Use of insects for food and feed

Scientific overview of the present knowledge on insect rearing, use of residual streams as substrates, and safety aspects

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Elise Hoek-van den Hil[1], Adriaan Antonis[2], Mike Brouwer[2], Marieke Bruins[3], Anita Dame-Korevaar[2], Johan van Groenestijn[3], Olga Haenen[2], Yvette Hoffmans[1], Nathan Meijer[1], Teun Veldkamp[4], Adriaan Vernooij[4], Marko Appel[1]

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P.O. Box 230, 6700 AE Wageningen, The Netherlands, T +31 (0)317 48 02 56, E info.wfsr@wur.nl, www.wur.eu/food-safety-research. WFSR is part of Wageningen University & Research.

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Contents

1 Introduction

This report gives a scientific overview of the present knowledge on insect rearing, use of residual streams as substrates, and safety aspects of insects used for food and feed. This overview will form the basis for experiments in the public-private partnership (PPP) project SAFE INSECTS (LWV20.102).

Background
An important ambition of the Dutch government and society is to get as much value as possible from all residual streams and by-products from the food system. Insects are by nature ideally suited for thriving on various biomasses and could therefore provide part of the solution for circular agriculture and economy. This is highlighted in the vision of the Dutch insect sector, that has been published in October 2020 (Venik, 2020). It states that by 2030 the sector wants to make 100% use of organic residual streams from the food chain as nutrients for its insect rearing.

Therefore, organic residual streams or by-products as possible insect substrates will be the main focus of the present study. Organic residual flows are released at different points in the agri-food and horticultural chain, you could think of the primary sector, processing industry, retail, catering and consumers. The safe use of these (legally prohibited) residual streams for the rearing of insects and further application in the food system will contribute favourably to circular agriculture and result in a reduction of CO_2 and nitrogen emissions. In order to enable legal application of this circular solution, it is essential to research and assure feed and food safety.

Focus of the project SAFE INSECTS
In the present project the feed and food safety for humans and animals and the technical feasibility of insects being reared on residual streams will be investigated. The selected residual streams that will be investigated in the project are: poultry manure, category 2 meat meal from animal rendering, organic waste from household kitchens (GFE) and supermarket mix (food products that passed the expiration date or are unsaleable for other reasons). Insect species of interest are the yellow mealworm (YMW, *Tenebrio molitor* (L.) Coleoptera: Tenebrionidae) and the black soldier fly larvae (BSFL, *Hermetia illucens* (L.) Diptera: Stratiomyidae) as the most commonly used species, for food and feed respectively. Therefore, the focus of the present report was mainly on these two insect species. If data in literature on certain aspects was missing, results on similar species was included – but it must be noted that there may be differences between species that hinder extrapolation to species of focus.

Aim of the present literature study
The aim of this literature study was to give a scientific overview of the present knowledge on insect rearing, use of residual streams as substrates, and safety aspects of insects used for food and feed. This knowledge is needed as input for the experiments within the PPP project SAFE INSECTS. The results of this literature study will guide the decisions on the next steps to be made in the project, for instance: selecting food and feed safety parameters to be included, and design and setup of insect rearing experiments and design and setup substrate optimalisation. Furthermore, this report highlights present knowledge and data gaps on insect rearing on residual streams.

2 Methodology

2.1 Scientific literature

The databases Scopus and Web of Science (WoS) were used for the scientific literature study. Search strings were focused on BSFL and YMW in combination with substrates/waste streams, or feeding or food safety or feed safety or microbiological hazards, viruses, pathogens or insect diseases and related synonyms. The specific search strings can be found in Annex 1. All hits obtained from the scientific search engines were exported into a reference managing program. In this program, the references were categorized into the groups "Relevant", "Maybe relevant" or "Not relevant" after reading the titles and abstracts. "Maybe relevant" papers would be read when the relevant articles are not giving relevant information after all. The references in the group "Relevant" were subject to full-text reading. Relevant information was extracted and inserted into a separate file. In this manner, all relevant information was structured in one overview.

2.2 Advanced Google Search

With the help of the Advanced search feature in Google, the search for grey literature was executed. In this way, it was sought to obtain documents, such as governmental report, risk assessment reports and websites with the latest news on the topic. The search terms were "Insect AND rear AND safety" and were sought for in the websites of the EFSA, the Netherlands Food and Consumer Product Safety Authority (NVWA in Dutch), the National Institute for Public Health and the Environment (RIVM in Dutch) and Groen Kennisnet. Except for the search in the EFSA website, the searches were done in Dutch being "Insect AND kweken AND veilig". An additional search was executed for the website of Groen Kennisnet to obtain literature relevant for other fields of expertise as well. The search terms for this additional search were Insect AND feed OR feed OR food (in Dutch: Insect AND voer OR voeder OR voedsel). These searches resulted in approximately 374 hits in total. All the hits were screened on the title and relevant hits were fully read. From these, approximately 20 were found relevant. Similarly, to the scientific literature, the relevant information is extracted into the literature overview.

2.3 Interviews with partners

In addition to the literature search, interviews were performed with partners who can deliver residual streams to be used as rearing substrates in this project. These partners were Avined (chicken manure), Darling Ingredients (meat meal (cat. 2) from animal rendering) and AMS Institute - Amsterdam Institute for Advanced Metropolitan Solutions (organic waste from household kitchens).

3 Insect rearing

3.1 History and place of insect rearing in Europe and the Netherlands

Insect rearing in Europe and the Netherlands is a relatively recent development. During the past five to ten years, industrial production and scientific knowledge have been growing strongly (Huis, Oonincx, Rojo, & Tomberlin, 2020). Insects have the potential to serve as a food and feed source globally with a lower negative impact on the environment (Govorushko, 2019). Insects are being reared both for food and feed with the YMW and the BSFL as the most commonly used species, for food and feed respectively (Council on animal affairs, 2018).

Recent figures on the scale of insect production in the European Union are provided in factsheets published by IPIFF (International Platform for Insects as Food and Feed) (IPIFF, 2020a, 2020b). Edible insect-based production in 2019 in the EU was approximately 500 tons and is expected to rise to 260.000 tons in 2030. As a result of existing legislation, main markets for insects for feed are currently petfood and aquaculture. Demand from the poultry and pig sector is expected to rise strongly after EU approval on the use of processed insect protein in poultry and pig feed in September 2021 (EU, 2021b).

Insects for human food are currently mainly applied in special products such as bars, snacks and sport foods. Bigger market shares in the future are expected from insects in pasta, burgers, bread and possibly also whole insects.

Insect production in the Netherlands has been given more recognition by the government, as laid down in the *Sectorplan Agenda Ontwikkeling en Innovatie in de Nederlandse Insectenketen* (Venik, 2020). This document describes the vision, challenges and expectations of the possibilities for insect production in the Netherlands in the coming years. Furthermore, it highlights the possibilities and changes for the insect industry to contribute to a circular economy.

Actual production figures in the Netherlands are not publicly available, but some information is available from the Entomospeed project (Anonymous, 2019). Commercial production can be divided in large scale industrial investments and startups of family size business types. Whilst there are only few large-scale industrial producers, the number of small to medium scale producers is around 30, with almost half of these focusing on the production of YMW, approx. one third produces grasshoppers, whilst BSFL and certain cricket species are produced by only four or five producers. The turnover in producers is high; several producers have closed their business again after trying for a few years. No detailed analysis has been made of the reason why producers discontinue their business, though it is believed by many that the complexity of production and difficulties in finding stable sales markets are amongst the main reasons (Marian Peters (NGN), personal communication 2020).

3.2 Relevant EU legislation for insect rearing and insect products

European Union (EU) law regulates the conditions for food and feed business operators, including insect producers, to produce and commercialize their products in the EU. Notably, EU policymakers have adopted – in the early 2000s – a package of legislative texts which define general principles and standards in the area of food and feed safety. These legislative texts are most commonly known as the 'General Food Law' (Regulation No 178/2002) and the 'Hygiene Package' (primarily Regulation No 852/2004 on the hygiene of foodstuffs and Regulation No 183/2005 laying down requirements for feed hygiene). According to this

general legislation, producers of insects – like any other food or feed business operator – are responsible for ensuring the safety of the marketed products. To this end, these texts impose general obligations on those actors, such as the registration or approval of their activities before national competent authorities, and establishing hygiene standards to be applied at the different stages of production (IPIFF, 2021).

EU decision makers have also established restrictions on the feed which may be given to 'farmed animals'; i.e. animals that are kept for the production of food, feed or other derived products (e.g. wool or hides). These restrictions also apply to insects intended for human consumption or for animal feed use. Consequently, the primary source of insect feed consists of materials of vegetal origin. Some exceptions are permitted for certain materials of animal origin such as milk, eggs and their products, honey, rendered fat or blood products from non-ruminant animals. The feeding of farmed animals with other slaughterhouse or rendering derived products, manure, or catering waste is prohibited, however. The same ban applies to the use of unsold products from 'former foodstuffs' ("*foodstuffs, other than catering reflux, which were manufactured for human consumption in full compliance with the EU food law but which are no longer intended for human consumption for practical or logistical reasons or due to problems of manufacturing or packaging defects or other defects and which do not present any health risks when used as feed*" (Regulation (EU) No 2017/1017)), if these products contain meat or fish.

Furthermore, obligations lie with insect producers to ensure that their animals are kept in good health so as to prevent the spreading of diseases among their production flock. To this end, EU policy makers have established the responsibilities of animal keepers in the area of health and biosecurity in the so-called 'EU Animal Health Law' (IPIFF, 2021).

In addition to the 'general food hygiene requirements', the production and marketing of insects as food in Europe is governed by the 'Novel Foods' legislation – Regulation (EU) No 2015/2283. This legislation applies to all categories of foods that 'were not used for human consumption to a significant degree' within the European Union before 15 May 1997, which is the case of insects (EU, 2021a). As such, insect producers who wish to market their products as food in the EU must first submit a detailed dossier on the safety of their products to the European Food Safety Authority (EFSA). After a positive evaluation by EFSA, the European Commission (EC) drafts an amending Regulation which is considered by the Standing Committee on Plants, Animals, Food and Feed (Novel Food and Toxicological Safety section), which is composed of representatives of all EU countries and chaired by a European Commission representative. If this Committee gives a favorable opinion, the Regulation is generally adopted, and the product may be legally sold on the EU market. Novel food authorizations are granted for products – not to individual companies, as was previously the case – which means that other organizations than the one that submitted the initial dossier may also market that product, if produced in the same manner as defined in the dossier. However, organizations submitting a dossier may apply for some of the data supporting the dossier to be kept confidential for 5 years; thereby preventing other organizations from following the described procedures, effectively restricting production (IPIFF, 2021).

Until September 2021, insect products for animal feeding were only allowed for petfood and aquaculture, whilst for poultry and pigs only the feeding of whole larvae was allowed, with the exception of insect oil for young animals such as piglets. As from September 7th, 2021, insect products are fully authorized for all non-ruminant feed in the EU (EU, 2021b).

3.3 Physiology, life cycle and nutritional composition of BSFL and YMW

3.3.1 Black soldier fly larvae

The BSF originates from South America, but has become naturalized in most tropical and sub-tropical countries (Makkar, Tran, Heuzé, & Ankers, 2014). The adult fly is black and 15-20 mm long. Larvae can grow up to 25-30 mm in length and 6mm in width, and may reach a weight of over 200 mg. The larvae are voracious eaters and can consume several hundreds of grams of organic matter per larvae per day. They

feed on almost all decaying organic matter and can mature in about six to eight weeks under ideal circumstances. At the end of the larval stage, they develop into their pupal stage, which last from 14 days to several months, depending on the surrounding circumstances. The life-cycle of BSFL is shown graphically in Figure 1.

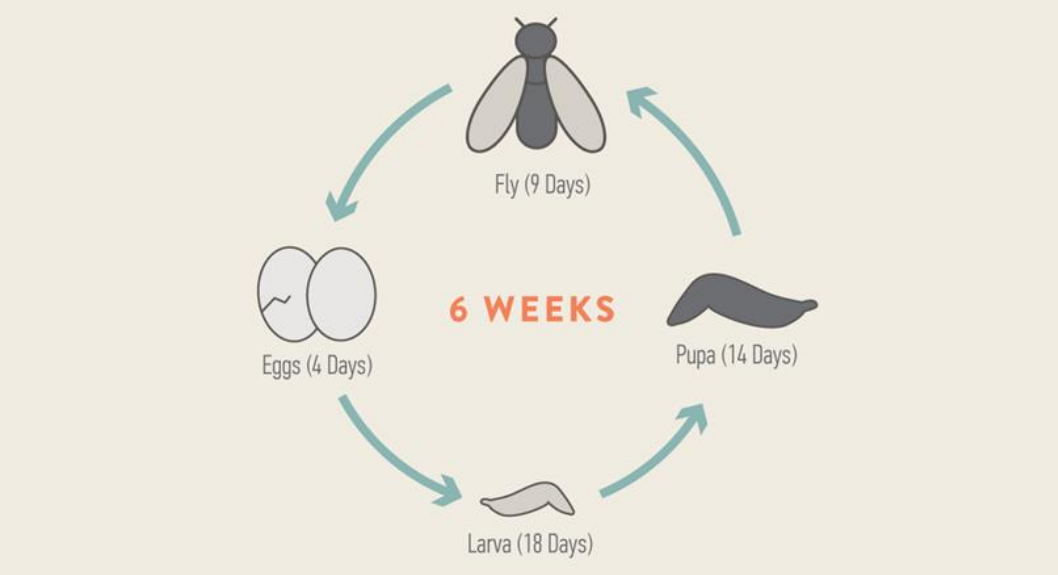

Figure 1 *Life cycle of black soldier fly (Skrobonja, 2021).*

Growth time of the larvae can be highly variable and depends on many factors (Van Huis & Tomberlin, 2017). The composition of the diet (especially moisture), but also environmental factors are important and as a result of this, the length of the larval growth period can differ from two weeks to several months. To what extent commercial insect rearing organizations value these different variables is unknown, and it is therefore not fully clear what the rearing cycle of BSFL under ideal industrial conditions looks like.

At the end of the larval stage (prepupa), the larvae empty their digestive tract and stop feeding and moving. The prepupae then migrate in search of a dry and protected pupation site (Diener et al., 2011). The duration of the pupal stage is about 14 days but can be extremely variable and last up to 5 months. The females mate two days after emerging and oviposit into dry cracks and crevices adjacent to a feed source (Diener et al., 2011). The adults do not feed and rely on the fats stored from the larval stage (Diclaro & Kaufman, 2009).

An overview of the chemical composition of BSFL, when provided with different substrate sources, is provided in Veldkamp and Vernooij (2021), copied here in Table 1.

Table 1 *Chemical composition of black soldier fly larvae (BSFL; Hermetia illucens) prepupae products, housefly (HF; Musca domestica) larval meal, yellow mealworm larvae (YMW; Tenebrio molitor), soybean meal and fishmeal (Veldkamp & Vernooij, 2021).*

	BSF prepupae grown on vegetable waste	**Full fat BSFL meal**	**Partially defatted BSFL meal**	**HF larval meal**	**YMW**	**Soybean meal**	**Fishmeal**
				g/kg			
Dry matter	410	884	939	920	381	877	913
Crude ash	96	74	68	65	24	64	168
Crude protein	399	425	408	533	491	467	629
Crude fat	371	325	128	203	352	15	98
Ca	28.7	20.8	5.8	-	0.4	2.9	40.3
P	4.0	4.7	7.6	-	7.5	6.4	26.0
				g/16 g N			
Lys	5.7	6.2	6.2	7.1	5.5	6.2	7.6
Met	1.9	2.5	2.9	2.5	1.3	1.4	2.8
Cys	0.5	0.7	1.5	2.8	0.9	1.5	0.9
Thr	3.9	4.2	4.0	5.3	5.1	3.9	4.2
Trp	1.5	-	-	6.5	4.1	1.3	1.1
Ile	4.3	5.6	4.9	3.6	5.0	4.6	4.2
Arg	5.0	5.7	5.2	4.8	5.2	7.5	5.9
Phe	4.1	5.5	4.4	6.0	3.5	5.2	3.9
His	3.1	3.7	3.0	2.9	3.2	2.7	2.6
Leu	7.0	8.6	7.6	6.1	10.6	7.7	7.3
Tyr	-	-	-	6.5	0.8	3.7	3.1
Val	6.2	7.2	6.7	4.3	7.3	4.8	4.9
Ala	6.1	7.3	8.2	5.5	8.2	4.4	6.3
Asp	9.0	11.0	10.3	9.9	8.1	11.6	9.3
Glu	10.4	11.6	15.3	13.4	11.3	17.8	13.0
Gly	5.6	7.2	6.2	4.5	5.6	4.3	6.5
Pro	5.4	7.2	7.8	3.8	7.0	5.1	4.4
Ser	3.8	4.8	5.0	2.5	5.1	5.1	4.0
Sum_AA	83.3	99.0	99.4	97.9	97.8	98.8	92.0
	Adapted from (Thomas Spranghers et al., 2017)	Adapted from (Crosbie, Zhu, Shoveller, & Huber, 2020)	Adapted from (Crosbie et al., 2020)	Adapted from (Hall et al., 2018)	Adapted from (Finke, 2002)	(CVB, 2019)	(CVB, 2019)

AA = amino acid, Ala = alanine, Arg = arginine, Asp = aspartate, BSF = black soldier fly (*Hermetia illucens*), Ca = calcium, Cys = cysteine, Glu = glutamate, Gly = glycine, HF = housefly (*Musca domestica*), His = histidine, Ile = isoleucine, Leu = leucine, Lys = lysine, Met = methionine, N = nitrogen, P = phosphorus, Phe = phenylalanine, Pro = proline, Ser = serine, Thr = threonine, Trp = tryptophan, Tyr = tyrosine, Val = valine.

3.3.2 Yellow Mealworm

YMW are the larval form of the mealworm beetle. The genus Tenebrio consists of two extant species, *Tenebrio molitor* and *Tenebrio obscurus*. Other notable species in the family Tenebrionidae that are also being reared for food and feed purposes are *Alphitobius diaperinus* (lesser mealworm, LMW) and *Zophobas morio* (superworms). Like most insects, they go through four life stages: egg, larva, pupa, and adult – as shown in Figure 2. Larvae typically measure about 2.5 cm or more, whereas adults are generally between 1.25 and 1.8 cm in length (Van Huis & Tomberlin, 2017).

