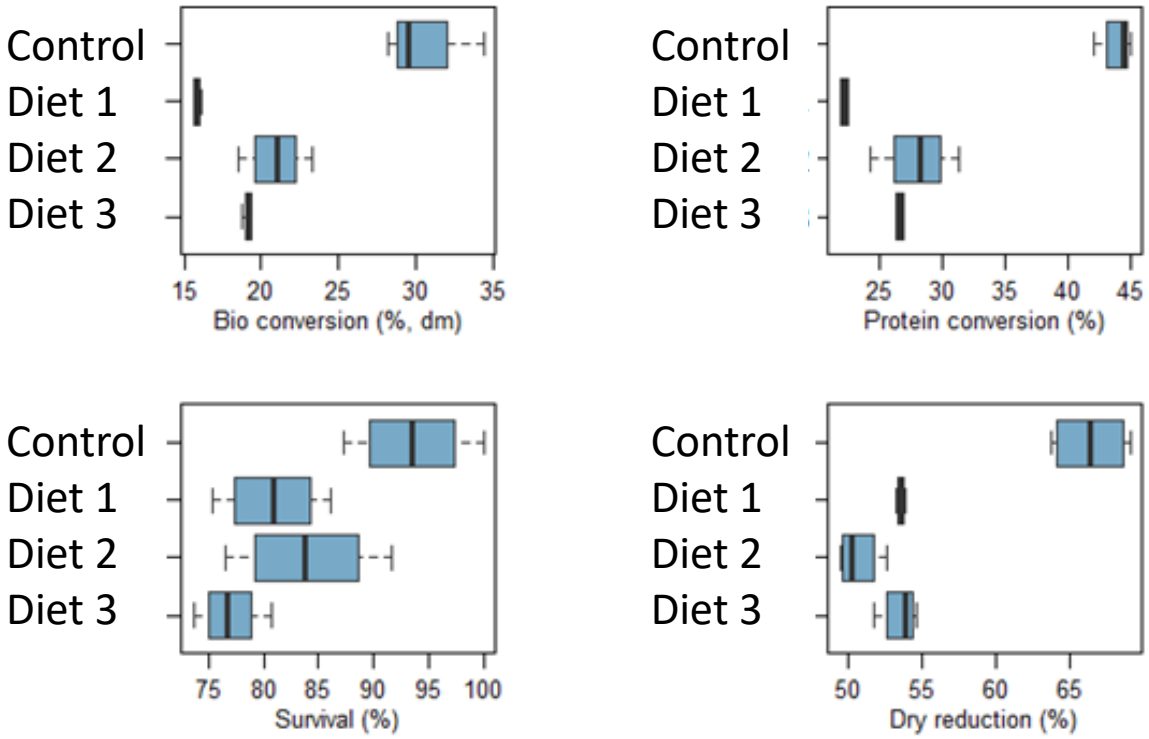

Figure 3.6. Box plots of the main performance indices of the first experiment.

Table 3.2. Summary of means (sd) of performance indicators from the first experiment.

Diet[1)]	Frass total (g)	Larva total (g)	Larva total (dm, g)	Larval protein (dm, g)	Wet reduction (%)	Dry reduction (%)	Bio conversion (dm, %)	Waste conversion (dm, %)	Protein conversion (dm, %)
Control 1	906 (46)	1432 (100)	599 (52)	203 (6)	82 (1)	66 (3)	30 (3)	46 (3)	44 (1)
Diet 1	1630 (26)	905 (17)	341 (5)	165 (2)	67 (1)	54 (0)	16 (0)	30 (0)	22 (0)
Diet 2	1655 (29)	1016 (72)	393 (35)	184 (18)	67 (1)	51 (1)	21 (2)	41 (3)	28 (3)
Diet 3	1541 (44)	974 (12)	370 (5)	172 (2)	69 (1)	54 (1)	19 (0)	36 (1)	27 (0)

[1)]See Section 2 for a description of the treatments.

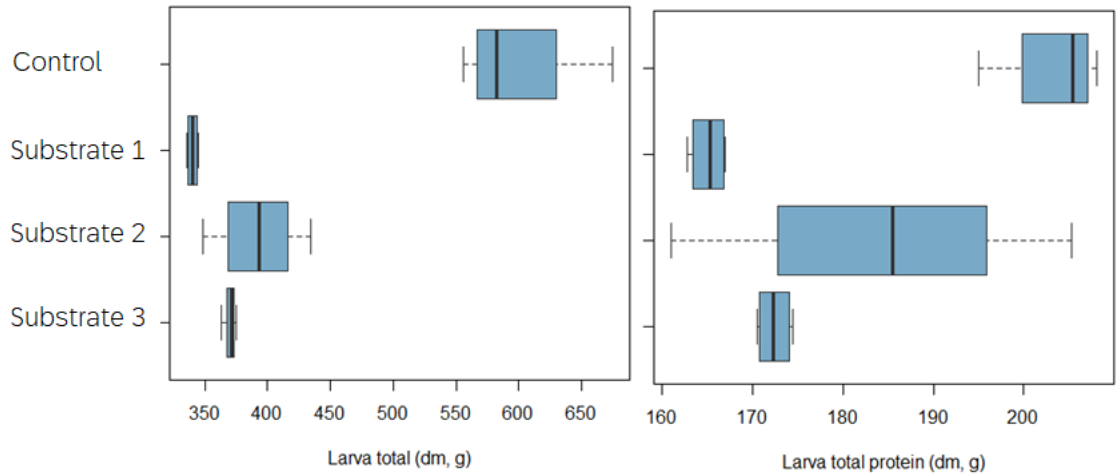

Figure 3.7. Box plot (showing median, lower and upper quartiles, minimum and maximum values of observations) of total larval biomass and protein as grams of dry matter (first experiment).

Focusing on the larval performance traits, the larval protein content (L_prot) had negative correlations with all other traits. Within the other traits, the correlations were positive and the correlations of larval survival rate (L_surv) and total larval protein amount (L_protW) differed from the other traits (Figure 3.8).

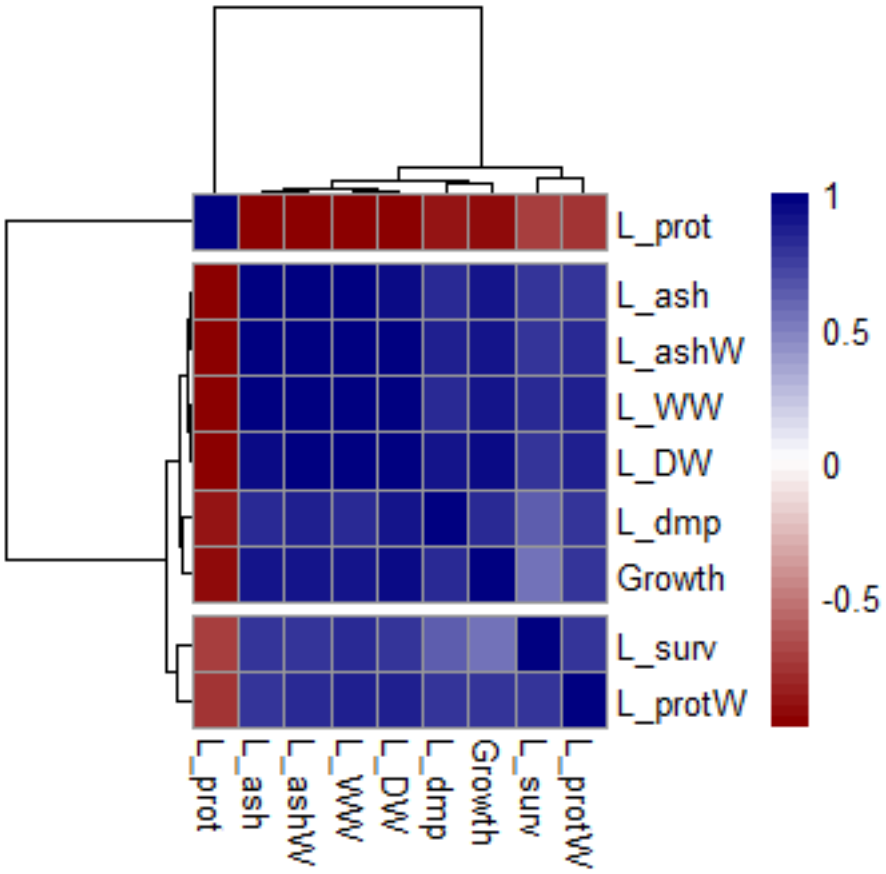

Figure 3.8. Heatmap of correlations between larval performance traits.

3.1.3.2. Quantitative analysis to explore reasons for the observations.

Co-inertia analysis was used to search for successive PCA axes from diet characteristic data and larval performance characteristics data with the maximum covariance. Invariable traits were excluded. Diet characteristics included: ADF, dm%, Ala, Arg, Asp, CyS, Phe, Glu, Gly, His, Ile, Leu, Lys, Met, Pro, Ser, Thr, Tyr, Val, Ca, Cu, Fe, Fat, K, Mg, Mn, NDF, Fiber, Prot, Trp, Ash, Zn, Carb, CtP. The larval performance data included:

L_WW Larval wet weight total
L_prot Larval protein content
L_ash Larval ash content
L_surv Larvla survival rate
L_dmp Larval dry matter content
L_DW Larval dry weight
L_ashW Larval ash weight
L_protW Larval protein weight
Growth Change in average wet weight of larva.

The analysis indicated a significant co-inertia (RV p = 0.002) and similar axis directions (Figure 3.9). Nearly all the projected inertia is differences between the control and the experimental diets along axis 1. The result suggested a clearer difference between experimental diets with different fresh plant components and a smaller difference between the two diets where only the concentrate part differed. While this is in line with the results about larval production, the more detailed results indicated that it depends on the performance characteristic.

The analysis suggested that larval protein content behaved opposite to other performance parameters, similar to the correlation heatmap presented in Figure 3.8. The second dimension adds depth to understanding trait similarities (e.g. total larval protein yield and growth behave similarly in two dimensions).