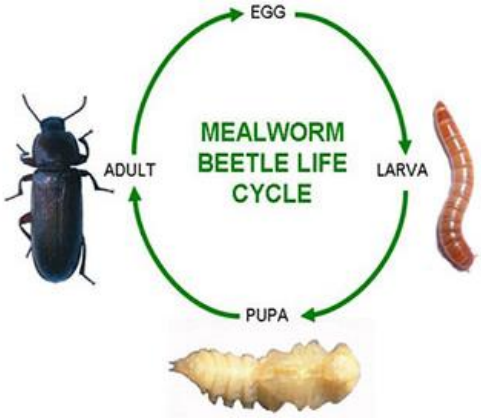

Figure 2 *Life cycle of Tenebrio molitor (Feedipedia, 2021).*

The life cycle of the YMW is of variable length, from 280 to 630 days, depending on circumstances such as climate and feed availability. Females can lay between 100 and 200 eggs at a time and may lay up to 500 eggs in their lifetime. Larvae hatch after 10-12 days (at 18-20°C) and mature after a variable number of stages (8 to 20), typically after 3-4 months (at ambient temperature), but the larval stage can last up to 18 months. The mature larva is of a light yellow-brown color, 20 to 32 mm long, and weighs 130 to 160 mg. The pupal stage lasts 7-9 days at 25°C and up to 20 days at lower temperatures. The adult *Tenebrio molitor* beetle lives for 2 to 3 months. The life cycle of *Tenebrio obscurus* is shorter, particularly in the larval stage. As mentioned for BSFL, the life-cycle YMW under industrial and commercial conditions may differ from the description provided here, but it is unclear what this cycle looks like exactly due to a lack of data (Breeding Insects, 2020).

An overview of the composition of different YMW samples was provided by Ravzanaadii, Kim, Choi, Hong, and Kim (2012): the proximal content (shown in Table 2) and the amino acid content (Table 3).

Table 2 *Proximal content of Tenebrio molitor larvae, adult, exuvium and excreta (percent, dry basis).*

Component	Larvae	Adult	Exuvium	Excreta
Moisture	5.33	3.54	13.02	12.2
Crude protein	46.44	63.34	32.87	18.51
Crude fat	32.7	7.59	3.59	1.3
Crude fiber	4.58	19.96	25.96	13.66
Crude ash	2.86	3.56	3.22	7.29

Table 3 *Amino acid content of Tenebrio molitor larvae, adult, exuvium and excreta (grams per 100 grams protein).*

Amino acid	Larvae	Adult	Exuvium	Excreta
Isoleucine	3.556	3.918	1.9	0.33
Leucine	3.405	5.165	1.981	0.368
Lysine	2.906	2.227	1.009	0.193
Cysteine & Methionine	1.189	1.134	0.426	0.251
Phenylalanine & Tyrosine	5.219	3.173	3.016	0.366
Threonine	1.807	2.153	1.124	0.276
Valine	2.439	3.368	2.423	0.253
Histidine	1.527	1.71	1.236	0.438

The study of Hermans et al. (2021) showed that ingestion of LMW–derived proteins are easily digested after meals and amino acid absorption and "*does not differ compared with ingestion of the same amount of milk protein in vivo in humans, which shows that insects can provide a viable, high-quality protein source for human consumption"*.

3.4 Nutritional needs of BSFL and YMW

The development of diets capable of supporting and, ideally, maximizing insect growth and development has been identified as one of the major challenges for the insect-producing industry in terms of achieving efficient, cost-effective and sustainable insect production for food and feed. Insect diets should meet the nutritional requirements of the different insect species and should be optimized to suit the different life stages to maximize the total larval biomass production and increase adult reproductive performance (Van Huis & Tomberlin, 2017).

Both BSFL and YMW have an extremely wide range of diet preferences, so finding a mixture of suitable components is a complex task and still subject to many research questions. The most important criteria to evaluate the nutritional potential and efficiency of the diets are feed conversion efficiency, survival rate and developmental (growth) time of the larvae. In general, it can be stated that knowledge on the nutrition of insects is still in its early stages. Further studies should focus on finding optimized combinations of insect species and diet composition, in order to efficiently produce insects that meet the nutritional requirements of humans and other animals (Dennis G. A. B. Oonincx, van Broekhoven, van Huis, & van Loon, 2015).

Also within the insect sector, further research and experience is needed to decide on the best diet under the optimal circumstances, as was shown in a survey with Dutch producers (Van der List, 2020).

3.4.1 Black Soldier Fly Larvae

The exact nutritional requirements of BSFL are not well identified yet (T. Spranghers, Schillewaert, & Wouters, 2018). There is a vast volume of literature available on tests with various kinds of substrates (individual and mixed sources) fed to BSFL under different (laboratory) conditions. In experimental trials, various variables have been investigated e.g. protein, energy contents, and several minerals. A particular result for e.g. the optimum protein percentage in one trial, can be different again in other trials if the protein is derived from different feedstock. In general, however, it is shown that BSF can indeed be efficient converters of a large variety of organic waste products, indicating that their minimal nutritional requirements are rather easily met. Moreover, their omnivorous nature allows them to substantially contribute to sustainability and circularity when using waste streams and residual streams as substrate to feed insects (Diener, Zurbrügg, & Tockner, 2009).

In most BSFL feed trials, either (broiler) chicken feed or the Gainesville diet (50% wheat bran, 30% alfalfa meal, 20% corn meal) are often used as a reference feed in order to compare the effectiveness of experimental substrates.

(Thomas Spranghers et al., 2017) found that 2.7% protein in a wet mixture (30% DM) was sufficient for BSF rearing. Higher amounts of protein did not improve the growth, whereas lower levels resulted in reduced yield of harvested larvae. These findings are quite interesting given that most waste streams from vegetal origin contain substantially less crude protein than chicken feed (15-18%), making them possibly suitable candidates for BSF rearing. Moreover, lower amounts of crude protein in the BSF rearing diet will result in less nitrogen emission to the environment in the form of ammonia (Parodi et al, 2020)

Studies of (Bonelli et al., 2020) showed that larval growth performance is only moderately affected by a nutritionally poor diet. Differences in the activity of digestive enzymes, midgut cell morphology, and accumulation of long-term storage molecules can be observed, indicating that diet-dependent adaptation processes in the midgut ensure increased exploitation of poor substrates. Apparently, genes with important functions in digestion and absorption are differently expressed under different circumstances, confirming the adaptability of the midgut.

Villazana and Alyokhin (2019) recorded “dramatic differences” in seemingly similar substrates. Their research focused on substrates from marine animals (finfish trimmings, wet sea cucumber, dry quahog, and sea urchin). They stressed the importance of thorough testing of specific substrates instead of extrapolating from published data.

Research on pH was amongst factors investigated by (Ma et al., 2018). This study examined the effect of pH on BSFL production, development time, and adult longevity. The BSF were reared on artificial diets with initial pH of 2.0, 4.0, 6.0, 8.0, and 10.0; the control was set at a pH of 7.0. Final larval weight was highest on 6.0, 7.0 and 10.0, with no significant differences between these treatments. Prepupal weight was highest on diets with pH from 6 to 10. Larval development time was fastest at pH 8.0, about 3 d shorter than that of those reared on diets with initial pH 6.0, 7.0 (control), and 10.0. Performance on low pH is low. pH can also increase or decrease, due to fermentation processes in the substrate during growth (Murta, 2021).

Research results on pH values are difficult to compare from the available sources, as also diets and feeding regimes differed between the various projects. In most trials it was observed though that larval activity increased pH values from the fourth day onward, with final pH values of around 8.9–9.4 in all the treatments (Meneguz, Gasco, & Tomberlin, 2018) and to 8.5 (Ma et al., 2018).

In general, the most commonly used composition for BSF feedstock used by (T. Spranghers, Schillewaert, et al., 2018) is chicken feed mixed with water (30/70), with 5.4% protein (21% on DM). In these trials, the larvae grew to their maximum weight in 18 days, and contained 41% protein and 36% fat on DM. The feed conversion ratio of kg dry feed/live insects was 1.2. Based on these findings, Inago (2020b) advised as the ideal composition per kg:

- 700 grams of water (70%)
- 50 grams protein (5%)
- 120 grams sugar or starch (12%)
- 5 grams fat (0.5%)
- 125 fiber-rich material (12.5%).

3.4.2 Yellow Mealworm

Rumbos, Karapanagiotidis, Mente, Psofakis, and Athanassiou (2020) evaluated forty-four different feed types of vegetable and animal origin for YMW. In this study, the highest amount of total larval biomass was produced in grain by-products, such as wheat bran, durum wheat flour, maize flour and white flour, as well as in the two compound feeds tested (egg-layer hen feed and milk-based feed). In contrast, low values were recorded for total larval weight produced, when several different legume flours were tested as substrates. In terms of individual larval weight, high values were recorded for the aforementioned feeding substrates, as well as for other substrates, such as rye and barley flakes, millet grains and lupine meal. Similar patterns were observed for the Efficiency of Food Conversion (ECI) values and the total dry feed consumed.

In another study by (C. Liu, Masri, Perez, Maya, & Zhao, 2020) the growth of YMW was monitored on a base diet of wheat bran, supplemented with carrots, oranges or red cabbage, which improved the growth rates by 49.3, 42.5 and 37.9% respectively. Final weight reached was equal with the different feeding regimes.

Effects of variety in diets does not only affect the larval growth, it also influences the fertility and reproduction of adult *Tenebrio molitor* (Van Huis & Tomberlin, 2017). Higher egg production was recorded on a diet high in protein as compared to low protein diets. However, studies on diet-reproduction effects are limited.

The composition of YMW can vary strongly: (Van Huis & Tomberlin, 2017) found the following variation:

- DM (28–39%),
- Crude protein (38–73% in DM),
- Total Fatty Acids (TFA) content (15–40% in DM).

Due to this variation, many researchers have proposed that diet can be used to influence the body composition of insects, in order to optimize their nutritional value to meet different nutritional demands for various uses (C. Liu et al., 2020; Rumbos et al., 2020; van Broekhoven, Oonincx, van Huis, & van Loon, 2015). According to (Ribeiro, Abelho, & Costa, 2018) optimal growth is achieved with diets containing 5–10% yeast, 80–85% carbohydrate, and the addition of B-complex vitamins.

3.5 Rearing methods, scaling, housing conditions and standardization

There are only few studies based on mass industry-scaled production: most information available depends on studies conducted in laboratories (Van Huis & Tomberlin, 2017). Various manuals to produce insects at a larger scale are available (Borghuis; Buhler, 2020; Caruso, Devic, Subamia, Talamond, & Baras, 2014; Inagro, 2020a, 2020b; Park, 2016), but these mainly describe the technical infrastructure and design of a production unit. The impact of, for instance, rearing substrates, insect population genetics, diseases pressure, etc. on an industrial scale level is not well published. The larger industrial companies conduct their own R&D which they do not share with the industry at large for competition reasons. The smaller producers do not have the possibility to conduct such research themselves, as most of them are usually small to medium scale startups.

The basic rearing conditions for YMW are described in relatively few sources, as compared to BSFL, which is probably a reflection of the fact that production methods for YMW are perceived to be simpler than for BSFL: adult *Hermetia illucens* are flies and need to be kept in special gauze cages for reproduction whilst the adult YMW are beetles, easier to manage and can be kept in the same crates as the larvae used for reproduction.

There is a strong correlation between climate conditions in the production cells and diets in their effect on growth of the larvae. Ribeiro et al. (2018) provided a wide overview of the optimal environment, physical and dietary conditions to make the production of YMW more competitive with other protein sources. The review of Ribeiro et al. (2018) identified that: (a) the optimum temperature for YMW growth is 25–28°C; (b) YMW growth rate is greatest at ≥70% relative humidity (RH) with an optimum range between 60–75% RH.

3.5.1 Black soldier fly larvae

Black soldier fly production consists of two sections; the reproduction unit where adult flies are kept in wire-netted cages and the larvae production unit, which are kept in crates. An image of an adult black soldier fly is provided in Figure 3.

Figure 3 *Black soldier fly (source; (Park, 2016).*

The range of ideal environmental conditions to keep BSF is quite narrow. The ideal temperature is 27°C, as was tested by Jeffery K. Tomberlin, Adler, and Myers (2009). Relative humidity (RH) should be around 60% (Park, 2016), though BSF are known to thrive in a wide range om RH from 30 to 90%, which is indicative of its ability to easily adapt to different circumstances.

Black soldier flies require direct sunlight to encourage mating. Thus, operations indoors require supplemented artificial lighting, with 85% of the mating activity happening in the morning. Adult flies do not feed, but do require frequent water misting (Caruso et al., 2014). Adequate ventilation is a necessity to ensure constant temperature (27°C) and humidity (60-70%).
After collection, the eggs are kept in pop-up nets for 5 days to develop into larvae, which are then transferred into crates, usually of 60x40x19 cm, where they are regularly fed on the selected feedstock, generally three times per week. After having grown to maturity, the larvae will stop eating and start to pupate. They are then harvested before pupation start, using various kinds of sieving systems, either small-scale by hand or automated for larger scale operation (Inagro, 2020b).

3.5.2 Yellow Mealworm

For starting YMW production, basic infrastructure and equipment are essential (Inagro, 2020a) in order to control abiotic conditions. It is essential to equip rearing rooms with temperature regulation, as the optimum rearing temperature is between 25-28°C as well as with moisturizing gear, to maintain RH at 65 to 75% (Ribeiro et al., 2018). Effective light regimes also need attention. Furthermore, adequate ventilation systems need to be installed to allow for a constant climate in the whole rearing room. For warmer periods or in warmer climates, cooling equipment is necessary. Proper control of the conditions is needed to synchronize growth and harvest time.

Open, stackable crates used to keep YMW, are usually 40 x 60 cm. Each crate produces ca. 13.000 worms, of ca. 0.15 grams each, a total yield of ca. 2 kg per crate. One production cycle (seeding of larvae to harvest of larvae) takes about 8 – 11 weeks. Feeding consist of filling the crates at the start of the growing period with 800 grams of mixture of wheat bran and additional ingredients. This will last for at least eight week and might have to be topped in the period after eight weeks. Additional moist feeding (carrots, cabbage leave or other vegetable material) should start after three weeks and be topped up three times a week. Harvesting (sieving) and processing equipment is needed (drying, chopping, pressing), the latter depending on the end product required.

In smaller production units, feeding is usually done manually, in larger units this is automated. For medium sized, family-based business, automation methods are being developed by various companies.

3.6 Insect potential for food and feed production

3.6.1 Potential for human food production

Insects are categorized by the EU as Novel Foods, which means that each product will have to get pre-market authorization before it can be commercialized. On May 3rd, 2021, the Standing Committee on Plants, Animals, Food and Feed (Novel Food and Toxicological Safety section), has given a favorable opinion on the draft legal act authorizing the placing on the market of dried YMW, as a novel food. This was followed up on June 1st by the European commission, allowing YMW products on the market, as a whole, dried insect in the form of snacks or as a food ingredient, in a number of food products (EU, 2021a). The second species, migratory locust (*Locusta migratoria* (L.) Orthoptera: Acrididae), was approved on 12 November 2021; and the third species house cricket (*Acheta domesticus* (L.) Orthoptera: Gryllidae) on 8 December 2021 (European Commission, ND).

3.6.2 Potential for animal feed production

Until recently, insect products were only allowed in pet food and fish feed. After the approval on September 7th, 2021 to use processed insect protein in poultry and pig feed (EU, 2021b), IPIFF (2020b) expects a rapid increase in the use of insect products in the animal feed industry. When additional, currently not yet allowed substrates will be authorized, it is expected that this will lead to a strong upscaling of the insects for feed industry and lead to lower cost prices.

IPIFF distinguished three scenarios (IPIFF, 2020a, 2020b). Scenario 1 is based on the approved use of a wider spectrum of authorized substrates before 2026. This will mean that more than 10% of the fish consumed in the EU will have been fed with insects, as will 1 in 40 eggs consumed, 1 in 50 chicken meat servings and 1 in 100 pig meat servings.

The second scenario is based on the intermediary step in approving former food stuff (food waste) as substrate for insects, which would lead to an increase in use of insects in pet food and fish feed.

The third scenario is based on the authorization of insect products for non-ruminants, without approval of new substrates. This will mean that more than 5% of the fish consumed in the EU will have been fed with insects, 1 in 80 eggs consumed and 1 in 100 chicken meat servings will be derived from insect-fed broilers.

3.7 Insect health

3.7.1 Insect diseases

Insect production in general can be affected by unwanted organisms which cause disease in insects or negatively influence the growth and production: viruses, bacteria, fungi, and parasites, competing organisms for feed, and predators (Eilenberg, 2015). Some of these hazards may also affect other animal groups or even humans (potential zoonotic bacteria for instance). Prevention and control of these hazards is important to ensure consistent production conditions.

Diseases of cultured edible insects are hardly studied so far. However, since this insect industry is growing rapidly and getting more attention; consequently more data is being generated (Maciel-Vergara, Jensen, Lecocq, & Eilenberg, 2021). The related diagnostics for diseases of insects is, in comparison to conventional livestock like poultry, swine and cattle, only starting: diagnostic protocols are available for only a handful of insect pathogens. Most challenging scenarios are related to the presence of covert infection, like protozoa and obligate bacterial pathogens. It is highly likely that, in the opinion of the authors, as the insect production sector expands and intensifies, outbreaks of certain diseases will occur, and more viruses will be discovered.

Insect species most studied in terms of insect disease are YMW (because they were most known as unwanted species in *e.g.* poultry farms), crickets (*Acheta domesticus, Gryllodes sigillatus*), and to a lesser extent the housefly (*Musca domesticus)*. Mainly the lack of knowledge on pathogens of *A. diaperinus*, *H. illucens*, and *Zophobias morio* – in particular the risk presented by viruses – needs to be addressed before routine diagnostics can be established. An overview of insect diseases described in literature for the most commonly reared species for food and feed purposes is given in Table 4 and Annex 2.

Table 4 *Number of known pathogens per insect species from this literature review (based on M. Boonstra, 2019, modified by O. Haenen, 2021, WBVR).*

Insect	bacteria	fungi	viruses	parasites
black soldier fly (*H. illucens*)	2	5	unknown	4
field cricket (*G. bimaculatus*)	3	4	6	4
housefly (*M. domestica*)	>14	>19	4	6
house cricket (*A. domesticus*)	6	3	5	6
lesser mealworm (*A. diaperinus*)	8	7	unknown	5
superworm (*Z. morio*)	2	1	1	3
yellow mealworm (*T. molitor*)	6	23	2	14

From this literature review, it was concluded that the most important threats to insect farming are viruses which cause an acute and often high mortality to the whole insect farm. For BSFL, no viruses are currently known; for housefly and YMW 4 viruses have been identified for both, and for crickets at least 8 viruses are known, some with up to 100% mortality. None of the investigated viruses are a risk for warm-blooded animals, including humans.

Various entomopathogenic bacteria have been identified. Some entomopathogenic bacteria are spore forming (genus *Bacillus*), others are non-spore forming (*Pseudomonas*, *Serratia* and *Rickettsiella*). Some are generalists, and others are specialists. In most cases, they infect their hosts via oral uptake (Maciel-Vergara et al., 2021).

The known number of pathogenic bacterial species per insect species is increasing. Most bacterial outbreaks are triggered by stress, like with *Serratia marcescens* and *Pseudomonas aeruginosa*. Some bacteria are very harmful to insects, and even sold as biological control, like *Bacillus thuringiensis*. There are a few bacteria potential zoonotic: *Proteus sp.*, *Paracolobacterum intermedium Borman*, *E. freundii* (Baak) Yale, *Pseud. aeruginosa* (Schroeter) *Migula*, *Serratia marcescens Bizio*, and *Klebsiella-Aerobacter* (De Las Casas 1971 in (Zabielska, 2008).

Fungi known so far to occur in insects are numerous for some species, like for the house fly and the YMW. Insect rearing requires relatively warm and very humid environments, where fungi can grow well both on substrate and the insects. *Metarhizium* and *Beauvaria bassiana* are serious pathogens, which also are used for biological control against insects. None of the fungi are primary zoonotic, although excess of fungi in feed and food will be unhealthy, regarding possible toxins formation, like *Aspergillis flavus* dominates and produces aflatoxins, harmful to humans (De Las Casas, 1971 in (Zabielska, 2008).

Parasites, in particular combined with other stressors, can have a mostly chronic, negative effect on animal health; and make the host more susceptible to other infections. Gregarines are only known to be parasitic to insects and are mostly non-lethal but can lower the insects' fitness. *Steinernema* is a genus with highly pathogenic parasite species, which, in permissive circumstances may cause high mortalities in insects. There are no recorded cases of zoonosis from insect parasites. However, insects may be intermediate hosts or vectors for poultry nematode infections, and for *Eimeria* spp., causing coccidiosis in poultry among others.

In contrast to fungi, bacteria and parasites, it is more difficult to prevent viral disease outbreaks by optimising culturing conditions and pre-processing of insect feed. Viruses can be latently present within a sub-population of insects and it will be difficult to determine and avoid conditions which would allow a virus to cause a big scale disease outbreak. In the opinion of the authors, it is unlikely that the insect sector will not encounter the same problems relating to (future notifiable) diseases as in intensive livestock keeping. It is therefore important that the current knowledge gaps are addressed. This will require investment of the insect production sector.

3.7.2 Insecticide residues

Pesticide residues may remain in or on feed materials after application in primary agriculture. Maximum residue levels (MRLs) have been set for pesticide / commodity combinations (Regulation (EC) No 396/2006), but these limits are set to protect the most vulnerable human consumers. Literature on the effects of pesticides on insects under industrial rearing conditions is scarce, which is especially problematic for compounds with insecticidal properties. Significant adverse effects on growth and survival by the insecticides spinosad and cypermethrin at concentrations in the diet equal to the MRL have been reported for BSFL (N. Meijer, de Rijk, van Loon, Zoet, & van der Fels-Klerx, 2021), and for the insecticides spinosad and imidacloprid in relation to LMW (N. Meijer, de Rijk, T., van Loon, J. J. A., Bosch, M. W., & Van der Fels-Klerx, H. J., In press). Additional studies on the effects of a variety of pesticides on BSFL have been published, including: cyromazine, pyriproxifen, λ-cyhalothrin, permethrin (J. K. Tomberlin, Sheppard, & Joyce, 2002), azoxystrobin, propiconazole (Lalander et al., 2016) and chlorpyrifos, chlorpyrifos-methyl, and pirimiphosmethyl (Purschke, Scheibelberger, Axmann, Adler, & Jager, 2017), but these studies found no significant effects on survival and growth. Although there is much literature available on the effects of pesticides on insects as pests – e.g. in the field or in storage – these results cannot easily be extrapolated to the conditions under which commercial insects are reared. The same is true for extrapolation to other species, because effects are believed to be species-specific.

3.8 Summary of insect rearing

Insect production has been growing strongly in Europe and the Netherlands over the past five to ten years. There are four industrial scale producers in the Netherlands, and around thirty medium- to small-scale producers. Research on insect production has also intensified over the past five years with focus on the suitability of the use of insect products as ingredients in food and feed.

The biggest challenge is in the development of insect diets capable of supporting and, ideally, maximizing insect growth and development of larvae and stimulating reproduction rates of adult insects. YMW and BSF both have wide, but also different diet preferences in terms of DM, energy, protein and minerals. The most important criteria to evaluate the efficiency of the diets are feed conversion efficiency, survival rate and developmental time of the larvae. In general it can be stated that knowledge on the nutrition of insects is still in its early stages (Barragan-Fonseca, Dicke, & Loon, 2017).

Rearing conditions differ between the two species of interest (BSF and YMW), especially at the reproduction stage: the adults of BSF are flies and need to be kept in gauge cage, whilst adult YMW beetles can be kept in the same crates as the growing larvae. Other important rearing conditions are climate control (temperature, humidity), vermin control and disease prevention. Options for automation of feeding, harvesting, and processing of larvae are being further developed.

Insect health may be impacted by disease or insecticide residues. Diseases may occur, like in other animal husbandry branches, by viruses, bacteria, fungi, and/or parasites, in mono- or multiple infections, causing mild to serious disease and losses. Relative to other animal husbandries, only few pathogens of edible insects are known so far. Therefore, it is expected that new pathogens and diseases will be discovered on the short-term. Most of the known insects pathogens are harmful to the insect only. No insect viruses are harmful to non-arthropods. There are a few bacteria potentially harmful to warm-blooded animals and humans. Fungi may, in excessive growth be harmful to insects, other animals, and indirectly even humans. Most parasites of edible insects are harmful for insect themselves, but some parasites may be vectors for animal disease to e.g. poultry. If standard hygiene measures are taken at the insect farm, and if the insect is appropriately processed before entering the feed-food chain, this risk is minimal. This is illustrated by the absence of zoonotic reported cases until today. However, spore forming bacteria, like *Clostridium perfringens*, are heat resistant, and need special attention to protect the animal production chain and man. Insecticide residues at concentrations equal to or below the legal limit have been found to affect insect growth and survival. More research is needed to determine which specific substances are of most concern.

3.9 Data gaps and research needs

The interest and demand for insect products has been growing strongly in recent years. Production rise has however not been able to keep up with the growth in demand. This has led to a situation whereby insufficient quantities of insect products are available. Insect products are also still more expensive than competing ingredients in the food and feed market. This shows the need for developing a more efficient production system, able to produce constant volumes at constant qualities for both food and feed industry. Research questions for insect development that still needs attention are diet composition/ration balancing together with the development of ration balancing tools, genetics, precision production methods to monitor reproduction and growth, disease prevention/control and use of frass.

Improving livestock production systems entails working on improvement of all elements of day-to-day management of livestock: feeding, housing, disease prevention and cure, genetics, and environmental issues etc. All these elements still need to be further developed to ensure a more efficient and sustainable insect production system:

- **Feeding**: the feed requirements of most types of insects are still not yet clear and efficiency of feed intake versus growth (feed conversion ration) is rather dynamic as insects have post-ingestive mechanisms to deal with imbalanced foods. This makes it extra complex to determine the most optimum feed ratio for insects. Developments such as software for ration balancing programs for insect feeding are therefore still in its very early stages.
- **Housing**: this means housing, regulating temperature and relative humidity as per the requirements of the insects. Larvae produce a lot of heat during the growing process. Moreover, substrates in combination with larvae emit various types of gasses, which also influence the climatic conditions in grower units. Balancing all this to a stable average temperature and RV is still challenging for most producers. Developing appropriate precision farming systems specifically for insect production can assist in making faster improvements. E.g. through using sensors monitoring larvae growth and the internal climate, production results will quickly become available to assist in improving production management.
- **disease prevention and cure**: in intensive insect rearing units this is unpredictable and has so far hardly been subject to research. Therefore, monitoring of diseases and developing prevention strategies and effective cures will become more important with the growth of the industry.
- **Genetics**: strategies and technologies for genetic improvement in insects still need to be developed. Existing livestock breeding strategies are based on the possibilities to assess the performance of individual animals, which cannot be applied to insects since they are kept in large numbers and performance evaluation cannot be done per individual animal.

- **Environmental issues**: the environmental effects of insect production under medium to large scale circumstances are not yet clear. It is not clear what types of gasses are emitted in commercial scale production units. Emission patterns will depend on various circumstances: type of substrate used, health of the insects, effectiveness of installation systems.
- **Working circumstances** insect producers and their staff are strongly affected by the need to properly regulate the internal climate in the production units. Moreover, in the case of YMW, air-borne dust particles can be a problem affecting workers' health.
- **The use of frass** for crop production also needs further investigation, as the soil fertilizing qualities of frass are not yet fully known.

3.10 Conclusions and research needs for current project

When rearing BSF and YMW on the different selected residual stream during the present project, nutritional needs should be taken into account. The ideal composition for BSF feedstock, according to Inagro, is: 70% water, 5% protein, 12% sugar or starch, 0.5% fat, 12.5% fiber-rich material (Inagro, 2020b). It is recommended to follow these guidelines for the experiments in this study. The ideal composition for YMW feed is less defined: it should have a dry matter content between 28–39%, crude protein (38–73% in DM), and total fatty acids content (15–40% in DM). According to (Ribeiro et al., 2018) optimal growth is achieved with diets containing 5–10% yeast, 80–85% carbohydrate, and the addition of B-complex vitamins. Water binding capacity, particle size, pH, frequency of feeding are important factors to take into account for substrate optimalisation for both species.

4 Use of residual streams as substrates

4.1 Composition of residual streams

In the current project, focus is on the following four potential substrate sources: 1) poultry manure, 2) Category 2 meat meal from animal rendering, 3) organic waste from household kitchens (GFE), and 4) supermarket mix. The latter material consists of food products that passed the expiration date or are unsaleable for other reasons. These residual streams will be delivered by the following partners: Avined for poultry manure, Darling Ingredients for meat meal (cat. 2) and collaboration partners of AMS Institute for GFE. Supermarket mix will be delivered by a party outside the project consortium.

4.1.1 Poultry manure

The most important types of poultry manure are laying hen manure and broiler manure. Sometimes manure is mixed with litter which is called litter manure. A photograph of poultry (laying hens) manure is shown in Figure 4. The wet manure is a mixture of urine and feces, contains 40% dry matter and is like a paste-like consistency. The white spots are the urine (communication with Avined). It should be mixed with more porous fibrous material to make it fit for insect rearing. The composition of poultry waste can be found in various papers and additional information was given by Avined and by Energiecentrale BMC Moerdijk (incineration plant) via Avined. These results are shown in Table 5. The presence of sufficient levels proteins, carbohydrates and minerals in principle make this manure suitable for insect rearing. Poultry manure contains more nutrients than dairy manure and it has a pH of 8 which is ideal for BSFL cultivation (Zhang et al., 2021).

Table 5 *Overview of composition of poultry (chicken) manure from differences literature sources. References are provided in the legend under the table.*

Parameter	**Reference**											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**12**	**12**
Country				China	China	USA	China		China			
% of wet mass												
Dry matter	40	57		23	25.18	23	25.2	52.3	23	66.3		
N		2.4										
P2O5		1.4										
K2O		2.0										
CaO		2.4									7.2	
MgO		0.7										
Na2O		0.3										
SO3		0.9										
% of dry matter												
Organic matter		86		67.5	66.9							
Ash		14					22.82	18.4			22.73	
Total protein										10.5	46.87	
Total-N					3.78		3.54	3.28	3.45			
NH4-N								0.55				
Fat										6	2.64	
Cellulose					7.51		14.49		14.19			12
Hemicellulose									21.66			24
Lignin					2.80							2
TOC							40.19					
Total-P							2.28		2.28		3.42	

Parameter	Reference											
	1	2	3	4	5	6	7	8	9	10	12	12
mg/kg DM												
B		52										
Co		< 5	0.83									
Cu		103	110									
Fe		1018										
Mn		506										
Mo		4.6	3.58									
Zn		460	405									
As		<3	0.27									
Cd		0.18	0.13									
Cr		14	6.03									
Hg		<0.05										
Ni		10.0	5.98									
Pb		<5.0	1.12									
Ba			22.4									
Sb			0.05									
Se			0.73									
U			0.30									
V			1.48									
pH				7.81	7.24			7.4	7.07			

1: Avined interview
2: (BMC, 2020)
3: (Klein & Roskam, 2018)
4: (Wang et al., 2021)
5: (Elsayed et al., 2020)
6: (Miranda, Cammack, & Tomberlin, 2020)
7: (Mazza et al., 2020)
8: (van Emous, Winkel, & Aarnink, 2019)
9: (Rehman et al., 2017)
10: (Riudavets et al., 2020)
11: Phyllis.nl - Sample 957
12: Phyllis.nl - Sample 2380

Figure 4 *Poultry (laying hens) manure.*

4.1.2 Category 2 meat meal from animal rendering

Slaughterhouse waste is an animal by-product and animal by-products are classified into 3 categories. This classification is laid down in the European Animal By-Products Regulation (Regulation (EC) No. 1069/2009) and is based on the risk to public and animal health. Category 1 material has proportionally the highest risk, category 3 material the lowest. There are regulations for each category about how the material must be destroyed or can be processed or used. An overview of the materials falling within each category, as laid down in 8-10 of Regulation (EC) No 1069/2009, is provided in Annex 3 of this report.

Category 1 material is considered to present too high of a risk to be used as animal feed. Category 2 material includes dead animals: these may be used as fertilizer or as feed for animals that are not in the food chain (mink), not for pets, but for pack animals (sled/hunting dogs). Darling Ingredients is the only company in the Netherlands that processes category 1 material. Currently Cat 1 and Cat 2 materials are processed together, but in the near future, category 1 material will be processed separately from category 2 material. In category 2 material, just like category 1 material, the animal species are not separated. The mixture contains pig and chicken. Category 2 material is processed by using method 1 from Regulation 142/2011: which entails heating of sufficiently shredded material for 20 minutes at 133°C and 3 bar. The sterility of the material is validated by measuring *Clostridium perfringens*. Subsequently, category 2 material is dewatered and separated. Three streams are then created: water (70%), meat meal (20%) and fat (10%). The meat meal is the product proposed as a raw material for insect breeding. It will contain processed animal protein (PAP) from various animal species including pig and chicken. The material is less odorous than fish meal. It is a dry powder (meal) with particles of a millimeter. The composition of meat meal as described by different sources is provided in Table 6.

Table 6 *Composition of category 2 meat meal.*

	Darling Ingredients Interview	**Gobbi, Martinez-Sanchez, and Rojo (2013)**	**Feedipedia (2021)**
Dry matter (% of wet matter)	95		95.8
Protein (% of wet matter)	63	50	
Fat (% of wet matter)	7-15	18	
Ash (% of wet matter)	16	17.9	
% of dry matter			
Crude protein			54.9
Ether extract			11.4
Ash			30.5
Ca			10.11
P			4.87

4.1.3 Organic waste from household kitchens (GFE)

Currently, household kitchen organic waste ends up in the fraction source separated organics (Dutch: GFT) which is digested (biogas) or composted. GFT also contains organic biomass from gardens and other non-edible sources, whereas GFE ideally only contains the organic leftovers from human food consumption. Much of it still ends up in the mixed municipal waste fraction, which is incinerated. In the Netherlands, various initiatives are running to separately collect household kitchen waste (GFE). Collection can be in bins or via kitchen sink grinders. In the latter the material will be highly diluted with water. The composition of household kitchen waste is known from (Bisschops, Melita, Nanninga, & Weijma, 2020) and Ansems et al. (Ansems, van Groenestijn, & van Horssen, 2018). Table 7 gives composition data of household kitchen waste and related sources; in Table 8 compositions of organic residues related to (but different from) the four selected substrates can be found. Photographs of unprocessed and processed GFE are shown in Figure 5 and Figure 6, respectively.

Table 7 *Composition of kitchen organic waste and vegetable residues.*

	(Bisschops et al., 2020)	**(Ansems et al., 2018)**	**(Hui Wang et al., 2017)**	**(Zheng et al., 2013)**
	Kitchen organic waste	Kitchen organic waste Deventer	Waste carrots	Decayed vegetables (market waste, China)
Dry matter (% of wet matter)	24.0	20		
% of dry matter				
Organic matter	91	98.3		
Total Kjeldahl N	3.1			
Total N	3.1			
P	0.4	0.46		
K	1.3			
Ca		2.1		
Mg		0.16		
Total carbohydrates		36		
Organic acids		22		
Starch and sugars	44.0			
Soluble sugars				8.7
Fat	13.8			0.7
Protein	19.3	17		1.2
Cellulose	5.5		4.32	32.5
Hemicellulose	6.4		4.29	21.8
Lignin	2.8		10.26	8.6
pH		5.2		

Table 8 *Composition of organic waste (and chicken feed as reference) according to (Moritz Gold et al., 2020).*

	Canteen waste	**Vegetable canteen waste**	**Cow manure**	**Poultry slaughterhouse waste**	**Chicken feed**
Dry matter (% of wet matter)	26	17.3	13	33.3	40
Organic matter (% of DM)	93.0	92.4	80.7	94.0	98.2
Protein (% of DM)	32.2	12.1	11.1	37.3	19.1
Lipids (% of DM)	34.9	28.9	4.4	42.9	4.8
Non fibre carbohydrates (% of DM)					
• Total	7.5	15.5	1.8	0.3	28.5
• Glucose	3.5	3.7	0.7	0.2	0.5
• Starch	4.0	11.6	1.0	0.1	27.5
Fibre (% of DM)					
• Total	36.2	31.5	58.4	20.8	22.0
• Cellulose + lignin	22.8	24.0	40.9	9.3	8.6
• Hemicellulose	13.4	7.5	17.4	11.5	13.5
pH	4.3	3.8	7.2	5.7	5.7

Figure 5 *Unprocessed GFE.*

Figure 6 *Shredded and homogenized GFE.*

4.1.4 Supermarket mix

Supermarket mix consists of products that were left unsold in supermarkets. It falls under the definition of the legal term 'former foodstuffs': "*foodstuffs, other than catering reflux, which were manufactured for human consumption in full compliance with the EU food law but which are no longer intended for human consumption for practical or logistical reasons or due to problems of manufacturing or packaging defects or other defects and which do not present any health risks when used as feed*" (Regulation (EC) No 68/2013). If these products are, or contain, products of animal origin, then the legislation on animal by-products also applies (Regulation (EC) No 1069/2009). The products may have been mechanically unpacked (Regulation

(EU) 2017/1017). In principle, the presence in feed of packaging from the use of products from the agri-food industry, and parts thereof, is completely prohibited (Regulation (EC) No 767/2009, Annex III). However, complete removal of packaging materials by mechanical means is not cost-efficient. It has been hypothesized that insects could be reared on former foodstuffs containing packaging materials, since the risks for conventional livestock animals (e.g. choking or plastic particles) is absent. Van der Fels-Klerx et al. (2020) found several contaminants such as dioxins and heavy metals to bioaccumulate in insects reared on supermarket waste containing packaging materials but concluded that concentrations of tested contaminants to be below the applicable legal limits.

The composition of supermarket mix as analysed by Groenestijn (van Groenestijn, 2015) and by Attero (2021) is shown in Table 9. With some effort, the nutritional value can be estimated from these data. The presence of meat, fat, fibres and carbohydrates makes this mix potentially suitable for insect rearing. The material is a thick viscous slurry (like pea soup), containing about 18-19% dry matter (Figure 7). The material is prone to spoilage and shortly after production, a biological acidification is needed to be applied and/or the product need to be stored in a refrigerator or freezer. Between production and processing of the supermarket mix, CO_2 gas is produced as well. Tankers (trucks) loaded with the mix often cannot even stay overnight, because they flood as foam is formed because the CO2 cannot escape from the liquid.