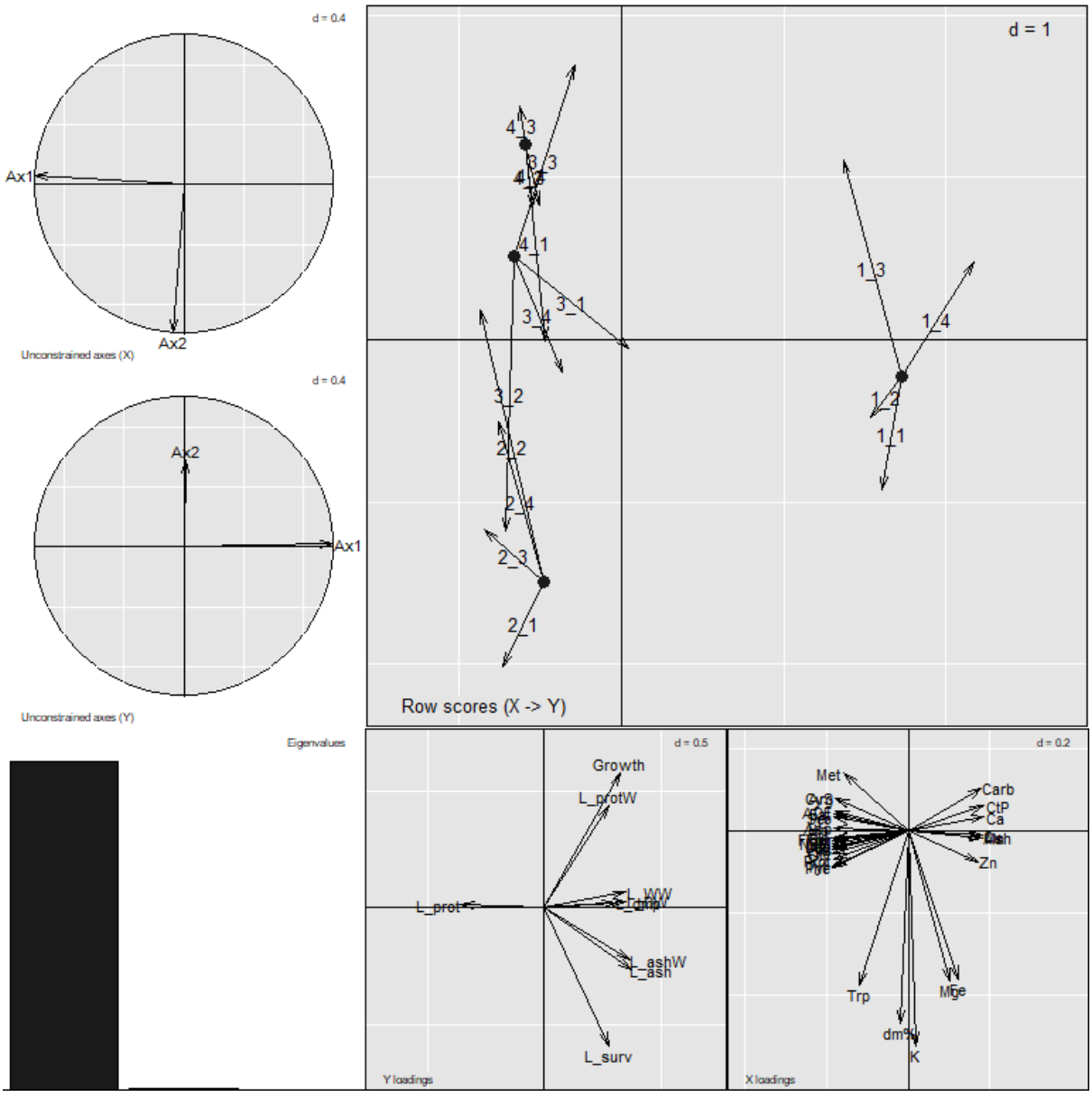

Figure 3.9. Co-inertia analysis results. The circles on left show rotation of the original PCA results in the analysis. On down left, the eigenvalue plot shows the amount of inertia explained by three first dimensions. The plot on top-right shows the matched plot. Dark small circle indicates multivariate plot of diet characteristics. The arrows beginning from this indicate matching multivariate results for larval growth performance characteristics. The two smaller plots on the bottom indicate clines of original variables in the plot with arrow pointing to direction of increasing value.

This exploratory analysis suggested that higher Ca, carbohydrate-to-protein ratio (CtP), Ash, Mn, Cu, Carb and Zn contents were associated with the most beneficial characteristics. High Fe and Mg were associated with higher survival rate. In this data set, higher protein had negative impact. Among individual amino acids, Trp and Met were ranked highest, supporting a further assessment of them in the second experiment.

In analysis of the impact of diet traits one trait per similarly varying group (see diet characteristics heatmap in Figure 3.8) was used as predictor candidate. Carbohydrate:protein ratio, Trp, Met, Fe and dm% were evaluated. For total larval dry matter yield, carbohydrates: proteins ratio, Trp, and Fe contents formed the best predictor set. No interactions were assessed. The adjusted r2 was 0.915 and all individual p-values were below 0.03. The predicted values (marginal effects) for the model terms (Figure 3.10) showed an increased carbohydrate:protein ratio (or increased share of carbohydrates) to have a strong impact, and the amount of Trp and Fe to have smaller impacts on larval biomass.

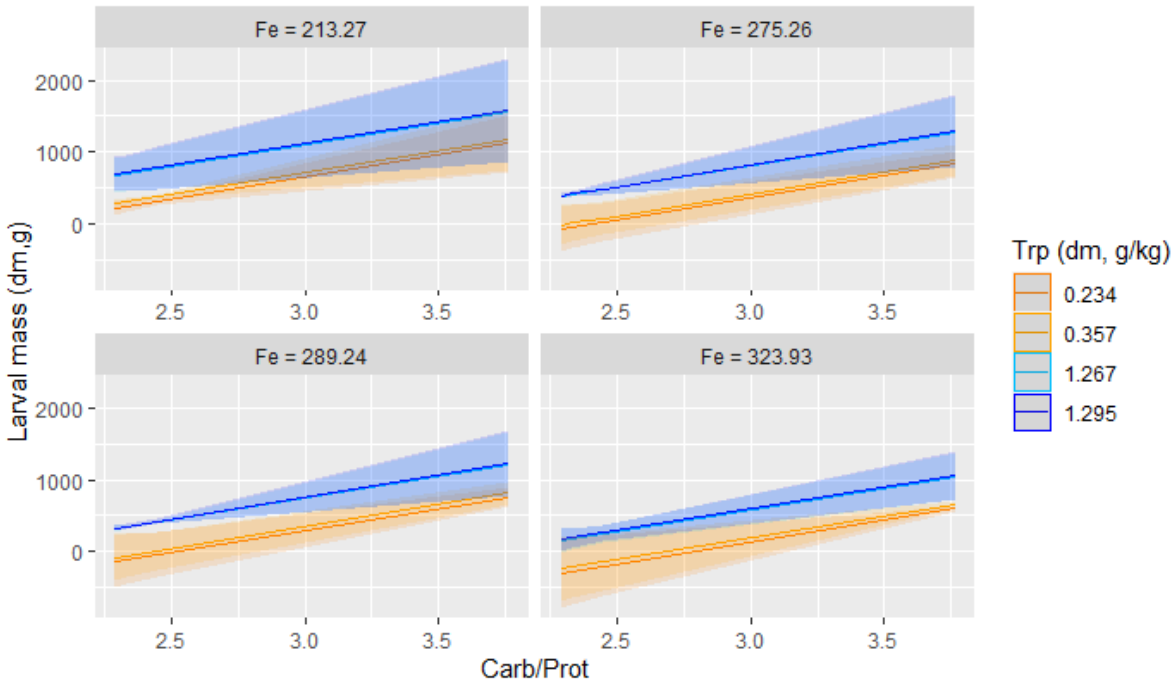

Figure 3.10. Interactions between Larval mass (dm,g), carbohydrates: proteins ratio and Trp (dm, g/kg) content.

For total larval survival rate, carbohydrate:protein ratio and Trp were the best predictor set. The adjusted r2 was 0.593 and all individual adjusted p-values were below 0.05. The predicted values (marginal effects) for the model terms (Figure 3.11 below) show increased carbohydrate:protein ratio (or increased share of carbohydrates) to have a strong impact, and the amount of Trp to have smaller impact on the survival rate.

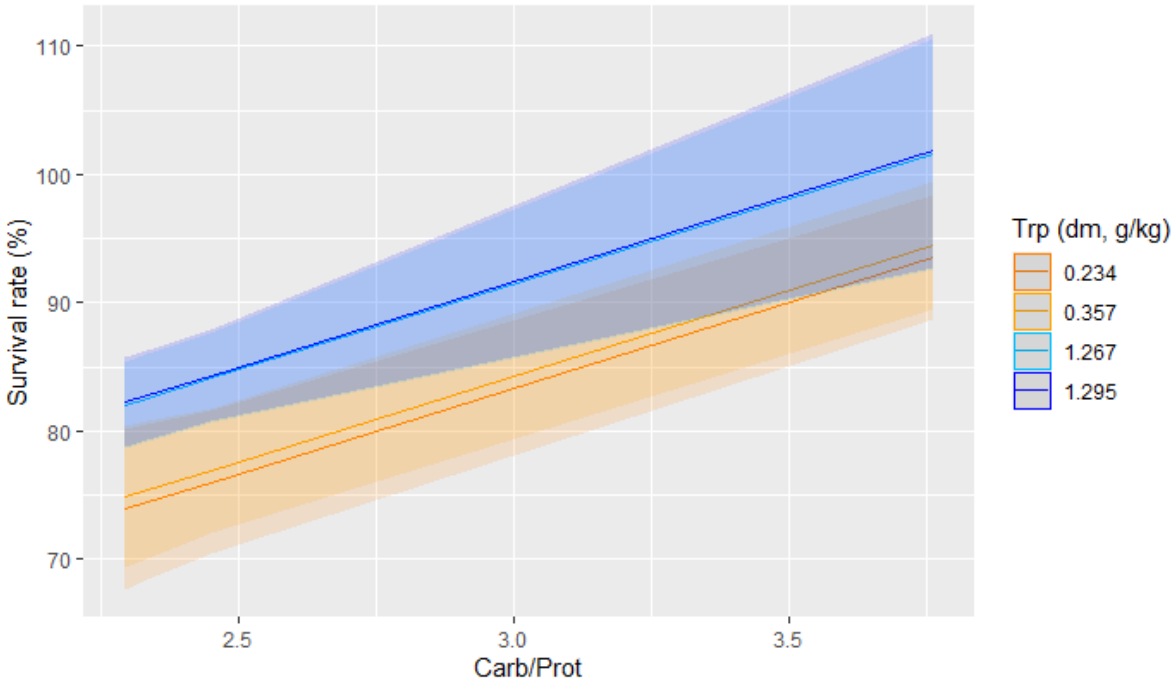

Figure 3.11. Interactions between survival rate, carbohydrate:protein ratio. and tryptophan contents

For larval growth, carbohydrate:protein ratio and Met were the best predictor set. The adjusted r2 was 0. 885 and all individual adjusted p-values were below 0.02. The predicted values (marginal effects) for the model terms (Figure 3.12) showed an increased Carbohydrate:protein ratio (or increased share of carbohydrates) to have strong impact, and the amount of Met to have smaller impacts on larval growth

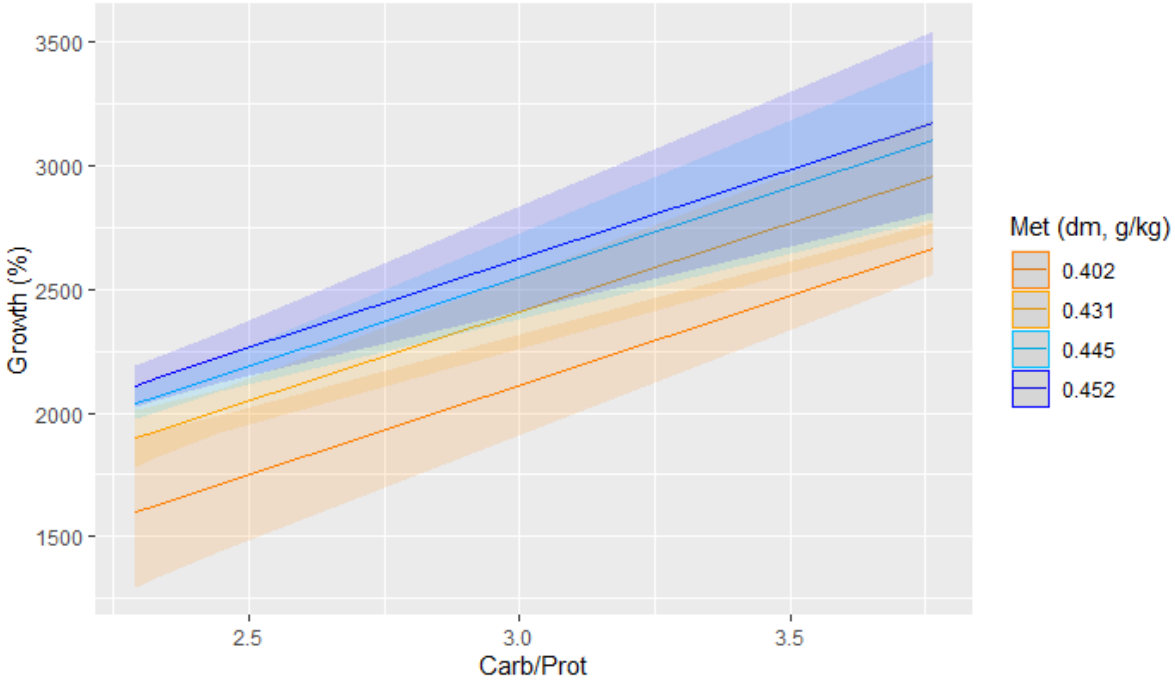

Figure 3.12. Interactions between growth rate, carbohydrate:protein ratio and methionine contents.

For larval protein yield, Carbohydrate:protein ratio, Met and Trp were the best predictor set. The adjusted r2 was 0.690 and all individual adjusted p-values were below 0.08. The predicted values (marginal effects) for the model terms (Figure 3.13) showed an increased Carbohydrate:protein ratio (or increased share of carbohydrates) to have strong impact, and the amount of Trp and Met to have smaller impacts on larval protein yield.

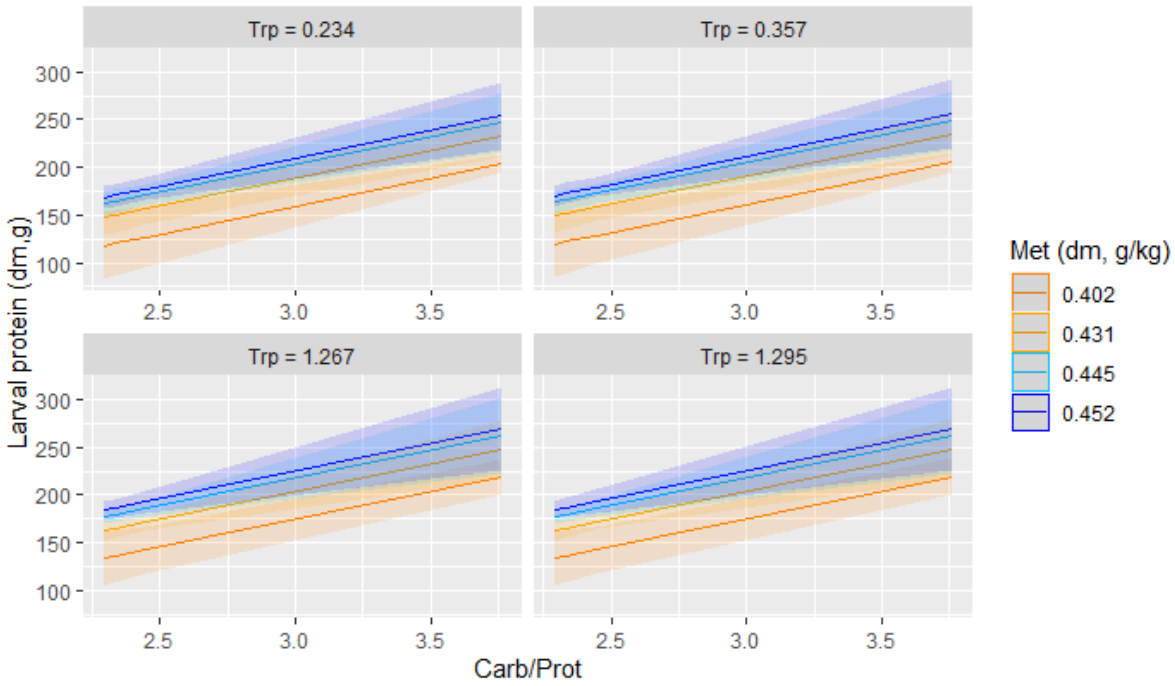

Figure 3.13. Interactions between larval protein, carbohydrate:protein ratio and methionine and tryptophan contents.

3.1.4. Performance of the second experiment

3.1.4.1. Performance indicators

Growth analysis results demonstrated a faster growth and a smaller final size of larvae for the experimental diets compared to the control diet (Table 3.3, Figure 3.14, Table A6 in Annex 2). At the end of the experiment the pH value of control 2 and diet 4 was lower than that of diet 3 and 5 (Table A6 in Annex 2).

Table 3.3. Modelled growth with maximum exponential growth rate (mumax), maximum weight (y_max), time (days) when larval size is within 95% of the maximum (time_for_max) and the final larva weight (FinalWeight).

Diet	mumax	y_max	time_for_max	Final weight
Control 2	0.66	294.9	[8.0,9.0]	232.03
Diet 4	0.52	109.1	[8.0,9.0]	108.83
Diet 5	0.66	150.1	[6.2,7.6]	107.67
Diet 6	0.65	214.1	[6.6,9.0]	168.67

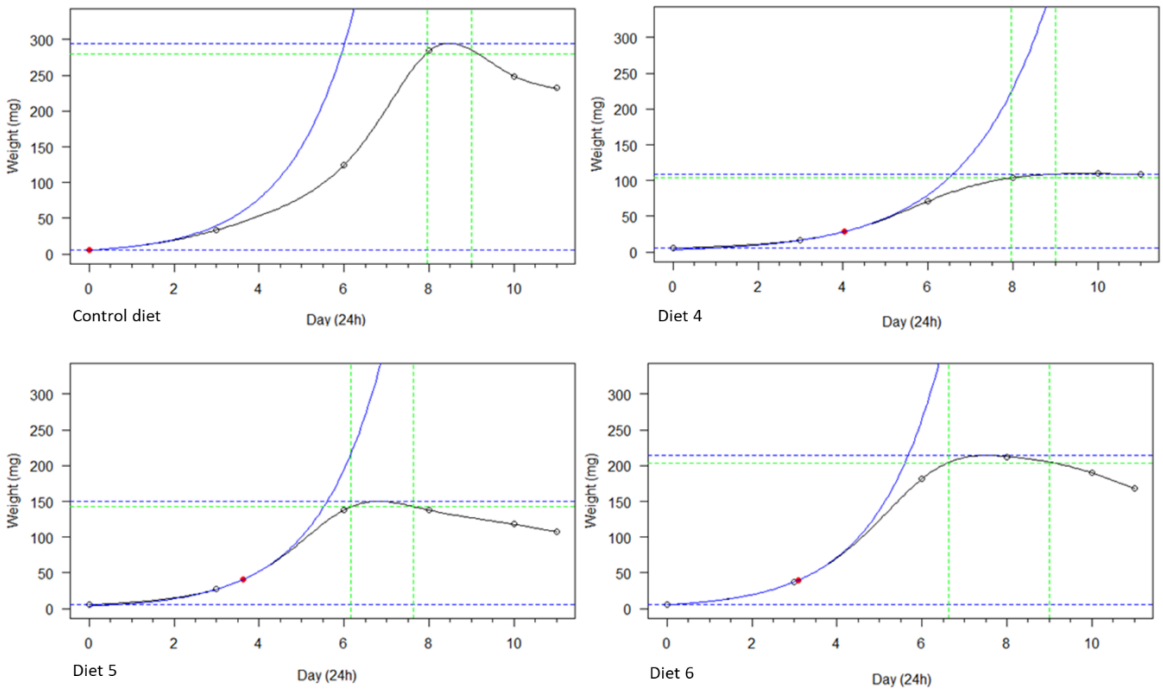

Figure 3.14. Plot of estimated growth based on subsamples. Plot indicates the maximum growth rate curve (blue solid) and maximum estimated size (blue hatched) and the region when larvae are estimated to be within the 95% of the maximum size (green hatched lines).

The diets differed e.g. regards to the total produced larval matter and protein, bio conversion and dry and wet reduction (Figure 3.15, Table 3.4) and there were clear differences in the survival rates as well. Mixed model test indicated very significant impact for the diet ($p << 0.001$). Random factors (column and row) did not had a significant impact.

The estimated impacts for the experimental diets compared to the control were Diet 4: -271 g; Diet 5: -229 g; Diet 6: -124 g (Figure 3.16). In other words, they were predicted to provide approximately (435-200)/ 435≈ 55 % of the dry larval material compared to the control.

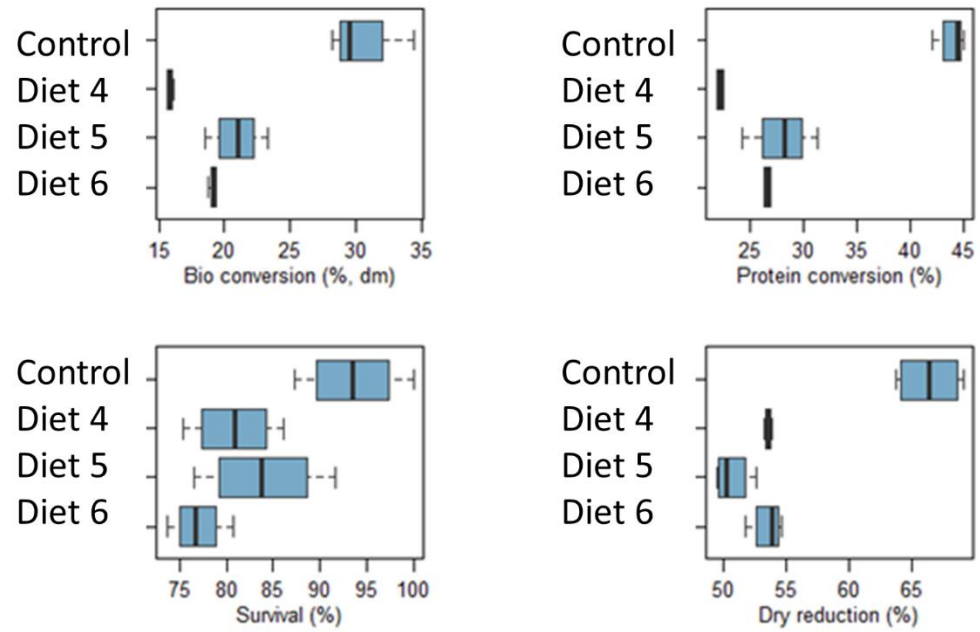

Figure 3.15. Box plots of the main performance indices.

Table 3.4. Summary of performance indicators (mean (sd)) from the second experiment.

Diet[1)]	Frass total (g)	Larva total (g)	Larva total (dm, g)	Larval protein (dm, g)	Wet reduction (%)	Dry reduction (%)	Bio conversion (dm, %)	Waste conversion (dm, %)	Protein conversion (dm, %)
Control 2	934 (53)	1216 (82)	434 (31)	165 (12)	81 (1)	65 (4)	22 (2)	34 (1)	36 (3)
Diet 4	1387 (27)	555 (19)	163 (8)	89 (6)	72 (1)	54 (1)	8 (0)	15 (1)	12 (1)
Diet 5	1161 (15)	631 (54)	206 (15)	101 (7)	77 (0)	61 (1)	10 (1)	17 (1)	14 (1)
Diet 6	1003 (69)	908 (93)	310 (28)	138 (12)	80 (1)	67 (2)	16 (1)	24 (3)	23 (2)

[1)]See Section 2 for a description of the treatments.

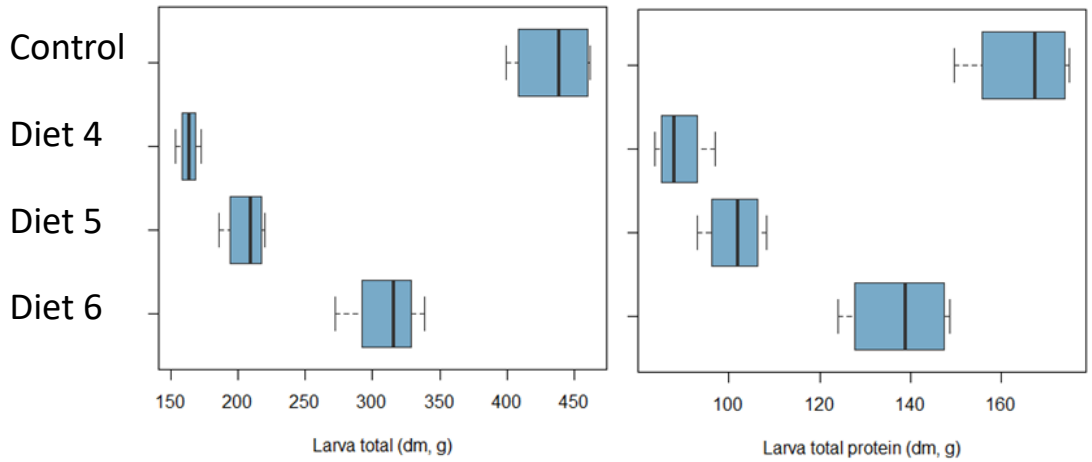

Figure 3.16. Box plot of total larval biomass and protein as grams of dry matter.

Focusing on the larval performance traits, the larval protein content (L_prot) is opposite to all other traits with the surprising exception of survival rate. This reflects the poorer result for the control treatment in this experiment, compared to experiment 1. The larval survival rate (L_surv) was again behaving differently than most other traits (Figure 3.17).