Table 9 *Composition of supermarket mix.*

Parameter	**Reference**	
	Attero 2021	**van Groenestijn (2015)**
% of wet matter		
Dry matter	18.8	18
% of dry matter		
Organic matter	93.03	88
Cl	1.6	
N	3.185	
P2O5	1.0	
S	0.33	
Lactic acid		11.5
Acetic acid		2.5
Propionic acid		1.4
Butyric acid		1.1
Valeric acid		0.1
Rhamnose after chemical hydrolysis		0
Galactose after chemical hydrolysis		1.0
Arabinose after chemical hydrolysis		0.7
Glucose after chemical hydrolysis		23
Xylose after chemical hydrolysis		0.6
Mannose after chemical hydrolysis		0
Fructose after chemical hydrolysis		0
mg per kg dry matter		
As	<5	
Cd	<0.4	
Cr	<5	
Cu	8	
Ni	<0.5	
Pb	<10	
Zn	46	
Hg	<0.10	
Ca	8.4	5.58
K	11	
Mg	1.2	1.16
Electric conductivity (mS/cm)	8.81	
Chemical oxygen Demand (mg/L)	269	
pH	4.49	4.1

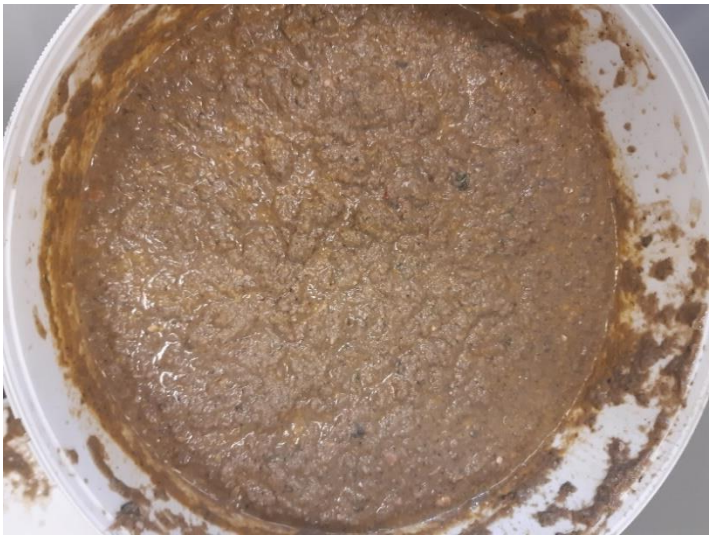

Figure 7 *Supermarket mix.*

4.2 Use of residual flows as substrate and approximate value calculation

BSFL and YMW can be grown on residual flows from agriculture and the food and feed processing and consumption chain. About half of the studied residues are legally not permitted in the Netherlands for insect rearing for feed and food application. Many papers only describe scientific studies. These studies generally describe the efficiency of rearing of insects on these residual streams and the relative potential of these substrates for specific insect species in comparison to a standard (optimal) substrate. Sometimes the larvae are used to extract chemical substances and sometimes it concerns a kind of composting process. In these composting studies, volume reduction of the waste is the primary goal. In such cases, the frass is the actual product of interest (compare this composting process with vermiculture: composting with the aid of worms).

An overview of the estimated value calculation for each of the four considered residual streams is provided in Table 10. This very rough estimation shows positive added values for each of the residual streams, while staying below or in range of the current prices for insect substrate. It will be interesting to investigate more accurately what the added value of the use as insect substrate will be, for each of the selected residual streams.

Table 10 *Estimated value calculation.*

Residual stream	Volume in NL (M ton/year)	Current estimated positive or negative value per ton (€)	Current total positive or negative value (M€)[1]	Postulated possible value as insect substrate per ton (€)	Estimated possible value of available volume (per year)[4]	Possible added value per residual stream (M€)[5]
Poultry manure	1.4	-€10	-14 M€	€50[2]	70 M€	84 M€
Meat meal (cat 2.)	0.2	€300	60 M€	€350[3]	70 M€	10 M€
Supermarket mix	0.016	€10	0.16 M€	€50[2]	0.8 M€	0.64 M€
GFE	1.4	-€70	-98 M€	€50[2]	70 M€	168 M€

[1]Volume x current value (M€); [2]assumed feasible price per ton, referenced to estimated current price of insect substrate of €300-€400/ton (i.e. poultry feed); [3]for meat meal (cat. 2): assumed added value of €50 per ton; [4]Volume in NL x postulated value (M€); [5]Estimated possible value minus current value.

4.2.1 Poultry manure

Currently broiler manure is incinerated by Energiecentrale BMC Moerdijk for energy production, since this manure is relatively dry (57% DM). The value of the material is about 7-10 euro/ton manure negative. Manure with a lower dry matter content (from laying hens) currently is dried (>70% DM) and exported for use as fertilizer. The wet manure has a value of 14 euros negative per ton and the dried manure 8-10 euros positive per ton. A small part goes to mushroom compost production (a few kton/year). Annually 1.4 million ton of poultry manure is produced in the Netherlands (communication with Avined). The sector is seeking applications with a higher value or circularity. Using poultry manure as a substrate for insect rearing is an option. Another motivation to go for a new application is the expectation that export of poultry manure will decline in future. For Avined, the implementation horizon for the insect rearing option is 5 to 10 years.

4.2.2 Category 2 meat meal from animal rendering

Currently meat meal is used as fuel in coal power plants (but this will stop soon), in the cement industry and as fertilizer. The value is 300 euro/ton positive. The sector is seeking higher values and follows the Lansink scale, but everything within the restrictions of the law. According to the law, category 2 material is not waste, which creates various opportunities such as the use of the material as substrates for insect rearing. Some experience with insect rearing on meat meal already has been gained by the Dutch sector (communication Darling Ingredients). Detailed information is not available since these experiences are part of confidential business-to-business work. In addition, Darling Ingredients USA has experience with BSF. In 2009, ca. 61 kton/year food grade meat/bone meal and ca. 47 kton/year Cat2+Cat1 meat meal was produced from the Dutch slaughterhouses. At that time, Dutch alive slaughter weights for different animal species were as follows: pigs: 1600 kton/yr; cows: 300 kton/yr; calves: 350 kton/yr; poultry 800 kton/yr; lamb: 30 kton/yr (Luske, 2009). A more recent rough estimation derived from the CBS slaughter weight data is that 200 kton of meat meal may be produced per year in the Netherlands.

4.2.3 Organic waste from household kitchens (GFE)

Currently, household kitchen waste is part of source separated organic waste or mixed household waste. GFE should contain only vegetables, fruit and food waste and not other organic material from gardens or other sources. A few pilot projects are running to collect GFE separately, e.g. by Circulus Berkel in Deventer or Meerlanden in the Schiphol area. Amsterdam is considering to collect this waste via kitchen grinders in high rise buildings, but the challenge remains to keep the dilution with water to a minimum, in order to keep the GFE fit for use as an insect substrate. Household kitchen waste has a negative value of 60 euro per ton. The plan, at least for Amsterdam, is that the material first will be converted into biogas. In the coming years the logistics can be organized and in a next stage the GFE can be used to produce products with a higher value. Most household kitchen waste already is converted into biogas as part of source separated organics (GFT), but now it will be collected separately from garden waste in the households and subsequently offered as GFE to the biodigester. For a potential use as insect substrate collection of solid GFE via bins would be preferred. The estimated volume of GFE in the Netherlands is 1.4 Mtons/year.

4.2.4 Supermarket mix

Dutch suppliers have different opinions on the feasibility of using supermarket mix for insect rearing. Some think that legal objections will persist because the mix contains meat, and it is believed that feeding animal proteins to animals has a risk of proliferation of spongiform encephalopathies. It is uncertain if insect species reared for food and feed can act as vectors for agents of such diseases, but some urge caution. The value of supermarket mix is 0-10 euro/ton (positive) (communication Attero) and the amount produced in the Netherlands ca. 16.000 tons/year (Schripsema, van der Burgh, van der Sluis, & Bos-Brouwers, 2015).

4.3 Examples of residual flows (permitted and prohibited)

Several substrates for BSFL are reported in literature. Growth was observed in the type of substrates given below, sometimes as part of a mixture and sometimes as a sole substrate. The growth observed was acceptable.

- Manure: Horse manure, cow manure, sheep/goat manure, chicken manure, duck manure and the solid phase of pig manure.
- Legally allowed, clean, high value substrates: Wheat bran, brewery spent grain, pineapple grain, maize bran, soybean bran, palm seed meal and poultry feed.
- Meat meal derived from slaughterhouse waste.
- Household and restaurant waste and related substrates: kitchen waste, canteen waste, restaurant waste, domestic or municipal organic wastes, agro-industry by-products, food processing waste, deteriorated fruits and vegetables, coffee grounds, chicken meat, eggs and banana peels.

Examples can be found in several papers (Alyokhin, Buzza, & Beaulieu, 2019; Gobbi et al., 2013; Moritz Gold et al., 2020; Isibika, Vinnerås, Kibazohi, Zurbrügg, & Lalander, 2019; Li et al., 2020; Cuncheng Liu, Wang, & Yao, 2019; Logan, Latty, & Roberts, 2021; Pamintuan, Agustin, & Deocareza, 2020; Raksasat et al., 2020; Zheng et al., 2013; Zurbrügg, Dortmans, Fadhila, & Diener, 2018).

Substrates for YMW reported in literature can be found below. In all cases growth, optimal or suboptimal was observed, sometimes after mixing the substrates with other substrates.

- Poultry litter (a mixture of manure, feathers and feed residue), hatchery waste, broiler's eggshells, mixtures of cattle manure and cereal straw, and horse manure and cereal straw and dry egg whites;
- Bread remains, cookie remains;
- Vegetable waste, green garden waste, apricots, decayed vegetables (market waste), potato steam peeling, potato, carrot, lettuce, cassava plant, olive pomace, banana peels, carrot waste, watermelon rinds and peanut oil;
- Spent grains and beer yeast, wheat bran, soy flour, rice bran, maize stover, wheat middlings, rapeseed meal and maize DDGS.

Examples can be found in several papers (Harsányi et al., 2020; Mattioli et al., 2021; Riudavets et al., 2020; Rumbos, Bliamplias, Gourgouta, Michail, & Athanassiou, 2021; Shu, Kok, & Jiun, 2018; Silva et al., 2021; Truzzi et al., 2019; van Broekhoven et al., 2015; Varelas, 2019; Hui Wang et al., 2017; Zheng et al., 2013).

Spent mushroom substrate can be used for YMW cultivation as well (Li et al., 2020). In this particular study, this substrate was based on saw dust and wheat bran. Therefore, it contained residues of saw dust, wheat bran and mycelium. Mixtures of spent mushroom substrate and wheat bran were more favourable for larvae development than spent mushroom substrate alone.

Some mealworms species have been found capable of digesting plastics. Cardboard polystyrene, oxo-degradable polyethylene, polyethylene regranulate and oatmeal waste have been used for mealworm cultivation (Przemieniecki, Kosewska, Ciesielski, & Kosewska, 2020). As such, it can be hypothesized that these mealworms could be reared on former foodstuffs containing packaging materials, as discussed in section 4.1.2.

4.4 Inventory of possible steps in substrate optimization

Pre-treatment technologies frequently mentioned for preparing substrates for BSFL and YMW are size reduction, mixing with other substrates and adjustment of the dry matter concentration to a desired value. The right dry matter concentration can be reached by drying, adding water (provided that sufficient water-binding capacity is present in the substrate) or mixing it with other substrates. In addition, mixing with other substrates can be used to reach a balanced diet or to increase porosity (decrease bulk density).

4.4.1 Pre-treatment to prepare substrates for BSFL

4.4.1.1 Poultry manure

With respect to chicken manure: drying is currently normal practice to reduce transport costs of the manure and to make storage possible. Storage of wet poultry manure gives rise to ammonia emission (personal communication with Avined).

Storage at -20°C in plastic bags are described in many papers on laboratory studies. For example, poultry manure (hens) from USA, less than 12 h old was stored at -20°C in bags (Miranda et al., 2020).

Not all studies involved a pre-treatment. Wang et al. (2021) took Chinese chicken manure, didn't carry out any pre-treatment and just mixed it with the larvae.

D. G. A. B. Oonincx et al. (2015) dried chicken, pig and cow manure in an oven at 60°C and subsequently ground the material in a cross-beater mill. Then it was mixed with water to 66% water content. In some parts of the world low-cost methods for drying are used. Chicken manure from Cameroon was dried in the open air and adjusted to 60% moisture by adding water (Dzepe et al., 2021).

Logan et al. (2021) mixed equal weights of chicken breast meat, chicken egg and chicken manure in a blender. They got higher yields of BSFL than on 100% manure. According to Xiao, Geng, Yang, Wang, and Xu (2020) chicken manure can be mixed with pig manure or rice bran or used pure. The authors did not use any pretreatment. Pure chicken manure could be used for BSFL cultivation, but best results were reached with mixtures of 15% rice straw and 85% chicken manure. Rice bran reduced the viscosity and increased ventilation.

4.4.1.2 Other types of manure

Alyokhin et al. (2019) used horse manure or food waste (cafeteria). These feedstocks were mixed 50%/50% with softwood shavings and sawdust to increase porosity and prevent accumulation of standing free water in the growing bins and water was added to reach 60% moisture. The many substrates used by Ganda, Zannou-Boukari, Kenis, Chrysostome, and Mensah (2019) were all adjusted to 20-30% dry matter content.

According to Julita et al. (2018) horse and sheep manure are suitable to grow BSFL. Mixtures of 50%/50% with vegetable waste are even better for larvae development.

Mixtures of quail manure and wheat bran were used by El-Dakar, Ramzy, and Ji (2021). Mixing can improve the growth rate and yield of BSFL. Up to 60% manure the same high growth rate larvae was obtained, but above 60% a slower rate was observed. The used pre-treatment was a removal of feathers. Moritz Gold et al. (2020) made mixtures of all kind of wastes and discovered that the protein/NFC ratio should be 1/1 for an optimal BSFL development. NFC is non-fibre carbohydrates (glucose, starch).

Raksasat et al. (2020) tried to mix various substrates: dairy manure, soybean curd residue, waste coconut endosperm, wheat bran. The ideal mixture was restaurant waste (431 DM parts) and fruit and vegetable residue (399 DM parts): in the first the required protein is present (meat).

There are many examples of mixing manure with fibrous organic matter. Another one is the work of Pamintuan et al. (2020) in the Philippines. They took duck manure, sun-dried it to remove moisture, then mixed it with fermented rice straw (1 inch, soaked in water, one week) which was dried.

4.4.1.3 Category 2 meat meal from animal rendering

With respect to category 2 meat meal: the dry product can be stored for at least half a year and probably 3 years (communication with Darling Ingredients). In the summer, slightly more free fatty acids can be found in the product due to the breakdown of fats, but fat oxidation can be counteracted with antioxidants. If a more moist product is made, it can be advised to acidify it to pH 4 to inhibit microbial degradation (Darling Ingredients interview). According to experts from Darling Ingredients one should be careful with freezing: this damages the fat cells, resulting in faster oxidation. It concerns the oxidation of polyunsaturated fatty acids and it results in rancidity.

Meat meal can be used for BSF cultivation. Gobbi et al. (2013) mixed 500 g meat meal with 800 mL water. However, they got a poor performance. It has to be mixed with hen feed. The mixture contained 250 g hen feed, 250 g meat meal and 800 mL water. The meal contained 50% protein, 18% fat and 17.9% ash.

4.4.1.4 Supermarket mix

van der Fels-Klerx, Meijer, Nijkamp, Schmitt, and van Loon (2020) studied cultivation of BSF on supermarket mix (former foodstuffs) and found that BSF larvae can be reared on former food products containing 3-6% of plastic fragments or carton packaging materials without negative effects on growth or survival. A lot of experiences using kitchen waste may also be applicable to supermarket mix. Removal of packing material may be an additional necessity before this type of material can be used to rear BSFL on.

4.4.1.5 Organic waste from household kitchens (GFE)

BSFL avoid to migrate through a fat/grease layer and in some substrates, there is too much fat. Raksasat et al. (2020) skimmed the excess fat/grease/oil top layer of batches of restaurant waste, otherwise the larvae had to dig a lot. Zheng, Li, Zhang, and Yu (2012) also recommend to first remove grease from restaurant waste. This 3% w/w fraction can be used to produce biodiesel; the solid residue may be used for BSF cultivation.

Kitchen waste moisture content was 60% (Cuncheng Liu et al., 2019). At this level, the larvae exhibited the highest survival rate and the highest conversion rate in converting kitchen residue.

Most studies have been done on laboratory scale, but the paper of Yang and Tomberlin (2020) describes a full-scale plant in China in which organic waste from restaurants is used to grow BSFL. The waste is collected within 24 h, an automatic sorter is used to remove nonfood waste items (plastic, cans). Then the material is ground into a slurry with particle size under 5 mm. Subsequently it is cooked for 4 hours at 80°C and centrifuged to yield three fractions: lipids, liquids and solids. Only the solid part is used for BSFL cultivation. But first these solids were put in 1000 L containers and inoculated with 30 L *Lactobacillus* culture. In the subsequent 24 h fermentation the pH drops from 6 to 4. This material was mixed with wheat bran to reduce moisture content to 70%.

Vegetable refuse can be shredded to reach particles 0.5-1 cm (Parra Paz, Carrejo, & Gómez Rodríguez, 2015). Moritz Gold et al. (2020) used a kitchen blender to homogenize various types of wastes. The material was stored frozen at -20°C in plastic bags. Zurbrügg et al. (2018) used municipal waste. Hazardous inorganic substances were removed, and the particle size was reduced to 1-2 cm (ideal for BSF). The material was mixed with other materials to end up with 70-80% moisture or it was dewatered.

4.4.1.6 Substrate improvement by fermentation

Several studies are dedicated to introduction of micro-organisms to the substrates. These micro-organisms are sometimes derived from the gut of the BSFL and can help to degrade fibres inside or outside the gut. For example, introduction of bacilli in the substrate helped to enzymatically hydrolyse fibres in the gut of the larvae (Zhang et al., 2021). A similar study is from Raksasat et al. (2020). Chicken manure was fermented in situ with *Bacillus subtilis* (simultaneously with larvae cultivation). *B. subtilis* originated from the BSFL gut and it digested protein and organic phosphorus in chicken manure. The result was a higher yield and faster development of the larvae. According to the authors *B. subtilis* additionally can protect BSFL from pathogens. Another example is the inoculation of *Lactobacillus buchneri* in soybean curd residue (Raksasat et al., 2020). Mazza et al. (2020) grew micro-organisms from the gut of larvae on a medium. This mixture of bacteria was added to Chinese chicken manure. BSFL growth (rate and yield) was enhanced by the bacteria addition. The authors stated that the mechanism of this stimulation is unclear. According to Rehman et al. (2019) addition of certain bacteria to poultry waste improves the BSFL gut microbiome. The result is a better conversion of lignocellulose and as a consequence a higher yield and faster growth of BSFL.