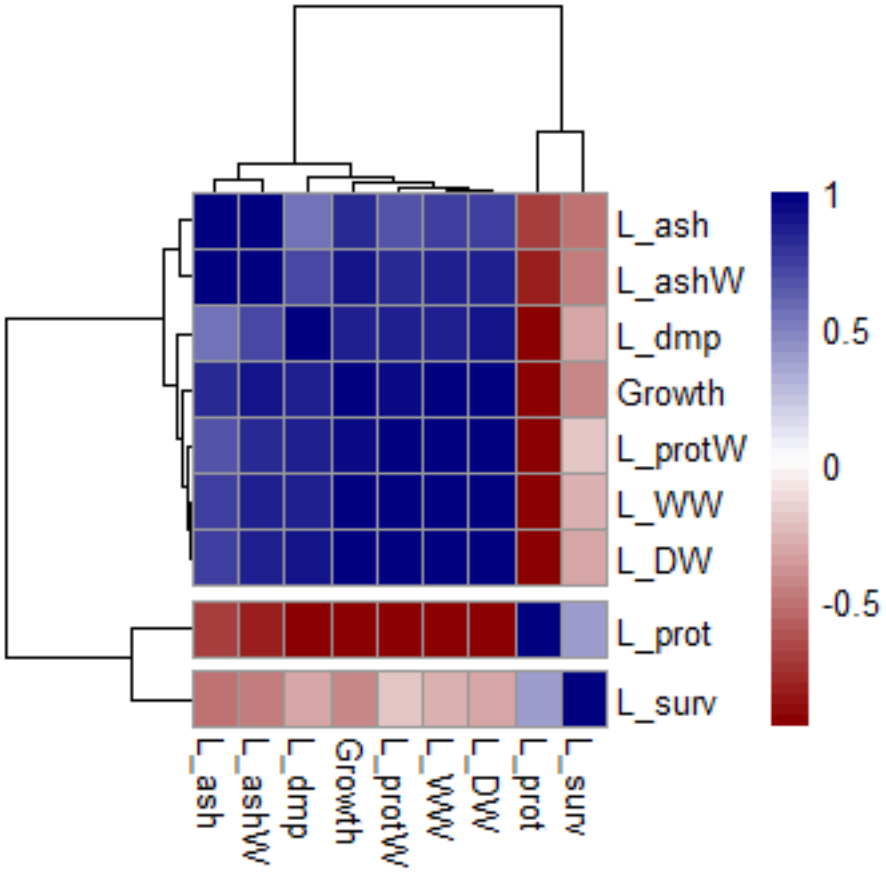

Figure 3.17. Heatmap of correlations between larval performance traits.

3.1.4.2. Quantitative analysis to explore reasons for the observations

Co-inertia analysis was used to searched successive PCA axes from diet characteristic data and larval performance characteristics data the with maximum covariance. Invariable traits were excluded. Diet characteristics included: ADF, dm%, Ala, Arg, Asp, CyS, Phe, Glu, Gly, His, Ile, Leu, Lys, Met, Pro, Ser, Thr, Tyr, Val, Ca, Cu, Fe, Fat, K, Mg, Mn, NDF, Fiber, Prot, Trp, Ash, Zn, Carb, CtP. The larval performance data included:

L_WW Larval wet weight total
L_prot Larval protein content
L_ash Larval ash content
L_surv Larvla survival rate
L_dmp Larval dry matter content
L_DW Larval dry weight
L_ashW Larval ash weight

L_protW Larval protein weight
Growth Change in average wet weight of larva.

The analysis indicated significant co-inertia (RV p = 0.001) and similar axis directions (Figure 3.18). Most of the projected inertia was differences between the control and the experimental diets along axis 1. The three experimental diets were approximately equally separated along the second dimension. Their separation along the first dimension was less, but also equally distanced. In general, the multivariate plots of the larval performance traits and the diet traits agreed.

The analysis suggested again that larval protein content behaves opposite to most of the performance parameters, similar to the presented correlation heatmap earlier (Figure 3.17). Larval survival rate and Larva ash (content or mass) were another cluster of traits opposing to each other and were orthogonal to the axel of larval protein content and most of the performance traits.

This exploratory analysis suggested that higher CtP, possibly accompanied by Carb, Fiber, Arg, Ala, Zn, Ca, Trp, Mn, Ash, ADF, Phe were associated with the most beneficial characteristics, while Prot and Tyr were associated more with high larval protein content (not Larval protein yield). High fiber and dry matter were associated with high ash (mineral) content of the larvae. Survival was associated with Arg, ADF, Asp, Lys. High Fe and Mg were associated with higher survival rate. In this dataset, a higher protein content had a negative impact on insects. Among individual amino acids, Trp and Met were ranked the highest in terms of importance to insect performance, supporting further research of these amino acids.

In analysis of the impact of diet traits one trait per similarly varying group (see diet characteristics heatmap above, Figure 3.17) was used as predictor candidate. Carb, Trp, Met, dm%, Prot% were evaluated.

For total larval dry matter yield, Met and dm, were the best predictor set. No interactions were assessed. The adjusted r2 was 0.962 and all individual p-values were below 0.001. The predicted values (marginal effects) for the model terms (Figure 3.19) showed an increased Met to have a strong negative impact, and the amount of dm to have positive impact on the dry matter yield.

For total larval survival rate, dm was the only chosen predictor. The adjusted r2 was 0. 306 and adjusted p-value was 0.015. The predicted values (marginal effects) for the model terms (Figure 3.20) showed an increasing dm% to have negative impact on survival rate in this dataset.

For larval growth, protein percentage was the only predictor selected. The adjusted r2 was 0. 941 and p-value was below 0.001. The predicted values (marginal effects) for the model terms (Figure 3.21 below) show increasing protein % to reduce growth.

For larval protein yield, Met and dm% were the best predictor set. The adjusted r2 was 0.920 and all individual adjusted p-values were below 0.001. The predicted values (marginal effects) for the model terms (Figure 3.22) show increasing Met to decrease protein yield and increasing dry matter content to increase it, in this data set.

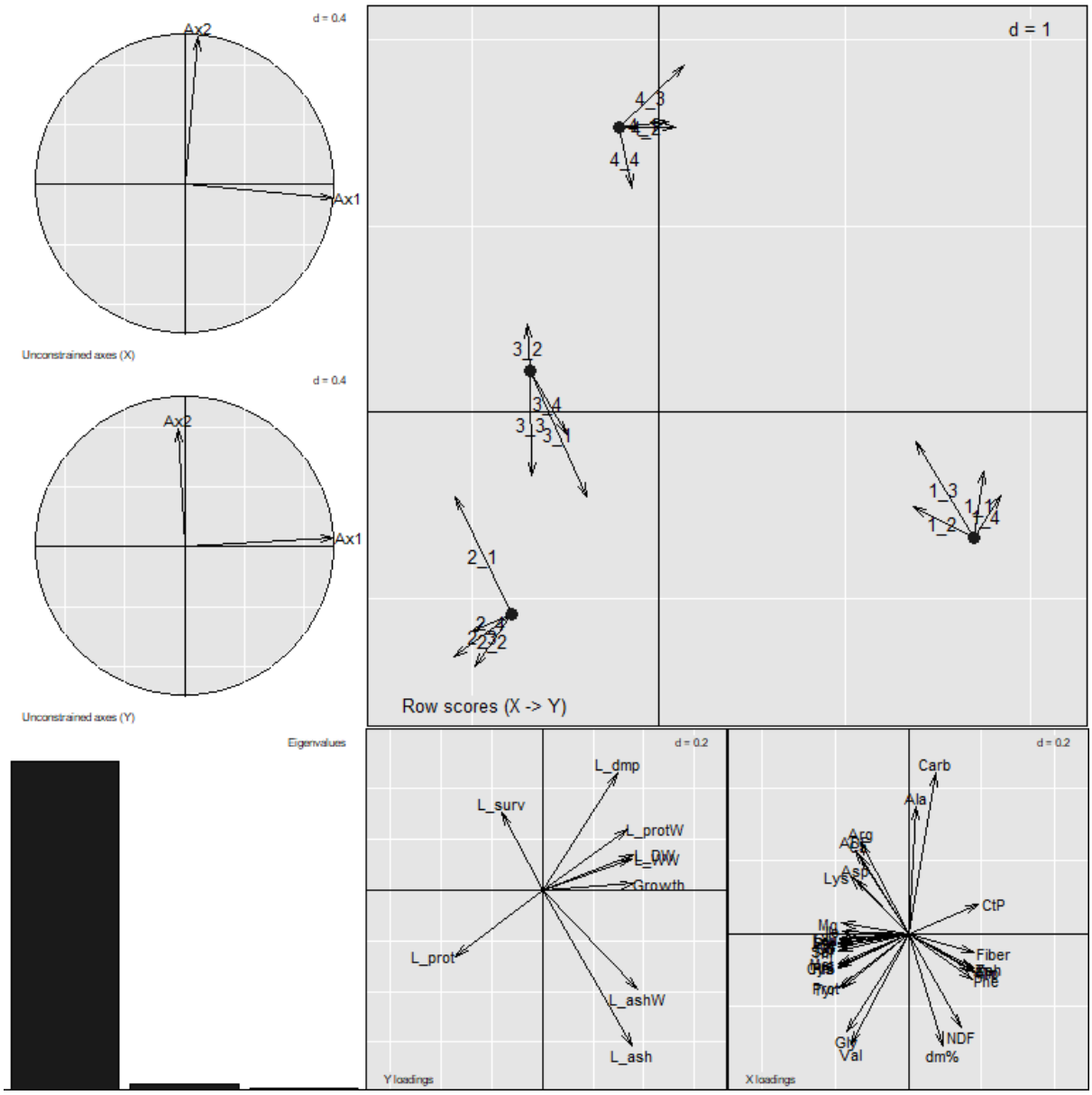

Figure 3.18. Co-inertia analysis results. The circles on left show rotation of the original PCA results in the analysis. On down left, the eigen value plot show the amount of inertia explained by three first dimensions. The plot on top-right shows the matched plot. Dark small circle indicates multivariate plot of diet characteristics. The arrows beginning from this indicate matching multivariate results for larval growth performance characteristics. The two smaller plots on the bottom indicate clines of original variables in the plot with arrow pointing to direction of increasing value.

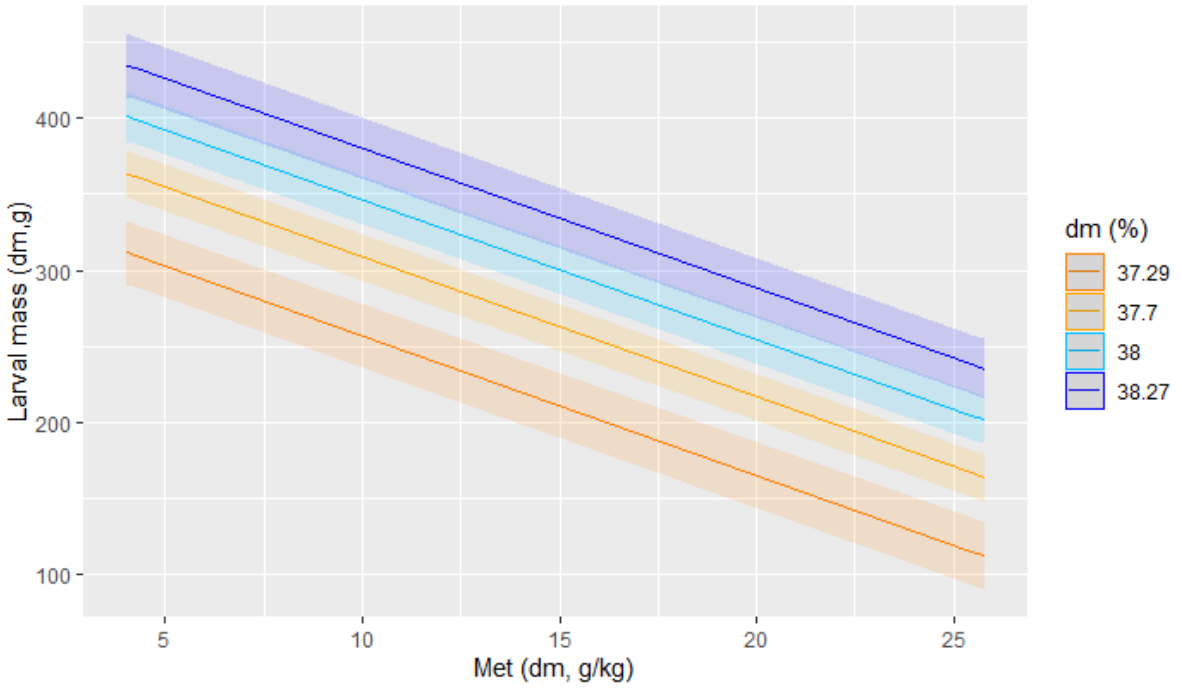

Figure 3.19. Interactions between larval mass (g dry matter), methionine and dry matter content of diet.

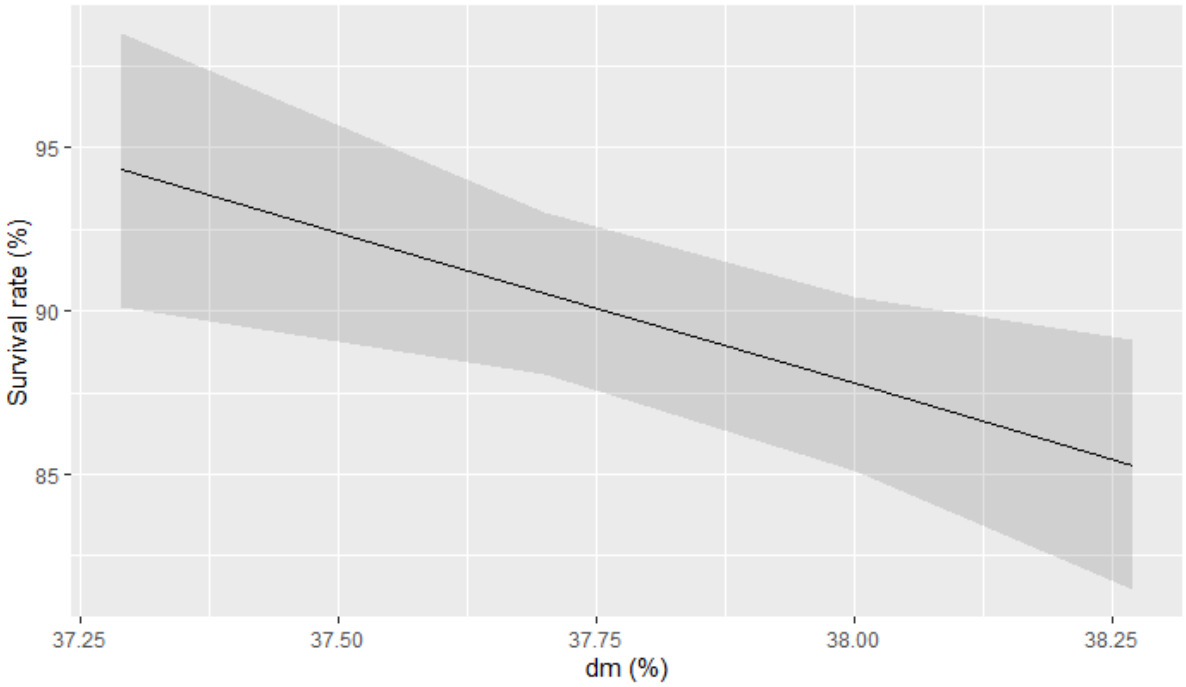

Figure 3.20. Interactions between larvae survival rate and dry matter content of diet.

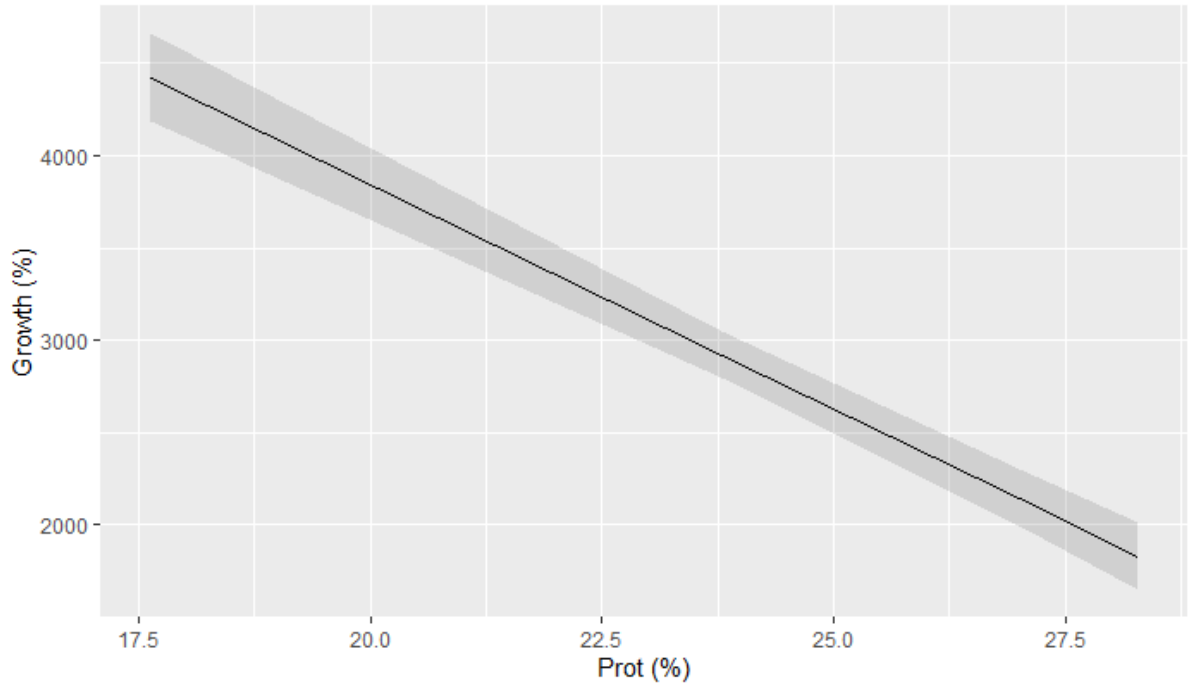

Figure 3.21. Interactions between larvae growth rate and protein content.

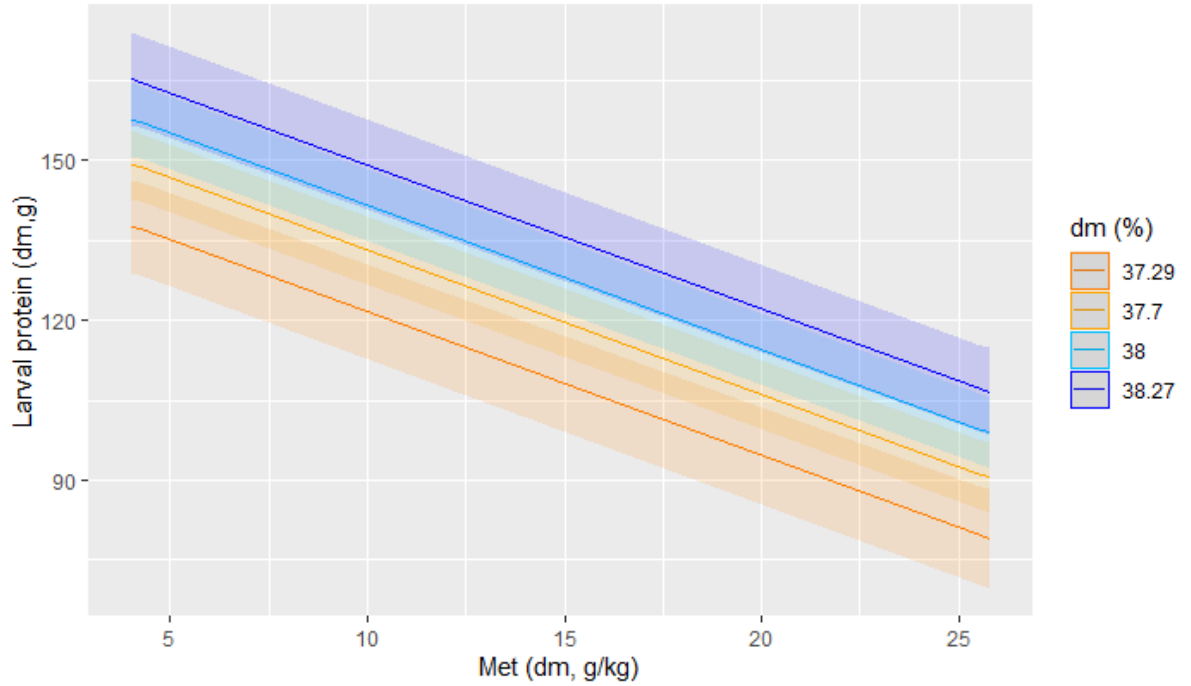

Figure 3.22. Interactions between larval protein content and methionine content.

3.1.5. Concluding remarks

The best-performing diets (after the control diet) in the first experiment were diets 2 and 3 and in the second experiment diet 6.However, the performance of diets 3 and 6 was weakened by quite low survival rate. Nevertheless, the total biomass in these diets were closest to the control diets.

These diets included self-prepared concentrates (containing dried brewer's spent grain, feed yeast and potato protein), and at least some of three sidestream-like feed materials, namely crushed tomato, breadcrumbs and fresh apple. In general, the diets containing potato and broad bean did not perform as well as other tested diets. Potatoes contain glycoalkaloids (mainly α-solanine and α-chaconine) and broad beans contain vicin, convicin and phytic acid, which are harmful substances and they may have reduced larvae performance in the *H. illucens* test. These substrates had also quite dense structure and that may have caused some difficulties for *H. illucens* larvae. This issue requires further research. The concentrate could be supplemented also with other side streams than those tested in the experiments, but that would require further research.

The results also suggest that the amino acid composition of the diets fed to *H. illucens* is an important factor for the performance of *H. illucens*. At least the level of tryptophan and methionine in the diet affects the performance of *H. illucens*. Further research on the amino acid requirements of *H. illucens* is needed. Moreover, the addition of higher amount of tryptophan (4.7–5.2%) was found to have a favourable effect on *H. illucens* larvae growth and survival. However, the addition of extra methionine did not improve growth, development, or mortality of *H*. *Illucens* larvae, and very high concentration of methionine reduced the performance of larvae. Hence, the results suggest that a methionine content of 1.54% in the diet is sufficient for *H. illucens* larval growth. Besides tested amino acids, also other nutrients influence the performance of larvae. Hence, further research under controlled conditions is needed to identify the contribution of other amino acids and micronutrients on *H. illucens* growth.

Sensors tested in the experiment turned out to function as expected and they provided useful data, such as temperature information that was able to signal the most appropriate timing of terminating a batch. However, insulating the boxes from leakages and ensuring that the sensor is protected properly from the feed, the insects and the frass was also important.

3.2. *T. molitor* experiment

3.2.1. Experiments with single by-products

Besides the control diet of wheat bran, the best performances were observed for tomato peel and brewer's spent grain diets (Table 3.5, Figure 3.23). The differences were tested with a Kruskal–Wallis test, followed by Mann-Whitney U for multiple pairwise comparisons with Bonferroni corrected p-values (α=0.05). Even if the time of development, based on the appearance of the first pupa, was longer compared to wheat bran, survival, weight gain and first pupae weight were not significantly different from the control. This means that both tomato and brewer's spent barley grain had a good effect on larvae growth and moult, comparable to the standard diet. A smaller quantity of brewer's spent grain was consumed when compared to wheat bran and tomato peels, but it produced the same effects on larval development. This could be due to the high content in non-fiber carbohydrates.

Olive pomace and potato diets displayed incontrovertibly negative results. In none of the replicas the pupa stage was reached and the survival rate was substantially lower than that of wheat bran diet. Hence, these replicas were interrupted after 109 days of experiment. Data of these two diets were recorded both at 81 and at 109 days. The results suggest that brewer's dried spent barley grain and dried tomato pomace are potential alternatives to wheat bran, although they required a longer time of pupa development. These diets showed a good performance also in the main experiment.

3.2.2. Experiments with mixed by-products

In the main experiment, complete (mixed) diets were tested. In the main experiment, some treatment diets were not completed before day 56 (i.e. when this report was prepared). The data on day 56 are shown in Table 3.6. At day 56, only diets A (control), F, H and I were concluded since all the replicas had the first pupa. Diets C, D and G had the first pupa only in half replicas, while diets B and E were far from being concluded. Table 3.6 summarises the results. The statistical analysis was performed only for the diets with complete replicas.