More conventional (not derived from gut) pre-fermentation of substrates can improve digestion as well, as has been proven by van Huis (2020) using maize straw. An extreme example can be found in the study by Elsayed et al. (2020). Chicken manure was first anaerobically digested (biogas, 37°C, 30 days) and then centrifuged. The solid fraction (30% DM) was used for BSFL cultivation.

Isibika et al. (2019) compared three different pre-treatment methods for banana peels. In all cases the banana peels were homogenized and stored at -10°C. The three pre-treatments tested were:

- Microbial pre-treatments using *Trichoderma, Rhizopus* and bacteria in a 7-21 days solid state fermentation at 28°C;
- Ammonia pre-treatment adding 0.8% NH3-N and 7-14 days incubation to balance C/N ratio;
- Heat treatment at 120°C and 2 bar during one hour; the hypothesis was that this treatment degrades macromolecules and enhances availability of micro-nutrients.

Microbial and ammonia treatment resulted in larger larvae. The heat treatment yielded a lower larvae weight and is therefore unfavorable.

4.4.2 Pre-treatment to prepare substrates for YMW

4.4.2.1 Poultry manure

Silva et al. (2021) used poultry litter, which is a mixture of manure, feathers and feed residue, for YMW cultivation. According to the authors this mixture is high in nitrogen, fibers, energy and minerals. The litter was autoclaved at 104°C at 1.2 atm for 60 minutes, then dried in oven at 65°C, ground with a knife mill and stored at -20°C.

4.4.2.2 Meat meal (cat. 2), supermarket mix, organic waste from household kitchen waste

No public information sources on the use of meat meal, supermarket mix and GFE is known, but information on the use of other (related) organic residues can be found in various papers.

Cookies, bread and brewery spent grains were dried in an oven at 90°C and finely ground (Mattioli et al., 2021). Watermelon rinds, broiler's egg shells, banana peels were oven dried at 70°C for 24 hours (Shu et al., 2018). Vegetable garden waste was chopped to 2-4 cm pieces. A mixture was made of 90% vegetable and garden waste plus 10% chicken feed (Harsányi et al., 2020).

Spent mushroom substrate can be used for YMW cultivation. This substrate was based on saw dust and wheat bran. The pre-treatments started with natural air drying followed by crushing (pulverization) into powder. Then the powder was sieved and the <1.25 mm fraction was used for YMW cultivation (Li et al., 2020).

Hui Wang et al. (2017) used corn stover dried at 60°C for 6 days, chopped, ground and sieved (< 3 mm) and carrot waste (92.5% water content, sliced). They made mixtures: every month corn stover was added to the mealworm culture and every two days carrots were added. This way biodiesel could be produced from corn stover, via the larvae lipids.

In a review Varelas (2019) mentions the use of wheat bran, soy flour, spent grain, spent brewer's yeast, bread remains, potato steam peeling, potato, dry egg whites, rice bran, carrot, lettuce, cassava plant, peanut oil. The liquids and slurries were dried and mixed with other substrates. The semi-liquids were used to make pellets or extruded forms. The solids were ground, extruded and pelletized. The solids could optionally be encapsulated with complex coacervation technology using proteins and polysaccharides.

Again mixing can solve many problems. Riudavets et al. (2020) tested substrates for mealworms: apricots, brewer's spent yeast, hatchery waste. They had no success with sole apricots and sole BSG, but by mixing it with other substrates suitable mealworm substrates were obtained.

Spent grains and beer yeast, bread remains, cookie remains, potato steam peelings, maize DDGS were tested by van Broekhoven et al. (2015). These feedstocks were lyophilized, ground and mixed. Sole cookie remains resulted in a mortality of larvae because of a too low protein content and maybe the cinnamon and clove vapours were toxic. The solution proven was to mix these feedstocks with protein rich substrates. Three other interesting observations were made in this study: (1) potato starch is more resistant to digestion than starch from wheat and maize, (2) potato glyco alkaloids can have a toxic effect and these persist after processing (3) selective carrot consumption by mealworms.

In all papers growth of YMW was acceptable, sometimes because of the right mixing of substrates to create a nutritionally balanced diet.

4.5 Summary the use of residual flows as substrates

For BSFL, it can be concluded that for the substrate the particle size should not be larger than 1 cm. Moreover, the dry matter content should be adjusted to 20-40% and the substrate should be porous. Methods to carry out these adjustments are known. Substrates should have a certain balance between protein and carbohydrates and mixing substrates is common practice. Improvements can be attained by fermentation of the substrate and removing excess grease.

For YMW, particle size of the substrate can be much smaller than 1 cm: YMW is used to flour. The dry matter content of the substrate should be adjusted to 85%, which is often reached by mixing dry carbohydrate-rich substrates with vegetables. Also, the YMW should get a balanced diet, which mostly mean that protein sources and carbohydrate sources are mixed (see section 3.4.2 for detailed information).

The four selected residual streams (poultry manure, meat meal (cat. 2), household kitchen waste and supermarket mix) are comparable to residual streams which have been described to be appropriate as substrate for insect rearing.

4.6 Data gaps and research needs

Variations of composition depending on the season, location and type of supplier are not known. Knowledge of the extent of these variations can be used to make substrate pre-treatment processes robust and flexible. Although slurries are mostly dried or mixed with dry fibrous materials, there is no expression of the desired porosity. A combination of the particle size and bulk density may give the right information on this porosity, but standards, recommendations or rule of thumbs are absent in literature.

Research is needed to develop scalable substrate preservation methods. Freezing is only realistic on lab scale. On full scale drying, acidification, adding preservatives, pasteurization and sterilization may be a few options. We also should determine what will happen with the substrate when not preserved and how fast undesired properties will develop (protein and carbohydrate degradation, acidity, ammonia, sulfides, micro-organisms). This will give indications of the shelf life.

4.7 Conclusions and research needs for current project

In the preparation of substrates for BSFL, the following best practices were identified:
Size is reduced to particles near 1 cm and visible contaminations (with sufficient size such as pieces of plastic) are removed. Dry matter content is adjusted to a value between 20% and 40%. This can be reached by drying (not too hot, e.g. in an oven at 60°C or in open air), by adding water or adding another substrate, taking care to retain sufficient water-binding capacity in the substrate. The porosity can be increased and the water-binding capacity can be regulated by adding fibrous material such softwood shavings, saw dust, wheat bran and rice bran. Fermentation can be carried out to acidify the substrate (preservation) or make it better digestible. Grease is removed in particular when accumulated as floating layers on top of a batch in a bucket. Forced grease removal (using a centrifuge) is an option. Substrates are mixed to make a balanced diet, e.g. protein/NFC of 1/1, or meat plus vegetables or manure plus vegetables.

Storage at laboratory scale is mostly done at -20°C in plastic bags (except for meat meal (cat 2.), for which freezing is not recommended), while large scale storage of residual stream before processing can be reached by drying or acidification (< pH 4).

For YMW less information was found. Substrates are mixed to get a balance in proteins, carbohydrates and fibers. Sometimes substrates are dried, powdered and pelletized. For YMW, the substrate should be drier (85% DM) than for BSFL.

5 Chemical and physical food and feed safety aspects

5.1 Allergens

The consumption of insects may trigger an allergic reaction in consumers who are sensitised to house dust mites and crustaceans. The allergens arginine kinase and tropomyosin were identified as the allergens responsible for the cross allergic reaction to insects. Other allergens in insects reported are glutathione S-transferase, glyceraldehyde-3-phosphate dehydrogenase, haemocyanin, hexamerin1B, and sericin (EFSA Panel on Nutrition et al., 2021; FAO, 2021).

The presence of the allergens arginine kinase and tropomyosin has been reported to be present in YMW (EFSA Panel on Nutrition et al., 2021; FAO, 2021; Seves, Verkaik-Kloosterman, Temme, & van Raaij, 2016). Primary sensitisation to the allergens in YMW mostly occur through inhalation or skin contact, but is was also shown in a clinical controlled oral challenge that an allergic reaction can be triggered upon ingestion of mealworms (German Federal Institute for Risk Assessment, Garino, Zagon, & Braeuning, 2019). Especially the cuticles in mealworms are suspected to cause allergenicity (FAO, 2021). EFSA approved YMW as a Novel Food in 2021, but recommended further research on allergenicity to YMW, including cross-reactivity to other allergens (EFSA Panel on Nutrition et al., 2021). Nonetheless, tropomyosin and arginine kinase have been detected in BSFL as well. Hydrolysation of the proteins in the BSFL was able to reduce immunoreactivity, but did not deplete it completely (Bessa, Pieterse, Marais, & Hoffman, 2020). Thermal processing resulted in a decrease of allergenicity to arginine kinase and enolase, while the allergenicity to glyceraldehyde-3-phoshate dehydrogenase increased (FAO, 2021).

Chitin, a polysaccharide, is mostly present in the shell and cuticles of insects. Chitin is known for its immunomodulatory characteristics. It may enhance the immune reaction to the other allergens and therefore enhance the risk for specific consumer groups. When these specific consumers are allergic to some allergens, but the allergic reaction becomes more severe, it may become an even higher risk. However, it has been found that doses up to 5 grams of chitin-glucan are not of concern for public health (EFSA Panel on Nutrition et al., 2021).

It should be kept in mind that allergens from other sources, present in the substrate, on which the insects are reared, can be transferred to the insects. An example thereof is gluten. Several grains containing gluten were used as substrate, resulting in the detection of gluten in the insects (5.5 mg/kg, the EU limit for gluten-free food products is 20 mg/kg)(EFSA Panel on Nutrition et al., 2021).

5.2 Heavy and trace metals

Besides the ingestion of heavy metals by the insects when present in the substrate, heavy metals can also be absorbed by the chitin in insects. Moreover, several heavy metals are shown to accumulate in insects (FAO, 2021). Therefore, it is important to consider the environment of insect rearing, when these insects have the purpose of food and/or feed (FAO, 2021). The accumulation of heavy metals in insects is also dependant on the stage the insect is in. Especially larvae have a high capability of accumulation of heavy metals (Council on animal affairs, 2018; Raad voor Dieraangelegenheden, 2018).

In section 4.1, heavy metal composition is given for poultry manure and supermarket mix. For kitchen waste, levels are unknown to the authors. In meat meal (cat. 2), only very low levels of heavy metals are detected, well below legal limits (communication Darling Ingredients). Legal maximum values for feed exist and the levels in the residues studied in our project should be compared with these limits. For example, olive pomace (residue from olive fruit processing) contains metals, like arsenic, cadmium, lead, mercury, nickel, and selenium, which ends up in the YMW larvae. Tests with mixtures of olive pomace and wheat meal were

carried out. The heavy metal content of the feed was below legal limits. The larvae contained the metals, but levels were below the legal limits as well (Truzzi et al., 2019).

5.2.1 Arsenic

In a market basket survey in Canada, samples of whole, dried insects, insect powders and finished products with cricket and silkworm as ingredients were purchased from retail shops. All the samples consisted of products that were destined for human consumption. Arsenic was detected in all samples of cricket powder and silkworm pupae (n=19) contained arsenic with a maximum concentration of 0.34 mg/kg for the cricket powder and 0.03 mg/kg for the silkworm pupae (Kolakowski, Johaniuk, Zhang, & Yamamoto, 2021). An experimental study (Biancarosa, Liland et al., 2018) tested BSFL on their concentrations of several heavy metals in the larvae after being fed with the addition of *Ascophyllum nodosum* seaweed to plant based feed (0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%). In general, the more seaweed was added to the insect feed, the higher the concentration of arsenic was measured in larvae. The highest concentration of total arsenic measured was 23 mg/kg DW in the larvae fed with seaweed (100%). This diet all consisting of *Ascophyllum nodosum* seaweed contained 36 mg/kg DW total arsenic. According to the calculation method for the bioaccumulation factor (BAF) by Van Der Fels-Klerx, Camenzuli, Van Der Lee, and Oonincx (2016), the BAF here is 0.64, which indicates that no accumulation occurred in these larvae. Contrarily, the BAF for inorganic arsenic in these larvae is 2.3, indicating that there was accumulation of inorganic arsenic in these larvae. The initial level inorganic arsenic in the feed, consisting completely of *Ascophyllum nodosum* seaweed, was 0.09 mg/kg DW and the level measured in the larvae was 0.21 mg/kg DW. With regards to the EU ML for total arsenic, being 2 mg/kg (on the basis of 12% moisture content, Directive (EC) 2002/32), larvae fed with 10% seaweed (3.4 mg/kg DW) would not comply with legal limits for arsenic in complete feed (Biancarosa et al., 2018). A study measured arsenic in larvae of YMW and of BSFL, which were fed with arsenic spiked insect feed (spiked with 1 mg/kg, 2 mg/kg and 4 mg/kg in a substrate with moisture content of 12%). After converting the measurements of the chemical analyses into BAFs, the results showed that arsenic accumulated in YMW, but not in BSFL. The arsenic taken up in the spiked insect feed was for the largest part excreted by the BSFL after they were transferred to a clean substrate for 2 days. Remarkably, the highest BAF in BSFL for arsenic was 0.58 mg/kg on the substrate spiked with 1 mg/kg. For YMW, the highest BAF for arsenic was 2.6 on the substrate spiked with the highest arsenic concentration of 4 mg/kg. The levels of arsenic remained well elevated over the EU ML, even after 2 days of feeding a clean substrate (FAO, 2021; Van Der Fels-Klerx et al., 2016). Another study on BSFL did show a slight accumulation of total arsenic. After feeding a substrate containing 2.5 mg/kg DW total arsenic, the larvae showed a concentration of 3.3 mg/kg DW of total arsenic (Schmitt et al., 2019).

5.2.2 Cadmium

Cadmium has been reported to accumulate in BSFL by many studies and is for this reason also identified by many authors as a compound of concern for food and/or feed safety (FAO, 2021). In the market basket survey, cadmium was detected in the majority of the insect samples (crickets and silkworms; total n=19) with a range in concentrations of 0.031 mg/kg to 0.23 mg/kg (Kolakowski et al., 2021).

When fish meal was replaced by insect meal for the purpose of feed for salmon, the level of cadmium was higher in feed with insect meal compared to the feed with fish meal. The highest concentration of cadmium in insect meal of BSFL was 0.30 mg/kg DW, following feeding on a plant-based substrate with 60% of *Ascophyllum nodosum* seaweed (Biancarosa et al., 2019). In the previous study by Biancarosa et al. (2018), the levels of cadmium were measured in the BSFL and in the substrate. The level of cadmium was the lowest in the control larvae (only plant-based substrate), being 0.41 mg/kg DW. The level of cadmium in the plant-based substrate was 0.09 mg/kg DW. The high amount of cadmium in the larvae compared to the substrate indicates the accumulative character of the BSFL for cadmium. The highest cadmium concentration obtained in this study was for the BSFL reared on a substrate solely consisting of the *Ascophyllum nodosum* seaweed, being 2.3 mg/kg DW. The substrate with seaweed contained cadmium with the concentration of 0.34 mg/kg DW. These results are indicative of BAFs around 4 – 7. In a spiking study by Van Der Fels-Klerx et al. (2016), the cadmium was taken up from the spiked substrate and even from the control (unspiked) substrate by BSFL. The BAF among the groups ranged from 5.8 to 9.5 for cadmium, which indicates high accumulation of cadmium in BSFL. In this study the cadmium concentration reached around 7 mg/kg DW in

BSFL. Also the results in the study by Schmitt et al. (2019) suggested that cadmium is accumulated in BSFL. The level of cadmium was 1.4 mg/kg DW in the larvae, but 0.5 mg/kg DW in the substrate. Another study investigated the concentrations of the metals cadmium, chromium and copper in the BSFL with *Lactobacillus buchneri* added and reared on a substrate of soybean curd residue. The authors concluded that the concentrations of these metals were not influenced by the addition of the bacterium, since no significant differences were seen. Average concentrations of cadmium in BSFL (n=3) were between 0.027 mg/kg and 0.045 mg/kg, while the concentrations in the substrates were 0.05 mg/kg and 0.06 mg/kg. It was measured that a part of the cadmium remained in the larvae and a part was excreted into the rest material (Somroo et al., 2019). Cadmium is found to have a negative influence on the gut microbiome of the BSFL, which may support the growth of pathogenic bacteria in insects. In one study, larvae of mealworms showed a high accumulative character regarding to cadmium. Cadmium was detected in the larvae in a feeding trial with several organic soil substrates, in which cadmium was naturally present. The concentrations of cadmium in larvae were much higher than those in the several substrates. The highest cadmium concentration in substrates was 28.9 µmol/kg, but was 188.8 µmol/kg in the larvae reared on that substrate (Vijver, Jager, Posthuma, & Peijnenburg, 2003). It is observed that each time the insect moults, the concentration of cadmium in the insect decreases. Nonetheless, the decrease in the cadmium content in insects after moulting should be investigated more closely (Claeys, Ulens, & Witters, 2014). In a spiking study by van der Fels-Klerx et al. (2016), the opposite was observed: cadmium was taken up from the spiked substrate by YMW, but the BAF remained below 1 (range: 0.4-0.7). The cadmium concentration reached ca. 0.75 mg/kg DW in YMW.

5.2.3 Chromium

The presence of chromium in insects is not investigated by many studies. One feeding trial with solid aquaculture waste as substrate for BSFL measured chromium amongst several other metal compounds. The study found that chromium was 4.4 mg/kg DW in the substrate, but was 1.7 mg/kg DW in the larvae (Schmitt et al., 2019). Another study investigated soybean curd residue as substrate to rear BSFL. The level of chromium in this study was 0.04 mg/kg in the larvae, which was 0.22 mg/kg in the start substrate where the larvae were reared on (Somroo et al., 2019). According to the results from both studies, chromium does not have an accumulative character in BSFL.

5.2.4 Cobalt

Cobalt is not often investigated in studies on BSFL and YMW. As a result of this literature screening, one study reporting data on cobalt in BSFL. Schmitt et al. (2019) investigated the feasibility of the use of solid aquaculture waste for the rearing of BSFL. The substrate of solid aquaculture waste contained 0.5 mg/kg DW cobalt. The concentration of cobalt in the insect larvae was 0.2 mg/kg DW. These results indicate that there would be no accumulation of cobalt in BSFL.

5.2.5 Copper

As cadmium, copper negatively impacts the gut microbiome of the BSFL and stimulates pathogenic bacteria growth. Copper was also investigated in BSFL reared on a substrate of solid aquaculture waste. The amount of copper was 15.1 mg/kg DW in the substrate and 12.7 mg/kg DW in the larvae (Schmitt et al., 2019). In a study, which investigated the influence of *Lactobacillus buchneri* on metals in BSFL, the highest concentration of copper was 0.08 mg/kg. However, the initial concentration of copper in the soybean curd residue substrate was 0.39 mg/kg and 0.31 mg/kg was traced back in the frass (Somroo et al., 2019). The results of these two studies suggest that copper does not accumulate in BSFL. Vijver et al. (2003) investigated larvae of mealworm reared on several kinds of soils. The highest concentration of copper (3000 µmol/kg) was measured in larvae reared on a substrate with a copper concentration of 18.7 µmol/kg. In contrast to BSFL, larvae of mealworm seem to accumulate copper according to the results of this study.