Results of Table 3.6 indicated that diets F, H and I showed the best performances among the tested diets, comparable to diet A in terms of larval and first pupa weight. These diets were comparable to diet A for other parameters excepted for the quantity of diet consumed. As already mentioned, these diets showed the shortest time of development until the first pupa. Although no statistically significant differences in the development time were observed, the mean development times of diets F, H and I can be considered quite long. Diets F, H and I, were made of 50% wheat bran, that could be important for the yellow mealworm development. Wheat bran contains the necessary nutrients for mealworm's growth, including B-complex vitamins and fatty acids. Because these nutrients are not contained in optimal proportions, adding supplementary feed sources can improve insects' development (Morales-Ramos *et al.*, 2010). Diet F and H contained brewer's yeast, that is an important source of proteins, B-complex vitamins, and minerals. Diet F contained spent grain, diet H contained tomato and diet I contained both of these. This part of the experiment seemingly confirmed the experiments with single by-products. Among the by-products tested, tomato peels and spent grains were the most eligible by-products to rear *T. molitor*.

Fundamental for *T. molitor* development is carnitine, a vitamin part of the B-complex. The larva retains more than 50% of the carnitine contained in the diet (Fraenkel, 1953). Yeast, wheat bran and tomato all contain carnitine.

Brewer's spent barley grain's success as a diet could be due to the high amount of carbohydrates, because *T. molitor* diet is suggested to contain 80-85% carbohydrates (Fraenkel, 1953). Brewer's spent barley grains are wide and all-year-round available in most countries. It is estimated that brewer's

spent barley grain constitutes 85% of total by-products generated by brewing, representing approximately 20 kg per 100 liters of of beer produced (Mussatto, 2006).

3.2.3. Concluding remarks

The best-performing *T. molitor* diets contained wheat bran (control diet) tomato pomace orbrewer's spent grain. In the first experiment these three diets were equally good in al other respects except the 1st pupa appearance, which took shorter time for the control than other diets. The development time was 31% longer for tomato pomace and 52% longer for brewer's spent grain than for wheat bran, which was concluded to have a major economic impact on the costs of rearing facilities per kilogram of yield. In the second experiment, statistically significant differences were observed only for the amount of consumed diet which was lower for diet F containing 50% wheat bran, 45% brewer's spent grain and 5% yeast, equally high for diet I containing 50% of wheat bran, 23% brewer's spent grain and 27% of tomato pomace, and higher for diet H containing 50% wheat bran, 41% tomato pomace and 9% yeast when compared to the control diet,

The results therefore suggest that on one hand wheat bran is a reasonable feed for *T. molitor*, and on the other hand that wheat bran can be replaced with other feed materials, especially brewer's spent grain without compromising the production results. While being a simple diet, wheat bran actually contains the necessary nutrients for mealworm's growth, including B-complex vitamins and fatty acids. Because these nutrients are not contained in optimal proportions, adding supplementary feed sources can however improve insects' development. As mentioned above, other diets that were performed similarly to control, all contained 50% of wheat bran and in addition, different combinations of brewer's spent barley grain, tomato pomace or yeast. These feed materials could therefore be considered as the promising feed materials to be included in *T. molitor* diets. Yeast, wheat bran and tomato all contain carnitine, which is an important nutrient for *T. molitor*.

Brewer's spent barley grain's success as a diet could be due to the high amount of carbohydrates. CArbohydrates deriving from the brewing industry are very variable in composition. In the current experiment Brewer's spent grain contained a fairly low level of protein and high level of carbohydrates. A "light" beer typically has a higher level of carbohydrates and a "strong" beer typically has a low level of carbohydrates, although this depends on the specificities of the processo of brewer. In the brewing industries the largest amount of the gain is the external part of the seeds. *T. molitor* usually chooces the feed materials that is consumers during the eating. For this reason, to ensure that all feed is consumed, the "biscuit" preparation was carried out during the experiment.

Brewer's spent grain is also a feed material that is constantly available in most countries. However, because wheat bran is in general quite affordable feed material, other options may not be able to improve the competitiveness of mealworm rearing process. While diet F was found the most appropriate diet when considering the byproduct tested and their "local" availability, further research is needed to investigate locally available side streams, such as side streams from milling and maize processing. A further step could be a study on avoiding the use of some products that have a real cost, such as yeasts.

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Table 3.5. Results of T. molitor rearing with the single byproducts.

Parameter	Wheat bran	Tomato pomace	Brewer's spent grain (SG1)	Olive pomace (day 81)	Potato peels (day 81)	Olive pomace (day 109)	Potato peels (day 109)
1st pupa appearance (days)	44.1±7.2a	67.3±11.7b	57.9±13.7b	NA	NA	NA	NA
Survival (%)	98.0±4.2a	99.5±1.6a	96.0±4.6ab	94.0±3.2b	68.5±9.1c	61.5±12cd	49.5±12.1d
Final weight per larva (g)	0.09±0.01a	0.09±0.02a	0.088±0.014a	0.015±0.002c	0.016±0.002b	0.017±0.003b	0.022±0.006b
1st pupa weight (g)	0.124±0.017a	0.119±0.020a	0.114±0.021a	NA	NA	NA	NA
Weight gain per larva (g)	0.084±0.012a	0.083±0.013a	0.081±0.013a	0.007±0.001b	0.004±0.001d	0.009±0.003bc	0.01±0.006c
Daily weight gain per larva (g)	0.0019±0.0002a	0.0012±8.7E-05a	0.0014±0.0002a	NA	NA	7.9E-05±0.00002b	1.3E-04±0.00005b
Consumed diet (g)	4.5±0.6a	4.5±0.7a	2.5±0.3c	2.1±0.2c	0.9±0.4b	2.2±0.2c	0.9±0.4b

Statistically significant differences between columns are marked with different letters in each row (Kruskal-Wallis test for equal medians, $p<0.05$).

Table 3.6. Results of the second experiment with T. molitor (mean (sd)).

	Diet A (concluded)	Diet B (day 56)	Diet C (day 56)	Diet D (day 56)	Diet E (day 56)	Diet F (concluded)	Diet G (day 56)	Diet H (concluded)	Diet I (concluded)
1st pupa appearance (days)	32±5.477a	-	-	-	-	40±8.834a	-	38±5.034a	39±3.922a
Survival (%)	99.50±1.581a	99.50±1.581*	100±0*	99.50±1.581*	96.50±4.116*	99.50±1.581a	99.50±1.581*	99.50±1.581a	100±0a
1st pupa weight (g)	0.118±0.014a	-	-	-	-	0.108±0.004a	-	0.127±0.020a	0.114±0.013a
Final weight per larva (g)	0.100±0.015a	0.054±0.005*	0.069±0.008*	0.071±0.005*	0.043±0.004*	0.104±0.016a	0.061±0.003*	0.110±0.012a	0.104±0.009a
Weight gain per larva (g)	0.072±0.015a	0.026±0.005*	0.041±0.007*	0.043±0.005*	0.014±0.004*	0.077±0.016a	0.033±0.003*	0.083±0.012a	0.077±0.010a
Consumed diet (g)	3.922±0.987a	-	-	-	-	2.71±0.616b	-	6.297±0.848c	4.836±0.644a
Concluded replicas at day 56 (number)	10	2	4	4	0	10	5	10	10

All replicas of diets A, F, H and I were concluded by day 56 of the experiment. Data of treatments that were unfinished by day 56 were calculated only on the concluded replicas so must be considered partial. Data marked with asterisks were not included in the statistical analysis. Statistically significant differences between columns are marked with different letters in each row (Kruskal-Wallis test for equal medians, $p<0.05$)

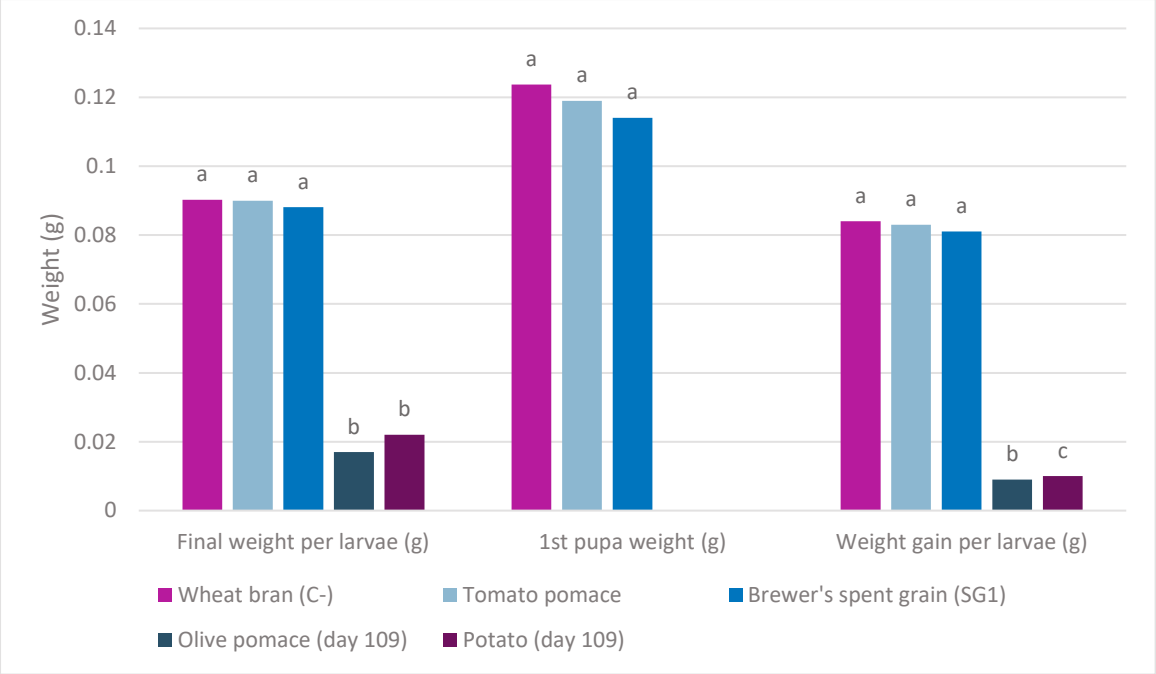

Figure 3.23. Results of first experiment on single products with T. molitor. The bars with the same letter do not differ statistically significantly, based on Mann-Whitney U test for multiple pairwise comparisons.

3.3. *A. domesticus* experiment

3.3.1. Results

The total weight gain of A. domesticus fed with the control diet (chicken feed) was on average 0.46 grams and the final weights of an insect was 0.26 grams. Only 27% of crickets fed with the control survived until adulthood. Although the means of reported results varied in numerical terms, No statistical difference was found between all diets in terms of survival and development compared to control (Table 3.7, Figure 3.24), when the performances of diets on insect growth were analyzed with a Kruskal–Wallis test (α=0.05).

The mortality rate observed in this experiment was considered low when compared to ICF's farm standards. House cricket is a species with a strong cannibalistic behavior and only a small part of the nymphs may reach the adulthood. In intensive farming conditions, 150 grams of 1st stage nymphs, containing about 240,000 individuals, placed in a rearing box, lead to only 20,000 adults (Stefano Magnaghi, personal communication). Almost all the survived insects had reached adulthood already at day 51 in all diets, while the farm standards often require 60 days. However, 51 days rearing time is not exceptional when appropriate feed and rearing conditions are provided (see e.g. Pastell *et al*., 2021). Hence, this could result from the experimental rearing conditions, which provide better accessibility to resources for the insect than industrial rearing methods.

The control diet in this experiment was chicken feed, which has been found to be able to result in high growth rates. Other diets were included different mixes of chicken feed, yeast, wheat bran, tomato, potato and brewer's spent barley grain. The results suggest that chicken feed in *A. domesticus* diets can be partially replaced by side streams originating from food supply chains without compromising the production performance. Overall, diet E, which was the most diverse diet and contained the lowest amount of chicken feed, was the most promising diet although it did not differ statistically significantly from other tested diets..

It was not possible to measure the feed intake in the *A. domesticus* experiment. The residual feed was not weighed, because the species tends to leave excrement on the feed that would have altered the final weight. Even if the mix of by-products was not merged to form a biscuit, no evidence of feed selection was observed. Crickets seemed to eat all components of the diets.

Chicken feed was present in all diets, together with the yeast and the wheat bran. Yeast, as stated above, contains B-complex vitamins, including B12, but also sterols and choline. Thiamine, pyridoxine, nicotinic acid, pantothenic acid, choline, and biotin are essential to house cricket (Ritchot, 1961). Intake of vitamin C, sterol, manganese, and vitamins B1 and B5 have a significant impact on crickets' biomass production (Morales-Ramos *et al.*, 2020). Desiccation of by-products at low temperature permitted the preservation of thermolabile components such as vitamins. Hence, it was expected that tomato pomace still contained choline, vitamins C and A, folic acid and various minerals that are also important for house cricket (Morales-Ramos *et al.*, 2020). Sorjonen *et al.* (2019) found the barley mash to be the overall best by-product diet for *A. domesticus* among the feeds tested in his experiments.

Working with *A. domesticus* is challenging because of occasionally high mortality rates. For this part of the experiments, replicas were limited to 5 with a countable number of crickets to allow the validation of the method. The results of these experiments show that the methodology here applied is effective and the by-products performances promising. Further studies with larger number of replicas and of individuals are scheduled for the last year of CoRoSect when the pilot studies with *A. domesticus* will take place.

Table 3.7. Results of A. domesticus experiment with five different diets (A, B, C, D, E).

Parameter	A (Control)	B	C	D	E
Survival (%)	27.5±6.6	44.0±11.5	31.0±9.1	19.5±11.9	33.0±11.1
Crickets reaching adulthood (%)	26.5±7.4	38.5±8.6	31.0±9.1	16.5±9.6	28.0±13.2
Final weight per insect (g)	0.56±0.06	0.59±0.04	0.61±0.03	0.58±0.05	0.63±0.10
Total weight gain per insect (g)	0.46±0.07	0.48±0.06	0.51±0.04	0.48±0.05	0.53±0.10
Daily weight gain per insect (g)	0.022±0.003	0.023±0.003	0.024±0.002	0.023±0.002	0.025±0.005

No statistically significant differences between groups were found (Kruskal-Wallis test for equal medians, $p<0.05$). Tested diets included different mixes of chicken feed, yeast, wheat bran, tomato, potato and brewer's spent barley grain.

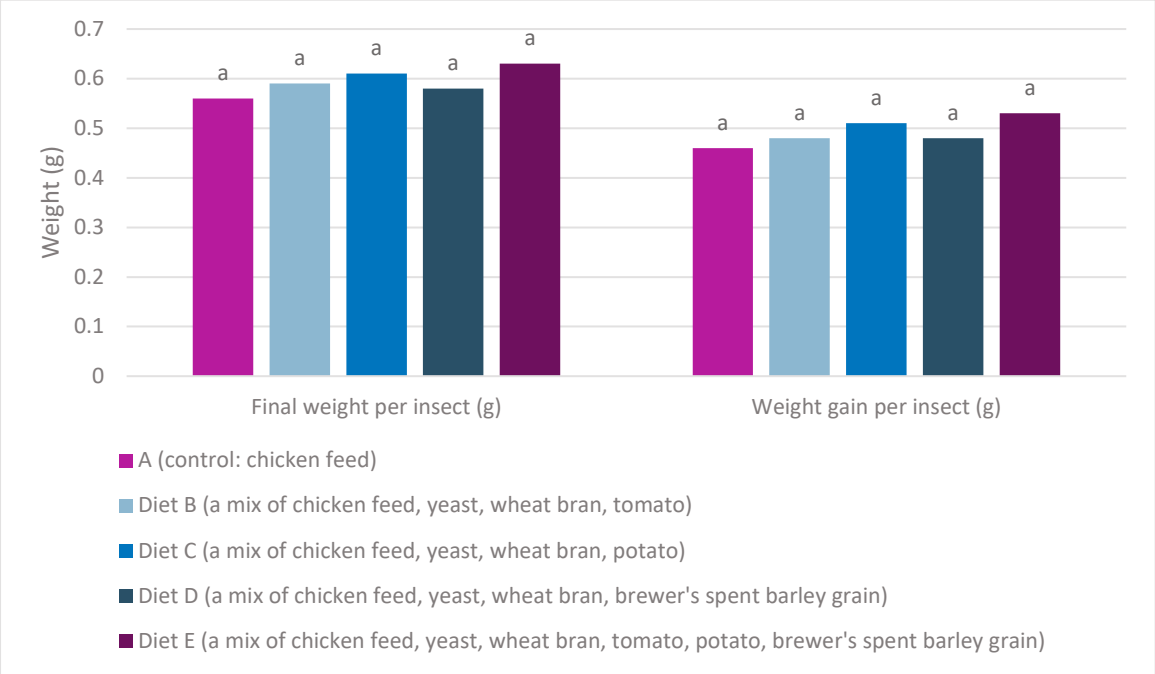

Figure 3.24. Final weight and weight gain of A. domesticus by experimental diet. The bars with the same letter do not differ statistically significantly (Kruskall-Wallis test).

3.3.2. Concluding remarks

Tested diets performed equally well with respect to all performance parameters measured in the experiment. Even though no statistically significant differences between the diets were observed, the results lead to important conclusions. The results suggest that at least 60% of chicken feed often used in *A. domesticus* diets can be replaced with byproducts of agriculture and food value chains, without compromising production performance. The results suggest that at least yeast, wheat bran, tomato, potato and brewer's spent barley grain can be used instead of chicken feed. In fact, the most diverse diet which contained the lowest amount of chicken feed was the most promising diet in the *A. domesticus* experiment although it did not differ statistically significantly from other tested diets. As side streams, especially when sourced locally, are often more affordable than the chicken feed, this offers promising avenues to improve the competitiveness of *A. domesticus* farming. Indeed, crickets can be reared on by-product-based diets, when the diet is in balance with all nutritional components (Sorjonen *et al.*, 2019). Even just mixing chicken feed with by-products may enable feeding cost

reductions to farmers. Qualitative feedback from the ICF farm, which carried out the experiment, suggested at least brewer's spent barley grain and yeast performed well in the rearing process.

Earlier studies (e.g. Pastell et al., 2020) have shown that chicken feed can yield good production results. Hence, the results suggest that at least yeast, wheat bran, tomato, potato and brewer's spent barley grain can be used to partially replace chicken feed and good production results can be achieved.

When taking into account the mean percentage of crickets reaching adulthood and the average weight of an adult cricket, computationally the highest total biomass of cricket yield was obtained for diet B (+53% when compared to the control) and for diets C (+27%) and E (+19%). Although the means of individual parameters were not statistically significantly different, the relative variation was fairly large. Diet B included 50% of chicken feed, 10% of both yeast and wheat bran and 30% of tomato pomace. Hence, these were concluded to be promising feed materials to be included in *A. domesticus* diets. Diet C was otherwise similar, but instead of tomato pomace it included 30% of potato peels. Diet E by contrast included 10% of all these feed materials and in addition 20% of brewer's spent grain.

3.4. Review report feedback

Review report concerning the first periodic reporting of CoRoSect was obtained in November 2022, when the main experiments reported in this deliverable were already running. The review report included two recommendations concerning task 3.2. Firstly, the consortium was encouraged to also look at feed combinations and not only single feed materials as a diet. This comment referred to the first *T. molitor* experiment, which was the only experiment that gave the insects only single feed materials as feed. This comment was already taken into account in the experimental design. All *H. illucens* and *A. domesticus* diets were based on feeding the insects with combinations of different feed materials. The same applied to the second *T. molitor* experiment which considered mixed diets. However, the current results suggested that the highest daily gain was achieved with the oligidic control diet that included only wheat bran. Moreover, mixed diets performed better than other oligidic control diet except wheat bran diet.

Secondly, the consortium was encouraged to investigate more than a single generation of insects fed with the new diets. This comment was to take into account the possible adaptation of insects to the new diets. Insect feeding experiments are typically carried out with only one generation of insects. Two-generation feeding experiment was not included in the current deliverable, mainly because of the short time between receiving the review feedback and the deadline to submit this deliverable. However, the consortium has planned to carry out a validation pilot in connection with the validation of model to be developed under task 3.3. This validation pilot shall include also an experiment where overall best-performing feed is given to insects for two consecutive generations. With this approach, the final experiment can be scoped to testing the most promising feed materials and the results of the second feeding experiment can be validated.

4 Conclusions

The purpose of CoRoSect task 3.2 experiments was to study the suitability of local side streams in the feeding of *H. illucens, T. molitor* and *A. domesticus.* Regarding the tested byproducts, dried brewer's spent grain, yeast and (crushed) tomato in the diets did not indicate major issues in any of the three species. Overall, the diets containing these byproducts performed quite well with all insect species and therefore it was concluded that brewer's spent grain, yeast and (crushed) tomato are promising feed materials to be considered in insects' diets.

In the *H. illucens* experiment, the control diet resulted in the best performance. The second best diet included a self-prepared concentrate (containing dried brewer's spent grain, feed yeast and potato protein), and three sidestream-like feed materials, namely crushed tomato, breadcrumbs and fresh apple. In general, the diets containing potato and broad bean did not perform quite as well as other tested diets. Potatoes contain glycoalkaloids (mainly α-solanine and α-chaconine) and broad beans contain vicin, convicin and phytic acid. These are harmful substances and they may have reduced larvae performance in the *H. illucens* test. These substrates had also quite dense structure and that may have caused some challenges for *H. illucens*. This issue requires further research. The concentrate could be supplemented also with other side streams, but that would require furhter research. The results also suggest that the amino acid composition of the diets fed to *H. illucens* is an important factor for the performance of *H. illucens*. At least the level of tryptophan and methionine in the diet affects the performance of *H. illucens*. Further research on the amino acid requirements of *H. illucens* is needed. This could be one of the aspects to be considered also in the forthcoming validation pilots.

Moreover, the addition of higher amount of tryptophan (4.7–5.2%) was found to have a favourable effect on *H. illucens* larvae growth and survival. Hence, the results suggest that *H. illucens* may benefit from high tryptophan concentration in the feed. However, also other amino acids and micronutrients may have contributed to the favourable result and therefore further research to exclude the contribution of other potentially important nutrients on *H. illucens* growth and survival are recommended. The addition of extra methionine, by contrast, did not improve growth, development, or mortality of *H. Illucens* larvae, and very high concentration of methionine reduced the performance of larvae. Hence, the results suggest that a methionine content of 1.54% in the diet is sufficient for *H. illucens* larval growth.

Regarding *T. molitor,* the control diet yielded the best performance results. While being a simple diet, wheat bran contains the necessary nutrients for mealworm's growth, including B-complex vitamins and fatty acids. Because these nutrients are not contained in optimal proportions, adding supplementary feed sources can however improve insects' development. Other diets that were closest to the control diet's performance, all contained 50% of wheat bran and in addition, different combinations of brewer's spent barley grain, tomato pomace and/or yeast. These feed materials could be considered to be included in *T. molitor* diets. Yeast, wheat bran and tomato all contain carnitine, which is an important nutrient for *T. molitor*. Brewer's spent barley grain's success as a diet could be due to the high amount of carbohydrates. In our case, there was a low level of protein and high level of carbohydrates in the spent grain. Because the brewing process influences the nutritional composition of brewer's spent grain, it is essential to take into account specific characteristics of grain that are used at each time. Major share of brewer's spent grain is often the external part of the seeds. Because *T. molitor* is selective when eating the feed, the "biscuit" preparation technique was applied successfully in the experiment to ensure that all parts of feed were consumed by the insects.

Brewer's spent grain is a feed material that is constantly available in most countries. However, because wheat bran is in general quite affordable feed material, other options may not be able to improve the competitiveness of mealworm rearing process.

The results suggest that at least 60% of chicken feed often used in *A. domesticus* diets can be replaced with byproducts of agriculture and food value chains, and without compromising production performance. At least yeast, wheat bran, tomato, potato and brewer's spent barley grain can be used instead of chicken feed. In fact, the most diverse diet which contained the lowest amount of chicken feed was the most promising diet in the *A. domesticus* experiment although it did not differ statistically significantly from other tested diets. As side streams are often more affordable than the chicken feed, this offers promising avenues to improve the competitiveness of *A. domesticus* farming.