Another study investigated copper concentrations in composite samples, a mix of buffalo worm, greater wax moth, mealworm beetle and migratory locust. The authors concluded that the amount of copper is comparable to the amounts present in meat and fish (Poma et al., 2019).

5.2.6 Lead

As cadmium, also lead is reported to accumulate in insects, in both BSFL (Purschke et al., 2017; Van Der Fels-Klerx et al., 2016) and mealworm (Claeys et al., 2014; Vijver et al., 2003). In the study of Van Der Fels-Klerx et al. (2016), the BAF for BSFL ranged from 1.1 to 1.8, indicating some, but limited accumulation potential for lead. For YMW the BAF remained well below 1 (range: 0.043-0.051), indicating negligible accumulation of lead in this species. In the study of Schmitt et al. (2019), lead did not seem accumulate in BSFL. In this study, the larvae were reared on substrates of solid aquaculture waste. The level of lead in the substrate was 0.5 mg/kg DW, while the lead concentration in larvae was 0.3 mg/kg DW. Concentrations of lead ranged between 0.019 mg/kg and 0.059 mg/kg in 58% of cricket powder and silkworm pupae samples (n=19) in a Canadian market basket survey (Kolakowski et al., 2021). In a study on BSFL reared on *Ascophyllum nodosum* seaweed, lead slightly accumulated in the larvae. The highest concentration of lead was 0.29 mg/kg in larvae, which was 0.25 mg/kg in the substrate. A follow-up study investigated the use of insect meal instead of fish meal for feed and the heavy metal concentrations. The concentration of lead was not higher than 0.08 mg/kg in insect meal of the BSFL after feeding a diet of 60% *Ascophyllum nodosum* seaweed (Biancarosa et al., 2019). Larvae of mealworm were reared on several types of soil. The highest concentration of lead was 260 µmol/kg in larvae, which were reared on a soil with a lead concentration of 63.2 µmol/kg (Vijver et al., 2003).

5.2.7 Manganese

Literature on Manganese in insects is scarce. It is reported that besides cadmium, copper and lead, also manganese accumulates in insects. BSFL provided with solid aquaculture waste contained 0.2 g/kg manganese. This level of manganese was 0.1 g/kg in the substrate made of the solid aquaculture waste (Schmitt et al., 2019).

5.2.8 Mercury

Mercury was tested in 19 samples of BSFL. Three quarter of the samples contained traces of mercury with concentrations ranging from 0.94 µg/kg to 28 µg/kg (Kolakowski et al., 2021). A similar pattern to manganese is observed for mercury in BSFL. The larvae, fed with solid aquaculture waste contained 0.2 mg/kg DW mercury, whereas the substrate contained 0.1 mg/kg DW of mercury (Schmitt et al., 2019). Samples of insect meal, grown on substrates with seaweed, were analysed for several metals. In general, levels of mercury were low. The insect meal from insects grown without the addition of seaweed contained 0.02 mg/kg mercury, while mercury could not be quantified in insect meal from insects with the highest inclusion of seaweed in the substrate (Biancarosa et al., 2019). Although mercury could not be quantified in the previous study by Biancarosa et al. (2018), the amount of mercury in larvae was slightly higher (0.023 mg/kg) than that in the substrate of *Ascophyllum nodosum* seaweed (0.021 mg/kg).

5.2.9 Molybdenum

Molybdenum has not been studied frequently in relation to its uptake or accumulation in BSFL or YMW. One study investigated determined the level of molybdenum in solid aquaculture waste, functioning as substrate, and in BSFL. The level of molybdenum in the start substrate was 1.2 mg/kg DW and 1.0 mg/kg DW in the larvae (Schmitt et al., 2019).

5.2.10 Nickel

To our knowledge, nickel has not often been the subject of research related to BSFL and YMW. One study determined the level of nickel in BSFL reared on solid aquaculture waste. The substrate contained 2.4 mg/kg DW of nickel and the larvae 0.8 mg/kg DW. This result indicates that nickel does not accumulate in BSFL (Schmitt et al., 2019).

5.2.11 Selenium

There is a paucity of literature on selenium in insects. One feeding trial with BSFL showed that the concentration of selenium in the larvae was lower than that of the substrate where the larvae were reared

on. The substrate contained 2.4 mg/kg DW selenium, whereas the larvae contained 1.2 mg/kg DW (Schmitt et al., 2019).

5.2.12 Silver

Also silver in insects was not often the subject of investigations. The authors believe that the first results on silver in insects were published in 2019. However, from these first results it does not appear that silver would be specifically of concern in insects, since no accumulation was observed in BSFL. The substrate, on which the larvae were reared, had a silver concentration of 42.6 µg/kg. The concentration of silver in the larvae was 13.1 µg/kg (Schmitt et al., 2019).

5.3 Mycotoxins

In contrast to many metals, there is no literature that suggests that mycotoxins accumulate in insects (M. Gold, Tomberlin, Diener, Zurbrügg, & Mathys, 2018). Even in studies, in which substrates were spiked with high concentrations up to 25 times the EU maximum limit of mycotoxins, these were barely detected in the insects, but particular in the frass (Camenzuli et al., 2018; DiGiacomo, Akit, & Leury, 2019). The mycotoxins were partly metabolised and partly excreted without metabolization. This was the case for the mycotoxins aflatoxin B1, deoxynivalenol, ochratoxin A and zearalenone in both BSFL and LMW (Camenzuli et al., 2018). Similar results were obtained in another study on BSFL only: none of the mycotoxins aflatoxins B1, B2, G2, deoxynivalenol, ochratoxin A, zearalenone accumulated in the larvae (Purschke et al., 2017).

Another study found some traces of the mycotoxins alternariol, alternariol methyl ether, HT2-toxin, nicarbazin, nivalenol, roquefortine C, and zearalenone in LMW. HT2-toxin was measured in quantities above the screening detection limit, however, no MRL is set for this toxin (De Paepe et al., 2019).

Bosch, Fels-Klerx, Rijk, and Oonincx (2017) studied the tolerance and accumulation of aflatoxin B1 (AFB1) in BSFL and YMW. They were fed poultry feed spiked with AFB1 up to 0.5 mg/kg dry feed. The AFB1 in the feed did not affect survival and body weight. Furthermore, AFB1 and aflatoxin M1 (AFM1) were below the limit of detection (0.10 µg/kg) in BSFL, whereas the YMW had AFB1 levels at 10% of the EU legal limit for feed materials. This study concluded that both species have a high aflatoxin B1 tolerance and do not accumulate aflatoxin B1.

5.4 Persistent organic pollutants

5.4.1 Dioxins and Polychlorinated biphenyls (PCBs)

Although not subject to many studies, dioxins and PCBs do not seem to accumulate in BSFL (M. Gold, Tomberlin, Diener, Zurbrügg, et al., 2018). Biancarosa et al. (2019) studied the dioxins PCDD and PCDF and DL-PCBs in BSFL. These were detected in small amounts (0.23 ngTEQ/kg), well below the EU ML for feed (0.75 ngTEQ/kg) (EFSA, 2015). Also in the study by van der Fels-Klerx et al. (2020), BSFL were analysed on the presence of PCBs and dioxins. The larvae were reared on substrates based on both meat and non-meat material with the addition of either shredded cardboard or plastic packaging material. The highest concentration of dioxins was detected in larvae reared on meat-based substrate with shredded carboard with 0.345 ngTEQ/kg. The concentration of the starting substrate was 0.175 ngTEQ/kg. The same counts for the concentration of the sum of dioxins and dioxin-like PCBs. The highest concentration of 0.381 ngTEQ/kg was detected in larvae reared on the meat-based substrate with cardboard with an initial concentration of 0.198 ngTEQ/kg. For the non-dioxin-like PCBs, however, the highest concentration (0.401 ngTEQ/kg) was found in the control group of larvae. The concentration in the substrate of the control group was 0.485 ngTEQ/kg. Even the highest concentrations given here were below the EU limits based on complete feed with a moisture of 12% (Directive (EC) 2002/32).

One study compared dioxins and PCBs (12 different PCBs) in a mix of buffalo worm, greater wax moth, mealworm beetle and migratory locust to common food products from animal origin. The concentrations of dioxins and PCBs were lower in the insect mix than in the animal products and were within EU MLs for feed (Poma et al., 2019).

5.4.2 Flame retardants

Samples of mealworm larvae were purchased in 2016 in both supermarkets in Antwerp, Belgium and online from European web-shops and analyzed for the presence of three halogenated flame retardants and 8 phosphorus flame retardants. None of the mealworm samples showed quantifiable amounts of any of the halogenated flame retardants. From the phosphorus flame retardants only triphenyl phosphate (TPHP), 2-ethylhexyl diphenyl phosphate (EHDPHP) and tris (1-chloro-2-propyl) phosphate (TCIPP) were measured in the quantities 8407 pg/g WW, 1159 pg/g WW and 1996 pg/g WW, respectively (Poma et al., 2017). After calculating the hazard quotients (estimated daily intake divided by relative oral reference dose factor) for these compounds, being lower than 1, it was concluded that these do not pose a food safety risk (Poma et al., 2019).

5.4.3 Mineral oils

A study on BSFL investigated meat- and plant-based substrates with the additions of shredded plastic or shredded paperboard carton. Mineral oil hydrocarbons were detected in high quantities in BSFL, ranging from 463 mg/kg DW to 533 mg/kg DW. The initial concentrations of mineral oils in the starting substrates were ranging from 91 mg/kg DW to 110 mg/kg DW. These would suggest that there is an accumulative mechanism present in the larvae. However, these quantities did not differ significantly between the experimental groups and the control group (<LOQ in the starting substrate and 453 mg/kg DW in the larvae). The presence of MOHs may not be dependent on the substrate (van der Fels-Klerx et al., 2020).

5.5 Pesticides

Not much literature reported results on pesticide analyses on BSFL and YMW. In general, it can be concluded from few studies that pesticides do not accumulate in these insects (M. Gold, Tomberlin, Diener, Zurbrügg, et al., 2018), not even when the substrates are spiked therewith (DiGiacomo et al., 2019). However, some literature report more contradicting conclusions in terms of YMW ability to not only excrete fungicides, such as benalaxyl, diniconazole, epoxiconazole, metalaxyl and myclobutanil, but also degrade or accumulate (FAO, 2021). Some insect samples from Belgium with the purpose of food showed traces of tributylphosphate (Poma et al., 2019). In another experimental study, mealworms were able to rapidly excrete the pesticide hexabromocyclododecane (HBCD) into the frass (Brandon et al., 2020). Another experimental study investigated the effect on both growth and mortality of BSFL after exposure to several insecticides: standard substrates for BSFL were spiked with the insecticides with levels equal to the maximum residue levels in the EU. Although some insecticides, such as cypermethrin and spinosad had a negative effect on growth, and increased mortality of BSFL; the insecticides did not accumulate in the BSFL. No accumulation was observed in the BSFL during the spiking experiments, since the bio-accumulation factor was below 1 for all insecticides with the highest bio-accumulation factor of 0.79 for cypermethrin (N. Meijer et al., 2021). Similar conclusions were drawn from a study investigating the performance of BSFL on substrates spiked with the pesticides chlorpyrifos, chlorpyrifos-methyl, pirimiphos-methyl; and the growth of the BSFL appeared unaffected by the addition of these pesticides to the substrate (Purschke et al., 2017). More information on the potential effects of pesticides on insect health are discussed in section 3.7.2.

5.6 Pharmaceutical active compounds

Rearing insects on manure is suggested as a potential process to reduce waste and to close the production loop in pork and poultry production systems, since current literature shows indications of detoxifying capability of several contaminants of insects (DiGiacomo et al., 2019; M. Gold, Tomberlin, Diener, Zurbrügg,

et al., 2018). Nonetheless, manure requires specific attention with respect to pharmaceutical active compounds; since these compounds may be administered to food-producing animals. Also the presence of antibiotic-resistant genes are hazards of concern in insect for food and/or feed, having regards to the substrate (Osimani et al., 2018). It is thought that the risk of antibiotic-resistant genes in insects is comparable to other food products and that antibiotic-resistant genes are not specifically of concern in insects only (Vandeweyer et al., 2019b).

BSFL were studied in composting studies using organic materials containing the pharmaceutical active compounds carbamazepine, roxithromycin, trimethoprim. No accumulation of these compounds was observed in the larvae. With regard to the frass of the larvae as compost, the half-life of these drugs was shorter compared the half-life in the control compost (van Huis, 2020), indicating that BSFL play a role in the breakdown of the pharmaceutical compounds, without accumulating them. For the compounds carbamazepine, roxithromycin, and trimethoprim; also no accumulation was observed in BSFL. BSFL are able to degrade other pharmaceutical active compounds, such as the antibiotic tetracycline. A great reduction of almost 96% was observed at the 12th day after being fed with a substrate spiked with tetracycline (Meyer, Meijer, Hil, & Fels-Klerx, 2021).

Another study analysed larvae of both BSFL (received from a research group) and the YMW (purchased from a pet shop) for the pharmaceutical active compounds metoprolol tartrate, nicarbazin, paracetamol and salicylic acid. The latter was found in each sample in concentrations of 3 µg/kg in BSFL samples and above the upper limit of quantification (>1 µg/kg) in LMW. Paracetamol was not detected in the samples of BSFL and LMW. Metoprolol tartrate was detected in samples of the LMW in levels above the upper limit of quantification (>1 µg/kg) and in samples of the BSFL in levels below the lower limit of quantification (<3 µg/kg). Nicarbazin was not detected in BSFL samples, but significant amounts above the upper limit of quantification (>3 µg/kg) were measured in all samples of the LMW (n=6). However, no limits are set yet in EU legislation for these compounds in food and/or feed (De Paepe et al., 2019). It was unknown what the rearing conditions of these samples were and on which substrates these were reared. As described earlier, the type of substrate can have a major impact on residues present in insects.

The antibiotic oxytetracycline was reduced by BSFL, reared on a wheat bran substrate (Cuncheng Liu, Yao, Chapman, Su, & Wang, 2020).

Although the promising utilisation of manure as insect substrate, current literature on the pharmaceutical active compounds in insects is not yet exhaustive and requires further research.

5.7 Processing contaminants

Chemical or thermal processes during processing of insects can result in several processing contaminants, such as acrylamide, chloropropanols, furans, heterocyclic aromatic amines (HAAs) and polyaromatic hydrocarbons (PAHs) (FAO, 2021). In the study by van der Fels-Klerx et al. (2020), PAHs were detected in BSFL reared on substrates with shredded carboard or shredded plastic. The highest concentration of PAHs was 2.17 µg/kg and was measured in the larvae reared on a plant-based substrate with shredded cardboard. The initial concentration of this substrate was 4.83 µg/kg.

Another study also showed indications that PAHs are not accumulated in BSFL (M. Gold, Tomberlin, Diener, Zurbrügg, et al., 2018). It is said that further research is needed to understand the extent of accumulation of several processing contaminants (FAO, 2021).

5.8 Microplastics

The topic of microplastics in combination with insects is only recently studied. A study investigated the effect on growth and survival of BSFL being reared on food waste as substrate. The food waste substrates contained 5%, 10% or 20% of the plastics polyethylene (PE) or polystyrene (PS). No significant differences

were observed in the growth nor survival of BSFL larvae fed with these substrates compared to the control (Cho, Kim, Kim, & Chung, 2020). Similar conclusions were drawn for BSFL fed on substrates containing the plastic material polypropylene (PP) (Romano & Fischer, 2021). Nonetheless, it was not investigated in either of these studies if microplastics are present in the larvae or even if these would accumulate in the larvae. Considering food safety, the presence of microplastics in insects should be investigated, when food waste containing rests of plastic materials is used as substrate.

5.9 Physical hazards

When insects are consumed as whole, it can pose a physical hazard. Hard particles of the insects, such as rostrum, spines, stings and wings can result in a physical obstruction (FAO, 2021). Nonetheless, this risk of obstruction may be minimised by the production of insect powder. Further, particles of all other materials and/or equipment used during the processing of insects, such as gloves, tweezers, plasters and/or packaging material may end up in the final product. These examples of materials are not fit for consumption and may be a physical risk.

5.10 Summary of chemical and physical food and feed safety aspects

Allergenicity
Some insect allergens, like arginine kinase and tropomyosin show similarities with allergens in crustaceans and dust mites and are, therefore, of concern especially to populations that are already allergic. Appropriate labelling of insect products should be implemented. Other allergens that can be found in insects are glutathione S-transferase, glyceraldehyde-3-phosphate dehydrogenase, haemocyanin, hexamerin1B, and sericin. Other allergens may be introduced in the insects from the substrate (e.g. gluten). It is required of all insect products that have thus far been approved as novel foods, that the allergic potential of insects for those sensitive to crustaceans is mentioned.

Heavy metals
Heavy metals are generally of concern for insect rearing, especially when substrates from residual streams are used. Several heavy metals may accumulate in BSFL and YMW, when present in the substrate, but this is species-specific. Literature describes that the metals cadmium, copper, lead, manganese, mercury and silver accumulate in insects. Especially the heavy metal cadmium was subject of several insect trials has shown a high accumulative character in BSFL.

Mycotoxins
Literature suggests that both BSFL and YMW have a high tolerance for mycotoxins (e.g. aflatoxin B1) in their feed and no indication exists that either species accumulates mycotoxins.

Dioxins and dioxin-like PCB's
Literature study shows that dioxins and dioxin-like PCBs do not seem to accumulate in BSFL and YMW. However, the number of references on this topic are not ubiquitous.

Flame retardants
Flame retardants are not often measured in BSFL or YMW. A study on mealworm larvae showed that halogenated flame retardants were present in low, unquantifiable amounts. Phosphorus flame retardants were detected in mealworm larvae, but the authors suggested that these amounts would not be of human health concern upon ingestion.

Mineral oils
As to date, mineral oils were subject in a single study in relation to insect rearing. Mineral oils were detected in high levels in BSFL. However, the study is not conclusive on the identity, uptake, metabolism, excretion or

accumulation of pesticides by insects, since there were no differences observed between the experimental groups and the control group.

Pharmaceuticals & veterinary drugs
Some references in the literature study indicate that BSFL may play a role in the breakdown of the pharmaceutical compounds (present in the substrate), without accumulating them. However, overall no conclusive results on uptake, metabolism, excretion or accumulation of pharmacological drugs by insects are given.

Processing contaminants
During the process in the production of insects or insect products, several processing contaminants could be introduced, like acrylamide, chloropropanols, furans, heterocyclic aromatic amines (HAAs) and polyaromatic hydrocarbons (PAHs). Although PAHs have been detected in a couple of insect studies, no accumulation of the hazard has been observed.

Microplastics
Microplastics seem not to affect growth or survival of insects. However, the presence of microplastics in insects should be investigated, when food waste containing rests of plastic materials is used as substrate.

Physical hazards
Several insects contain particles that might cause obstruction in the digestive tract. However, the BSFL and YMW are not known to have certain particles. Other physical hazards may be introduced during the production of insects, such as handers' gloves. However, this is not specifically for the insect production process, but counts for all food production processes.

5.11 Data gaps and research needs

The food safety of insects is not abundantly discussed and data on food safety hazards in insects is scarce. It has become clear from the literature study that certain food safety hazards in insects are not regarded as a concern for human health. This can be due to the fact that no accumulation occurs in insects or even that insects may be able to degrade or to metabolise certain compounds. Examples thereof are mycotoxins, pesticides and pharmaceutical compounds. However, it is not known yet what these metabolites or degradation products are and if these can pose a risk to human health upon ingestion. Further, several metals are studied and are shown to have an accumulative character in insects. Examples thereof are arsenic, cadmium, copper, lead and mercury in BSFL and copper in YMW. Overall, it has been noticed that more studies make use of BSFL in their research rather than YMW and thus more data has been reported on hazards in the former species.

Data on several hazards is scarce, like amongst others mineral oils and silver. Although the available data suggests that certain hazards would not pose a great risk to human health, the conclusion could only be drawn from limited data. This is the case for:
- Metals (cobalt, chromium, molybdenum, selenium, silver)
- Mineral oils
- (Unknown) mycotoxin metabolites (possible toxicity is unknown)
- Flame retardants
- (unknown) pesticide metabolites (possible toxicity is unknown)
- Processing contaminants (acrylamide, PAAHs)
- Microplastics

Furthermore, studies on PFAS and insects are lacking. There is a lack of information on hazardous substances in the substrates as well as examples of processes dedicated to remove these substances or neutralize the hazard, e.g. heat treatment or fermentation to degrade undesired chemical substances or washing with solutions of acid or alkali to clean the substrate. Pre-treatment processes are required for neutralizing or removal of anticipated hazardous substances, but the absence thereof would be the preferred option.

5.12 Conclusions and research needs for current project

In the present project, representative samples from each substrate that will be used in the insect-rearing experiments should be screened for the concentration of heavy metals before use. In case relevant heavy metal levels (Cd, As, Pb) are detected in the substrate at threshold levels that need to determined, the experiments will be extended to heavy metal screening of BSFL and YMW larvae that have been reared on each of these substrates. This should allow for calculation of the Bio Accumulation Factor (BAF) for Cd, As and Pb, which can serve (in combination with the actual concentrations of the heavy metals in substrate and insect) as a tool for selecting safe substrate-insect species combinations.

Former foods, meat meal (cat. 2) and probably chicken manure as well, will show low mycotoxin levels, unless these residual streams have become contaminated with toxin-producing fungi afterwards. Provided the spoilage of the residual streams is kept to a limited level, mycotoxins will most probably not be a high priority hazard of concern.

Since household kitchen waste and supermarket mix are derived from food for humans, the presence of undesired chemical substances normally will be unlikely. However, contamination with unfavorable substances may occur by for example accidental mixing with other type of waste.

For instance plastic and probably microplastics could be present in these residual streams, while information on the effects on insects is very limited, therefore microplastics are interesting to be studied in relation to these streams.

Persistent organic pollutants will probably not be present in residual streams originating from former food. The presence of dioxin/PCBs in meat meal is very unlikely and may occur only in very rare occasions. In poultry manure dioxin/PCBs may occasionally be present, but mostly in free-range hens that have foraged on soils that are contaminated by industrial activities, burning of waste or chemical spillage. PFAS are not studied in relation to insects, however they could be present in several residual streams, therefore it is interesting to include PFAS in our study.

Processing contaminants could have been formed during processing of food products as present in organic waste form household kitchens and supermarket mix.

Poultry manure could contain veterinary drugs, for example antibiotics, antiparasitics and coccidiostatica. Furthermore, the meat meal from animal rendering is also expected to contain veterinary drugs, which are allowed to be used in production animals.

Supermarket mix and household kitchen waste come from products in which pesticide levels are controlled in individual products, however some residue may be present in the these residual streams. One might consider checking poultry manure for pesticides, since poultry may have been exposed to pesticides through their plant-based diet.

6 Microbial hazards in edible insects and the effect of treatment methods

6.1 Overview of microbial hazards in cultured insects

Several articles describe the presence of microbial hazards: bacteria, viruses, parasites, and fungi in cultured insects. In this paragraph the results, main outcomes and recommendations of a recent extensive review (van der Fels-Klerx, Camenzuli, Belluco, Meijer, & Ricci, 2018) and a risk assessment from European Food Safety Authority (EFSA) (EFSA, 2015) regarding food safety issues related to the use of cultured insects for feed and food are summarized. Information on microbial hazards specifically for insect health is provided in section 3.7.

Several **bacteria** are mentioned (*e.g.* in mealworm) (van der Fels-Klerx et al., 2018). *Enterobacteriaceae*, bacterial endospores, *Proteobacteria*, *Firmicutes*, and *Actinobacteria* dominated the bacterial composition in meal-worm larvae. Counts of total viable bacteria and Enterobacteriaceae increased after pulverization of the insect, suggesting that most of the bacteria are present in the gut content. In general, high counts of micro-organisms are found in fresh insects. Heat treatment seems an important step to reduce these counts. The presence of psychrophilic organisms in mealworm larvae, able to reproduce at low temperatures raise concern for storage. According to EFSA (2015) potential pathogenic bacteria (such as *Campylobacter, Salmonella*, verotoxigenic *E. coli*) may be present in non-processed insects, but this is depending on the substrate and the rearing conditions. Active replication of these pathogens does not seem to happen in insects (EFSA, 2015). Effective processing will mitigate the transmission of these bacteria.

Viruses (*e.g.* norovirus, rotavirus, hepatitis E virus, hepatitis A virus) could be introduced via the substrate and transferred to primary production (van der Fels-Klerx et al., 2018). Specific insect pathogenic viruses are not considered as hazard for vertebrate animals and humans, however edible insects may act as vector for viruses (e.g. ASFV, see 1.3.3.2). Therefore, the type of substrate should be chosen and processed properly (EFSA, 2015).

Parasites, which may be a safety risk for feed and food can be present in edible insects (Galecki & Sokol, 2019) see also paragraphs 1.1.1 and 1.3). Proper management of rearing insects would prevent completion of the parasitic life cycle (EFSA, 2015).

Fungi and yeasts with potential hazards are found in fresh and frozen insects (*T. molitor* and *L. migratoria*). However, these risks can be mitigated by hygiene measures during the production chain (EFSA, 2015). Also for fungal communities differences are found between *H. illucens* fed on chicken waste versus on vegetable waste, indicating that the type of substrate is important for the selection of yeast and mould genera growing on the substrates (van der Fels-Klerx et al., 2018).

Prion related diseases have not been observed in insects. This is explained by the absence of the PrP-encoding gene in insects. However, insects might act as a vector transferring prions from substrates. Therefore, with regard to the choice of the substrate, EFSA recommends to take the following points into account (EFSA, 2015):

- In general, the use of food- and feed-grade substrates of non-ruminant origin (subcategories of substrate groups A, B, C and D not containing products of ruminant origin), non-ruminant animal manure and intestinal content (subcategory of substrate E), and organic waste of vegetable nature (substrate group F) should not pose any additional risk compared to use of other feed.
- The risk related to other substrates (substrate groups A, B, C D and E containing products of ruminant origin, and substrate group G) should be specifically evaluated. Some of this material is currently excluded from the feed chain because of prion-related concerns (*e.g.* certain tissues from ruminants because of the risk of spread of bovine spongiform encephalopathy (BSE) and other animal transmissible spongiform encephalopathies (TSE)), and material of human origin such as human faeces may pose a risk of transmitting human prion diseases to animals (*e.g.* variant Creutzfeldt-Jacob Disease to cattle).
- Inactivate prions by a proper thermal treatment of the substrate prior to rearing the insects.

Furthermore, other articles, mainly experimental papers, studied specific potential microbiological hazards in insects. In the following paragraphs the risks are described per type of insect.

6.1.1 Black soldier fly

In BSF prepupae (mature larvae), reared on standard vegetable substrates, viable Enterobacteriaceae (3.7 $\log_{10}$ CFU/g), *Bacillus cereus* (2.3 $\log_{10}$ CFU/g*, Campylobacter* (3.2 $\log_{10}$ CFU/g*, Clostridium perfringens* (0.8 $\log_{10}$ CFU/g, coagulase-positive staphylococci (3.9 $\log_{10}$ CFU/g, Listeriaceae (4.8 $\log_{10}$ CFU/g, and *Salmonella (2/6 positive samples)* were detected in prepupae. *Campylobacter* and *Listeria* counts increased with increasing rearing temperature (20 versus 33°C) (3.2 to 4.7 $\log_{10}$ CFU/g versus 4.8 to 5.8 $\log_{10}$ CFU/g (Raimondi et al., 2020). However, in another study on BSF reared on seaweed, faecal indicator organisms (FIOs) were low in larvae at the point of harvest, although larvae meal and extracted lipids were free of FIOs immediately after processing. During handling, distribution and storage the larvae meal and other externally sourced raw feed ingredients for larvae rearing and feed pellet formation (for Atlantic salmon) became contaminated with FIOs and *Listeria* spp. FIOs were also present, albeit at very low levels, in the feed pellets. Processing treatments provided effective decontamination, and FIOs and pathogen concentrations in final feed products never exceeded microbiological quality standards for insect processed animal proteins (Swinscoe et al., 2019). Although BSFL can decrease the pathogenic load in waste, the larvae themselves can contain *Salmonella* at the end of the rearing period, as reviewed by van Huis (2020). Muller, Wiedmer, and Kurth (2019) examined the effect of larval intestine extracts on the coccidian parasites *Eimeria nieschulzi* and *Eimeria tenella*, and on eggs of the nematode *Ascaris suum*. Furthermore, they focused on the question of whether the persistent parasite stages (oocysts and eggs) would be digested, pass through living larvae, or attach to the larval surface. Neither whole living BSFL, nor BSF larval intestine extracts had any effect on oocysts or eggs of the studied parasites. Although harbouring parasites in BSF is not well described, when used as animal feed, BSFL pose a risk of pathogen transmission to animals. To ensure feed safety, these larvae should be pre-treated properly to avoid this risk. A simple larval washing step is not sufficient for total removal of parasites (Muller et al., 2019).

A microbiological analysis of BSF residue used for soilless production showed presence of fungi and (coliform) bacteria. *Escherichia coli, Clostridium* spp. and *Salmonella* spp. were however absent, in line with the ecolabel criteria established by Decision 2001/688/CE (Setti et al., 2019).

6.1.2 Protaetia brevitarsis

A microbiological study was done in edible white-spotted flower chafer, *Protaetia brevitarsis* larvae flour (the freeze-dried beef flour (BF), insect flour (IF), and ethanol-defatted insect flour (IE)). The total aerobic bacteria count of IF was significantly higher than that of BF (8.71 vs. 1.52 log colony forming units (CFU) per gram; $p < 0.05$), while *E. coli* and coliform bacteria were not observed in the BF, but were present in IF (0.81 and 5.71 log CFU/g). The authors suggest that insects during breeding are contaminated via the substrate (from soil or sawdust) with bacteria. Defatting the larvae with 70% ethanol reduced the viable counts of total aerobic bacteria*, E. coli*, and coliform bacteria (Lee, Yun, & Goo, 2020).

6.2 AMR in substrates used for insect rearing and in edible insects

Many studies describe the presence of antimicrobial resistant bacteria and/or genes in substrates that can be used to rear insects, such as commercial composted domestic food and green waste material (Furukawa, Misawa, & Moore, 2018), raw meat, raw vegetables and fruit (Liang et al., 2020), untreated chicken manure and chicken litter (Glaize et al., 2020), pig manure (Kang et al., 2018; Pu et al., 2018), cattle manure (Zaheer et al., 2019), compost, and sewage sludge (Heck, De Marco, Duarte, Salamoni, & Van Der Sand, 2015). It should be noted that some of these antibiotic resistances could happen naturally as discussed by Liang et al. (2020).

Antimicrobial resistance genes were detected in edible insects (including YMW but no BSFL) (Milanović et al., 2016; Osimani et al., 2017; Vandeweyer et al., 2019a) and in laboratory-reared fresh YMW, their feeding substrates (wheatmeal), and frass. Based on the overall results, the contribution of feed to the occurrence of antibiotic resistance (AR) genes and/or antibiotic-resistant microorganisms in mealworm larvae was hypothesized together with vertical transmission via insect egg smearing (Osimani et al., 2017). Al lot of studies use PCR to detect antimicrobial resistance genes, which do not allow to determine in which bacterial species the resistance genes are present. This makes it difficult to appraise the potential risk of the presence of these genes. Most studies focussed on a broad set of resistance genes which are also commonly found in food substances, and presence of these genes in insects won't have additional implications for human or animal health. However one study particularly detected a low prevalence of some of the carbapenem resistance genes in mealworms from Belgium and a higher prevalence of some of the carbapenem resistance genes in edible grasshoppers from the Netherlands (Milanović et al., 2016). This type of resistance has currently not been detected in Dutch livestock, and as carbapenems are considered critically important for human health, it is essential to validate these results with in-depth molecular methods or by culture-dependent methods.

6.3 Microbial hazards in substrates used for edible insect rearing

Potential pathogens, including bacteria, viruses, parasites, and fungi can be present in substrates used to grow insects. We have focused on manure (animal excreta), slaughter waste, supermarket waste, and household kitchen waste. Although the number of scientific papers describing the presence of these potential pathogens in for example manure is large, the numbers of papers describing the potential hazards for insects are limited.

Potentially pathogenic organisms, including enteric bacteria, parasites, viruses, and fungi can be present in manure ((Urra, Alkorta, & Garbisu, 2019), i.e. *Salmonella, E.coli,* protozoa (*Cryptosporidium, Giardia*), viruses (HEV) (Manyi-Loh et al., 2013). Also newly assigned bacteria might be present in household kitchen waste (Vaz-Moreira, Faria, et al., 2009; Vaz-Moreira et al., 2010; Vaz-Moreira, Lopes, et al., 2009), however, these are not necessarily pathogenic. *Clostridium difficile* is an ancient and diverse species and therefore present in several sources: in meat, soil, agricultural by-products as compost, and in manure (Knight & Riley, 2019). *Enterococcus* spp. and also antimicrobial resistant Enterococci were present in pig manure (Ahmad, Ghosh, Schal, & Zurek, 2011).

6.3.1 Black soldier fly larvae

BSFL are well known effective decomposers of organic waste such as household kitchen waste (Jiang et al., 2020) and chicken manure (Bortolini et al., 2020). Larvae appeared to be helpful in reducing the pathogenic bacteria content of manure (Awasthi et al., 2020). Yu et al. (2011) showed that *Bacillus subtilis* strains could be isolated from the gut of BSFL after being fed with spiked chicken manure. These *Bacillus subtilis* bacteria were added into nonsterile fresh hen manure and homogenized. Treated manure was then inoculated with 4-d old BSFL. This illustrates that bacteria can readily be transferred to the gut of BSFL through the substrate.

6.3.2 Yellow mealworm

Different studies indicate the importance of avoiding the introduction of *Salmonella* into the production and thus the YMW via contaminated substrate (Crippen, Sheffield, Beier, & Nisbet, 2018; Jensen, Hansen, & Baggesen, 2020; Wynants, Frooninckx, Crauwels, et al., 2019). See also paragraph 1.5.

6.3.3 Other insects

Although outside the scope of this literature review, other insects in combination with pathogens and substrates came into the picture, and were included in this report. These included the LMW (*Alphitobius diaperinus*), all kind of flies (Diptera), and amongst them non-biting and biting flies, beetles, leafhoppers, and aphids.

6.3.3.1 Other flies (Diptera)

Most Diptera belong to the Brachycera, a suborder characterized by the reduction or fusion of antennal segments to eight or fewer, and by modifications to the larval head and mouthparts. With about 80,000 described species, this group contains many of the best known flies, such as houseflies and fruit flies.

Flies are well known pathogen transporters, including potential zoonotic pathogens. Baldacchino, Desquesnes, Duvallet, Lysyk, and Mihok (2018) summarized:

- nonbiting flies are mainly mechanical carriers of pathogens, especially bacteria (*e.g. Escherichia coli, Moraxella bovis, Staphylococcus aureus*).
- Tabanids and biting muscid flies are mainly mechanical vectors of pathogens including bacteria (*e.g. Bacillus anthracis, Anaplasma marginale*), protozoa (*e.g. Besnoitia besnoiti, Trypanosoma* spp.), and viruses (*e.g.* lumpy skin disease virus), whereas
- tsetse flies are biological vectors of trypanosomes causing African Animal Trypanosomosis. *Brachycera* flies are also developmental vectors of several nematodes (*Thelazia* spp., *Parafilaria bovicola, Stephanofilaria stilesi*).

Black, Hinrichs, Barcay, and Gardner (2018) drew attention to vinegar flies (*Drosophila repleta*) as potential vector of foodborne illness. *Drosophila repleta* were capable of transferring *Escherichia coli* O157:H7, *Salmonella* Saint Paul, and *Listeria innocua* from an inoculated food source to the surface of laboratory enclosures.

The horn fly (*Haematobia irritans irritans*) is an obligate blood-feeding ectoparasite, feeding almost exclusively on cattle. Olafson, Lohmeyer, Edrington, and Loneragan (2014) showed that adult horn flies can transmit *Salmonella enterica* (serovar Montevideo), but a transstadial carriage from larval to the adult stage is inefficient.

Bahrndorff, de Jonge, Skovgard, and Nielsen (2017) sequenced bacterial communities of 90 individual houseflies collected within and between ten dairy farms in Denmark. They showed that the microbiota of houseflies reflect the lifestyle of the housefly, that all kind of potential human pathogenic bacteria can be detected, which should be taken in account when addressing the transmission of pathogens by the housefly. Bahrndorff et al. (2017) also showed that *Campylobacter jejuni* was transmitted from infected larvae to pupae, but not to the adult stage. Wanaratana, Panyim, and Pakpinyo (2011) showed that houseflies are also potential vectors for avian influenza virus, and also African Swine Fever virus (ASFv) was transmitted via flies, that had fed on ASFv-spiked blood, to pigs (Olesen et al., 2018).

6.3.3.2 Other mealworms (Coleoptera)

LMW appears to be capable of vectoring pathogens. L. Zheng et al. (2012) exposed adult and larval *Alphitobius (A.) diaperinus* to two concentrations of *Salmonella enterica*, for different time periods and showed that if *Salmonella* is acquired, it commonly transits the gut, allowing the insect to disperse viable pathogenic bacteria within 2-3 h after exposure. In an experimental study, *A. diaperinus* beetles were also capable of mechanically transmitting Turkey Coronavirus (TCV). However, the virus, if present in beetle guts and on the whole beetle was not viable after 12 hours, indicating that *A. diaperinus* can be involved in transmission of TCV when an active outbreaks occurs, but it is less likely that it will play a role in transmission from *e.g.* contaminated field soils (Watson, Guy, & Stringham, 2000).