The experiments also produced observational data about the rearing process and its' parameters (such as temperature) which can be utilized in further steps of the project, and complemented with other data that are available prom project partners. Sensors can therefore be utilized to help monitoring the rearing process.

Because systematic testing of insects' nutritional requirement has not been carried out at large, further research on the importance of individual amino acids and micronutrients ais recommended in the future. In addition, experimental desings should pay attention to what is the best way of administering the diet. These aspects could be considered also in the forthcoming CoRoSect pilots.

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Annex 1. Analysed chemical composition of *H. illucens* diets

Table A1. Analysed chemical composition of H. Illucens larvae diets.

	C 2	S 1[1)]	S 2	S 3	S 4	S 5	S 6
Analysed chemical composition (%)							
Dry matter	38.3	41.0	36.0	37.0	37.7	38.0	37.3
Moisture	61.7	59.0	64.0	63.0	62.3	62.0	62.7
Crude protein	6.7	10.7	9.6	9.40	10.7	10.3	8.9
Crude fat	1.4	3.2	3.0	3.1	1.2	1.8	1.6
Crude fibre	1.8	4.9	4.6	4.4	2.3	2.8	2.5
ADF	2.0	5.0	4.9	5.0	2.6	3.0	2.9
NDF	4.8	14.5	12.6	13.0	6.4	7.9	7.6
Ash	5.0	2.0	1.6	1.6	1.4	1.4	1.2
Analysed mineral composition (g/kg fresh matter)							
Calcium	14.93	1.83	1.90	1.86	0.63	0.73	0.67
Copper	9.89	4.44	3.11	3.60	6.74	6.00	5.06
Iron	123.97	118.47	99.01	78.83	51.19	72.00	61.05
Magnesium	1.13	1.17	0.83	0.86	0.61	0.63	0.61
Manganese	53.95	18.19	12.81	13.21	7.14	9.20	8.55
Phosphorus	2.20	2.36	2.00	2.09	1.66	1.62	1.49
Potassium	2.29	3.03	1.51	1.44	5.03	3.66	3.53
Sodium	0.57	0.12	0.12	0.10	0.22	0.20	0.19
Sulphur	0.88	1.17	1.05	1.06	2.99	2.89	1.09
Zinc	41.19	36.38	29.51	31.21	20.24	23.60	21.00
Analysed amino acid composition g/kg fresh matter[2)]							
Alanine	2.69	4.97	4.35	4.44	4.68	4.68	4.12
Arginine	3.77	4.88	4.39	4.60	5.87	4.68	4.16
Aspartic acid	5.11	7.19	6.41	6.60	11.19	9.92	8.52
Cysteine	1.24	1.71	1.57	1.63	1.54	1.63	1.46
Glutamic acid	14.83	20.10	17.32	18.25	17.93	19.76	18.59
Glycine	2.86	4.20	3.70	3.73	4.44	4.28	3.76
Histidine	1.55	2.20	1.97	2.02	2.24	2.19	1.92
Isoleucine	2.51	4.01	3.51	3.57	4.56	4.44	3.84
Leucine	4.74	7.46	6.52	6.68	8.33	8.00	7.04
Lysine	3.10	4.53	4.04	4.04	5.91	5.16	4.36
Methionine	1.54	1.77	1.63	1.65	9.72	9.68	1.57
Phenylalanine	3.19	5.50	4.78	4.72	5.44	5.48	4.78
Proline	5.07	9.18	8.58	8.72	6.35	7.88	7.23
Serine	3.12	4.30	3.76	4.00	4.88	4.80	4.28
Threonine	2.58	3.78	3.28	3.43	4.37	4.40	3.81
Tryptophan	0.90	5.19	4.66	1.32	1.32	1.39	1.24
Tyrosine	2.03	3.23	2.80	2.80	3.76	3.74	3.23
Valine	3.18	5.42	4.82	4.88	5.59	5.52	4.82

[1)]See Section 2 for the description of the treatments. [2)] The essential amino acids for the rearing of insects are in italic (essential amino acids according to Cohen, 2004).

Table A2. Analysed chemical composition of H. illucens larvae (pooled sample) in control and experimental diets (fresh weight).

	C 1	S 1[1)]	S 2	S 3	C 2	S 4	S 5	S 6
Analysed chemical composition (%)								
Dry matter	41.4	38.8	39.2	39.8	35.7	29.8	33.1	34.1
Moisture	58.6	61.2	60.8	60.2	64.3	70.2	66.9	65.9
Crude protein[2)]	10.51	14.32	13.71	14.24	11.35	13.25	12.72	11.73
Crude fat	10.7	11.6	12.5	11.8	10.8	5.9	9.3	12.1
Crude fibre	2.8	2.6	2.6	3.0	1.6	2.2	2.6	2.4
ADF	2.9	3.6	3.2	3.5	2.2	2.4	2.7	2.5
NDF	5.0	5.6	5.5	5.9	3.9	3.4	4.3	4.0
Ash	6.6	2.8	3.2	3.3	5.9	2.1	2.1	1.7
Analysed mineral composition (g/kg fresh matter)								
Calcium	23.5	6.31	8.81	8.90	17.8	2.61	3.29	2.95
Copper	13.5	4.32	5.80	4.39	7.10	5.61	4.16	3.89
Iron	156.5	86.83	129.6	83.3	112.8	45.30	43.06	35.03
Magnesium	1.94	1.32	1.36	1.35	1.41	0.93	1.03	0.88
Manganese	129.51	66.68	57.54	61.41	119.48	26.32	35.51	33.48
Phosphorus	3.88	3.60	3.49	3.38	3.45	3.27	3.03	2.26
Potassium	4.88	5.65	4.92	4.81	4.64	5.71	4.21	3.48
Sodium	0.73	0.38	0.37	0.36	0.53	0.37	0.33	0.28
Sulphur	1.53	1.55	1.56	1.75	1.37	2.19	2.30	1.20
Zinc	63.83	47.50	45.46	43.37	47.63	33.22	34.00	33.48

[1)]See Section 2 for the description of the treatments. [2)]Used multiplier 4.76.

Table A3. Analysed chemical composition of H. Illucens larvae frass (pooled sample) of control and experimental diets (fresh weight).

	C 1	S 1[1)]	S 2	S 3	C 2	S 4	S 5	S 6
Analysed chemical composition (%)								
Dry matter	70.7	58.3	53.5	55.6	71.1	61.8	64.5	62.3
Moisture	29.3	41.7	46.5	44.4	28.9	38.2	35.5	37.7
Nitrogen	1.47	2.29	2.53	2.16	1.54	3.10	3.84	
Ash	17.3	4.7	3.7	3.8	18.1	4.5	4.7	4.7
Analysed mineral composition (g/kg fresh matter)								
Calcium	60.36	3.39	2.41	2.16	55.91	1.50	1.52	1.00
Copper	33.50	9.59	9.18	9.09	22.83	18.71	19.53	19.53
Iron	606.13	377.70	274.20	306.18	530.23	168.37	292.92	301.44
Magnesium	3.88	2.81	2.05	2.05	3.79	1.64	1.83	1.86
Manganese	115.74	26.20	16.40	15.76	115.62	15.48	18.18	11.07
Phosphorus	5.93	5.19	4.71	5.06	7.01	4.21	4.55	4.80
Potassium	6.33	7.75	2.97	2.85	7.85	13.29	11.25	12.08
Sodium	2.28	0.13	0.23	0.18	2.82	0.67	0.66	0.66
Sulphur	2.64	2.63	2.74	2.62	3.26	7.35	9.53	4.02
Zinc	116.50	83.08	73.47	78.82	136.98	56.12	74.75	68.36

[1)]See Section 2 for the description of the treatments.

Table A4. Description of H. illucens diet treatments used throughout the experiment.

Treatment	Description
C	Control diet, commercial store-bought laying hen feed (dry chicken feed, ME 11 MJ kg^{-1}) dissolved in water to 30% dry matter (DM).
S 1	Diet 1: Diet formulated to resemble same nutrient and amino acid profile as control feed: Concentrate 1 (29%), spinach (frozen product) 42% and potato (fresh) 29%. It was detedted later that tryptophan content was 7-fold compared to control diet.
S 2	Diet 2: Diet formulated to resemble same nutrient and amino acid profile as control feed: Concentrate 1 (27,5%), kale (frozen product) 60,5%, tomato (crushed) 8% and breadcrumbs 4%. It was detedted later that tryptophan content was 7-fold compared to control diet.
S 3	Diet 3: Diet formulated to resemble same nutrient and amino acid profile as control feed, ecept for tryptophan: Concentrate 2 (27%), kale (frozen product) 60%, tomato (crushed) 8% and breadcrumbs 5%. This diet was formulated to be tryptophan deficient compared to control, but it was detedted later that tryptophan content was at the same level as in the control diet.
S 4	Diet 4: Diet was formulated to resemble same nutrient and amino acid profile as control feed, ecept for methionine (7-fold concentration): Concentrate 3 (24.33%), potato (fresh) 29.0%, tomato (crushed) 41.82%, breadcrumbs 4.0% and methionine 0.85%.
S 5	Diet 5: Diet formulated to resemble same nutrient and amino acid profile as control feed, ecept for methionine (7-fold concentration): Concentrate 4 (22.0%), apple 28.4%, tomato (crushed) 42.0%, breadcrumbs 6.76% and methionine 0.84%.

Annex 2. Observed results' tables for *H. illucens* experiment.

Table A5. Observation day, total and mean weight, and the number of dead larvae in the subsample (30 larvae) and pH of the diet (NA=not available) in the first H. illucens experiment.

Substate[1)]	day	total weight mg	weight mg/larvae	dead	pH
Control 1	1	229	7.63	-	4.6
Diet 1	1	229	7.63	-	4.1
Diet 2	1	229	7.63	-	4.1
Diet 3	1	229	7.63	-	4.1
Control 1	4	2871	95.7	-	4.2
Diet 1	4	4666	155.53	-	4.2
Diet 2	4	4485	149.50	-	4.0
Diet 3	4	4495	149.83	-	4.1
Control 1	6	6395	209.83	-	-
Diet 1	6	5057	168.57	-	-
Diet 2	6	5014	167.13	-	-
Diet 3	6	5390	179.67	-	-
Control 1	8	5906	196.87	-	7.5
Diet 1	8	4915	163.83	2	8.4
Diet 2	8	4895	163.17	3	8.4
Diet 3	8	4960	165.33	1	8.3
Control 1	10	6082	202.73	NA	-
Diet 1	10	4928	164.27	NA	-
Diet 2	10	4704	156.80	NA	-
Diet 3	10	5230	174.33	NA	-

[1)]See Section 2 for a description of the treatments.

Table A6. Observation day, total and mean weight, and the number of dead larvae in the subsample (30 larvae) and pH of the diet in the second H. illucens experiment.

Substate[1)]	day	total weight mg	weight mg/larvae	dead	pH
Control 2	1	163	5.43	-	5.74
Diet 4	1	163	5.43	-	4.71
Diet 5	1	163	5.43	-	4.24
Diet 6	1	163	5.43	-	4.30
Control 2	3	1011	33.70	-	4.24
Diet 4	3	506	16.87	-	3.69
Diet 5	3	810	27.00	-	3.93
Diet 6	3	1120	37.33	-	3.73
Control 2	6	3720	124.00	-	4.20
Diet 4	6	2132	71.07	-	6.20
Diet 5	6	4155	138.5	-	5.45
Diet 6	6	5455	181.83	-	4.50
Control 2	8	8552	285.07	-	-
Diet 4	8	3125	104.17	-	-
Diet 5	8	4127	137.57	-	-
Diet 6	8	6375	212.50	-	-
Control 2	10	7445	248.17	-	7.76
Diet 4	10	3288	109.60	-	8.60
Diet 5	10	3540	118.00	-	7.96
Diet 6	10	5705	190.17	-	8.22
Control 2	11	6961	232.03	0	7.76
Diet 4	11	3265	108.83	0	8.60
Diet 5	11	3230	107.67	1	7.96
Diet 6	11	5060	168.67	3	8.22

[1)]See Section 2 for a description of the treatments.

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