Beetles (i.e. adult Coleoptera) as potential vector for the transmission of potential pathogens has been investigated in the poultry broiler production facilities. Hald, Olsen, and Madsen (1998) showed that the hairy fungus beetle (*Typhaea stercorea*) acted as a functional carrier of *Salmonella infantis* between successive broiler cycles. Hazeleger, Bolder, Beumer, and Jacobs-Reitsma (2008) showed that darkling beetles (*Alphitobius diaperinus*) and their larvae played a role in transfer of *Salmonella* and *Campylobacter* between consecutive broiler production cycles. In an experimental studies beetles were able to mechanically transmit mycobacteria, therefore this hazard should be considered when feeding captive animals with larvae (Fischer et al., 2004).

6.3.3.3 Leafhoppers

Soto-Arias, Groves, and Barak (2014) examined the role of plant feeding insects as vectors of *Salmonella (S.) enterica* to agricultural crops. They investigated the potential for transmission and retention of *S. enterica*. Leafhoppers (*Macrosteles quadrilineatus*) were capable of transmitting the bacteria, either by mechanical transmission or by contamination by excretion. Overall, their results suggested that phytophagous insects may serve as potential vectors of *S. enterica* in association with plants.

6.3.3.4 Aphids

Soto-Arias et al. (2014) included in their studies aphids (*Myzus persicae*), besides the leafhoppers, to explore the role of plant feeding insects as vector of *Salmonella (S.) enterica* to agricultural crops. They investigated the potential for transmission and retention of *S. enterica*. Aphids were capable of transmitting the bacteria in ways that are not limited to mechanical transmission. Overall, their results suggested that phytophagous insects may serve as potential vectors of *S. enterica* in association with plants.

6.4 Presence of microbial hazards in ready-to-eat insects and the effect of treatment methods

Several papers described finding the presence of microbial hazards in ready-to-eat insects. The presence of microbial hazards in ready-to-eat insects, *e.g.* collected from markets, is relevant for food safety and estimating the potential risks to human health.

Garofalo et al. (2019) extensively reviewed the microbiota of dried edible insects intended for human consumption. They tested powdered small crickets, whole dried small crickets (*Acheta domesticus*), whole dried locusts (*Locusta migratoria*), and whole dried YMW, through culture-dependent (classical microbiological analyses) and independent methods (pyrosequencing). In all insect batches they found low counts of total mesophilic aerobes, Enterobacteriaceae, lactic acid bacteria, *Clostridium perfringens* spores, yeasts and moulds. Furthermore, they found through pyrosequencing several gut-associated bacteria, some of which may act as opportunistic pathogens in humans. Bacteria that may cause food spoilage were also identified, as well as *Spiroplasma* spp. in mealworm larvae, which may cause neurodegenerative diseases in animals and humans. No viable *Salmonella* spp. and *Listeria monocytogenes* were isolated, but through pyrosequencing presence of *Listeria* spp., *Staphylococcus* spp., *Clostridium* spp. and *Bacillus* spp. (with low abundance) was found. They concluded that potentially harmful species *(i.e*. pathogenic, mycotoxigenic, and spoilage microbes) may be present in edible insects (Garofalo et al., 2019). However, treatment methods, of which heat treatment was the most efficient, can reduce microbial numbers. The authors recommend species-specific mitigation strategies. In general, the microbiota seems to be mainly influenced by the diet and environment. But other factors might also play a role, including vertical transmission of micro-organisms between generations. The authors identified lacking literature regarding fungal, including mycotoxigenic, species and spore-forming bacteria.

It should be noted that presence of microbial hazard in ready-to-eat insects can be a result of contamination along the chain, *e.g.* during storage, and therefore the indicated hazards described in this paragraph might be less relevant with regard to safe insect rearing.

6.4.1 Black Soldier Fly larvae

The presence of viable counts, of *Staphyloccocus aureus*, yeast mould, Enterobacteriacaea and *Salmonella* has been reported in BSF (Kamau et al., 2020; Klammsteiner, Turan, Juarez, Oberegger, & Insam, 2020; Larouche et al., 2019). However, some of the hazards were not detected (i.e. *Salmonella* spp. and *L. monocytogenes* (Vandeweyer, Crauwels, Lievens, & Van Campenhout, 2017); *E.coli* and *Salmonella*, (Kim, Kim, Yoon, Lee, & Kim, 2017)) and large variation between batches of individual rearers were found (Vandeweyer et al., 2017). Other authors also concluded that microbial counts in BSF after processing were absent or low, *e.g.* the absence of *Salmonella* and *E. coli* in compliance with hygiene criteria of European Commission for foodstuff (Cappellozza et al., 2019).

6.4.2 Mealworm

A study on edible YMW in which the effect of different treatments on the presence of microbes was determined showed that a blanching treatment of 60°C for 5 minutes seems most feasible time-temperature combination to reduce the microbial load (total viable aerobic count, Enterobacteriaceae, staphylococci, yeasts and moulds, lactic acid bacteria, aerobic bacterial endospores). Lower temperatures were unable to reduce microbial loads, higher temperatures did not improve product quality (pH and colour) and microbiological parameters. In the studied YMW larvae samples *E. coli, B. cereus, L. monocytogenes* and *Salmonella* spp. were never detected (Mancini et al., 2019). In another study 10 min boiling of YMW killed all Enterobacteriaceae (to not detectable levels), but 10 minutes roasting was less effective. However, bacterial spore formers were not completely inactivated, and may form a potential risk (Klunder, Wolkers-Rooijackers, Korpela, & Nout, 2012).

6.4.3 Adult Acheta domesticus and Ruspolia differens

In a study of Nyangena et al. (2020), in adult *Acheta domesticus* and *Ruspolia differens*, the prepupae of *Hermetia illucens,* and 5th instar larvae of *Spodoptera littoralis*, the effects of treatment methods were examined. All raw insects contained yeast, mould, *Salmonella*, Lac+ enteric bacteria, and *Staphylococcus aureus*. The authors concluded that processing improves the microbial safety: "Boiling (5 min in water of about 96°C) and toasting (5 min in cooking oil of about 150°C decreased aerobic mesophilic bacterial populations, lowered counts of *Staphylococcus aureus*, and eliminated the yeasts and moulds, Lac+ enteric bacteria, and *Salmonella*. Oven-drying alone marginally lowered bacterial populations as well as yeast and moulds, whereas solar-drying alone had no effect on these parameters. Oven-drying of the boiled or toasted products increased the aerobic mesophilic bacteria counts but the products remained negative on Lac+ enteric bacteria and *Salmonella*." However, nutritional values were decreased by these processing methods.

6.5 Transmission from substrate to larvae

Several studies showed transmission of different bacteria from the substrate to mealworms, *i.e. Salmonella typhimurium* (Jensen et al., 2020; Wynants, Frooninckx, Van Miert, et al., 2019). Jensen et al. (2020) found that, upon initial contamination of the substrate with different levels of *Salmonella typhimurium*, the larvae stayed *Salmonella* positive for at least 14 days during rearing at the contaminated substrate, when initial contamination levels were >3.4 log CFU/g. Although proliferation of *Salmonella* was not observed and the *Salmonella* level generally decreased during the rearing period, contamination of the substrate should be avoided and proper treatment of the larvae before consumption is recommended. Wynants, Frooninckx, Van Miert, et al. (2019) investigated whether transmission of *Salmonella* sp. to mealworms could occur, when reared on contaminated wheat bran. The survival of *Salmonella* sp. in larvae and bran depended on the contamination level. High contamination levels (7 versus 4 log CFU/g) resulted in presence of *Salmonella* sp. in larvae and bran after 7 days. The lowest contamination level of *Salmonella typhimurium* (2 log CFU/g) however, was present in most substrate samples even after 7 days of rearing, but not detected in mealworm larvae, indicating either competition with the larvae microbiome or due to their antibacterial effects (Wynants, Frooninckx, Van Miert, et al., 2019).

BSFL reared in seaweed-substrate artificially contaminated with *E. coli, E. coli* O157:H7, *Listeria monocytogenes* and *Vibrio parahaemolyticus* became contaminated by all four bacteria during rearing (8 days) (Swinscoe, Oliver, Ørnsrud, & Quilliam, 2020).

6.6 Antibacterial effects of BSF

Several studies describe the antibacterial effects of BSF on different pathogens: *Salmonella, E. coli Enterococcus* (DiGiacomo & Leury, 2019; Q. Liu, Tomberlin, Brady, Sanford, & Yu, 2008; Lopes, Lalander, Vidotti, & Vinnerås, 2020; Wu et al., 2021). Q. Liu et al. (2008) found that temperature significantly influenced the ability of the BSFL to develop and to reduce *E. coli* counts (biggest suppression at 27°C). Fat from BSF prepupae reduced lactobacilli and streptococci (T. Spranghers, Michiels, et al., 2018). Bessa et al.

(2020) mention in their review that BSF are innate decomposers and can reduce many microbial colonies, as *Enterobacteriaceae* and *Salmonella*. Regarding antimicrobial resistance however, in housefly antimicrobial resistance genes increased following vermicomposting (H. Wang et al., 2017).

However recently, in a series of rearing experiments, De Smet, Vandeweyer, Van Moll, Lachi, and Van Campenhout (2021) did not find any antibacterial effects of BSFL on *Salmonella* in the substrate. The authors recommend doing more inoculation studies with other bacteria, substrates and larvae, and advise to use *Salmonella*-free substrates, and not to count on the antimicrobial activities that BSFL may show in some circumstances.

6.7 Microbiome composition: pathogenic micro-organisms

Several studies investigated the microbiome composition of different insects, without studying specific pathogens. The presence of certain genera might be a potential risk, such as proteobacteria (Bruno et al., 2019), *Xanthomonadaceae* (plant pathogen) (Kawasaki, Kawasaki, Hirayasu, Matsumoto, & Fujitani, 2020). *Enterobacter asburiae* and an unclassified *Bacillus* species have been found in the gut of the waxworm, *Galleria mellonella* L. (Marshall, Dickson, & Nguyen, 2016). In a review of M. Gold, Tomberlin, Diener, Zurbrugg, and Mathys (2018): BSF gut bacteria are dominated by the phyla Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria. In BSFL, *Bacterioides, Dysgonomonas, Phascolarctobacterium* were abundant genera of bacteria. Vigneron, Jehan, Rigaud, and Moret (2019): The microbiota of *T. molitor* includes microbial taxa that may be pathogenic for human and animals, such as Enterobacteriaceae, Streptococcaceae, and Enterococcaceae.

6.8 Summary of microbiological hazards and the effect of treatment methods

In several edible insects, the presence of potential hazardous microbes is described. Assessing these risks is complex as risks also depend on the type of insect, substrate, production process, and treatment and storage methods. Two routes of transmission of pathogens to reared insects are suggested: transmission via the substrate and vertical transmission between generations. Transmission from the substrate is demonstrated for *e.g. Salmonella* sp., *E. coli, E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus*.

Microbiological hazards with zoonotic potential in insects are *Klebsiella pneumoniae* and *Serratia marcescens*. Microbiological hazards mentioned in substrates are bacteria, e.g*.: Campylobacter, Salmonella, E. coli (O157:H7), Bacillus spp., Clostridium spp., Listeria spp*.; viruses, e.g.: Norovirus, Rotavirus, Hepatitis (A and E) viruses, Turkey Coronavirus, African swine fever virus; parasites, e.g.: *Eimeria, Cryptosporidium, Giardia*. Listing the microbiological hazards mentioned in this literature study will not per se lead to the most important microbiological hazards, as some of the potential hazards are hardly studied and others (e.g. *E. coli, Salmonella*) are often included as reference bacteria but this does not necessarily reflect the hazard.

6.9 Data gaps and research needs

More research is needed to detect potentially pathogenic hazards to warm blooded animals and humans in the gut microbiome of edible insects, like reported by Garofalo et al. (2019). Some studies do report potential pathogenic genera, however, every insect farm is different and the microbe population of a farm changes in time, like stated by the Dutch Council on Animal Affairs in their report (Council on animal affairs, 2018), and therefore much information is lacking. A well based monitoring program on these hazards is recommended. We should thereby distinguish between pathogens that are only transmitted passively via the substrate and/or insect, or pathogens that infect the insect (the pathogen will replicate in the insect), or, like with parasites, enable the moulding of parasites in the insect into the next live stage, which may be

pathogenic to warm blooded animals, like poultry. This will also have implications for possible treatment methods, e.g. parasites might be affected by freezing the insects.

Some elements of insect rearing that need to be taken into account with respect to safe insect rearing and thoughts for further research are discussed in this paragraph.

It is suggested that low levels of contamination might be safe (Wynants, Frooninckx, Van Miert, et al., 2019), however studies are needed to investigate what "safe" levels are. Also environmental effects might play a role during rearing: e.g. temperature can cause an increase in pathogen counts (Raimondi et al., 2020), circumstances should be optimized to keep the potential infection pressure as low as possible. Further, one should not count on the antibacterial effect of BSF (De Smet et al., 2021). Although some studies describe antibacterial effects of BSFL on the microbes in the substrate, others do not confirm this (or only to a limited extent) (van Huis, 2020). Regarding treatment methods, these are able to reduce microbial counts, such as heating, or blanching, and the effect of alcohol should be studied. This is also important to avoid transmission of prions from *e.g.* household kitchen waste/slaughter by-products. The presence of psychrotrophic microbes should be studied and taken into account regarding storage of insects. The presence of spore-formers (often heat resistant) microbes requires extensive heat treatments, which are more intensive than currently advised by the European Commission for frass (insect manure) treatment: 1h 70°C is not enough for the spores of bacteria to be inactivated (L. van Campenhout, pers. comm. 2021). It should be noted that treatment as discussed in this paragraph are referring to treatment of microbiological hazards and not to chemical or physical hazards.

With respect to the risk of prions: the use of non-ruminant animal manure and intestinal content, organic waste of vegetable nature should not pose additional risks, assuming the food is prion free. If the source of substrates is not well defined, extensive heat treatment is needed to inactivate prions, and more research on these specific substrates is needed to eliminate this potential risk of transmitting prions.

Further should the process of harvesting be carefully executed, it might impact the risk of transfer from substrate to insects and final products (EFSA, 2015), and it should be according to existing guidelines/legislation. Regarding AMR: the detection methods are important: only PCR-testing to detect AMR genes is not sufficient, culturing or metagenomics are needed to study the presence of genes and potential AMR-risks.

In general it should be noted that biosecurity is very important. If insects can transmit/ take up microbial hazards from the substrate, also microbes present in their environment (from the rearing house or professionals) might form a potential hazard, for food and feed.

6.10 Conclusions and research needs for current project

It is recommended to get more insight in the transmission of specific pathogens. However, as long as the main route of transmission seems to be via the substrate, using a 'clean', specific pathogen free, substrate is most effective. This practice would also avoid vertical transmission. Insects processed under hygienic circumstances do not provide a source of human pathogens (Fanatico et al., 2018), however it may be obvious that all (untreated) organic waste streams may contain potential pathogens, including bacteria, viruses, prions, parasites, and fungi. When these untreated organic waste streams are used to grow insects, transmission of these potential pathogens from substrate to insect and from insect to the consumer of these insects at the end of the food chain may be an issue. All mentioned potential pathogens are summarized in this paper, which gives a start of potential hazards, but is by no means exhaustive.

Primary production should be managed in a way that reduces the likelihood of introduction of hazards and appropriately contributes to insects being safe for animal (feed) and human (food) consumption. This literature review will be helpful and can be used for the development of a risk-based hygiene programmes. Not only primary production is a significant source of hazards, also during further processing there are risks, if hygiene is not well taken into account. Like in other feed and food production facilities, strict hygiene conditions are required for hazard prevention.

In Table 11 the most relevant microbiological hazards for the four residual streams are listed. No pathogenic micro-organisms are expected in meat meal since this is sterilized. It is recommended that the presence of these microbiological hazards in the substrates is analysed. This includes mechanical and vertical transmitted pathogens with a zoonotic potential. Prions as transmissible spongiform encephalopathies (TSEs) are not listed as no suitable tests are available. Furthermore, it can be assumed that marketed European food is prion free, and it is therefore not relevant to test these in the selected substrates. Yeasts and moulds are prioritised for the present project and not listed as, although they might be present in the substrates.

Table 11 *Matrix of most relevant microbiological hazards for the four residual streams.*

Analysis	**Supermarket mix**	**Slaughter by-product**	**Manure of chickens**	**GFE**
Total bacterial count	X	x	X	x
Clostridium perfringens	X			X
Bacillus spp. (*subtilis*, *cereus*)	X			X
Campylobacter (*jejuni*)	X		X	X
Staphylococcus aureus (MRSA)	X			X
Microscopic study parasites	X	x	X	X
Hepatitis E virus (HEV)	X			X
Listeria monocytogenes	X			X
Salmonella spp.	X		X	X
bacterial (an)aerobic spore count	X	x	X	X
AMR resistance genes			X	
Avian Influenza virus (AIV)			X	

A draft longlist for the spiking of substrates in the experiments in this study has been made, which will be finalized based on the results of the tests of the 4 substrates and further considerations. For all substrates the same pathogens for spiking are suggested. This is based on the relevance of these pathogens/diseases in combination with the chance of finding these pathogens in substrates. This list is based on the opinion of veterinarian experts of WBVR: Hepatitis E virus (HEV), Avian Influenza virus (AIV), African or Classical Swine fever (ASF/CSF), *Toxoplasma* spp., *Salmonella* spp., *Clostridium perfringens*, multi resistant *Staphylococcus aureus* (MRSA), and *Klebsiella pneumoniae*. Moreover, mentioned pathogens represent different groups of microbial hazards. Depending on the final choices, it is advised to look closely at the method of spiking. For specific intracellular pathogens, the choice of infected tissue material might be preferable to single pathogens.

7 Overall conclusions

The present literature study provides an overview of the present knowledge on insect rearing, use of residual streams as substrates, chemical and microbiological food and feed safety hazard in insects. This extensive overview should form the basis for experiments in the PPP project SAFE INSECTS.

Optimal rearing conditions and specific nutritional needs for BSFL and YMW should be taken into account.

Next to nutritional needs, water binding capacity, particle size, pH, frequency of feeding are other important factors to take into account for substrate optimalisation. Rearing conditions (seeding density of the larvae, size of experimental units, climate control) should be optimal and well controlled.

The selected residual streams will be treated to obtain optimised and safe substrates for BSFL and YMW rearing. Possible treatments are heat treatment, washing/extraction, fractionation, or dilution by mixing.

Possible food and feed safety hazards will be analysed in the selected residual streams and will be taken along in follow up insects rearing and spiking experiments.

Chemical hazards were identified of being important to include in our experiments are heavy metals, veterinary drugs, pesticides, and possibly some POPs, and PFAS. Furthermore, microplastics are also important to consider to analyse in our studies. Selected microbiological hazards with zoonotic potential in insects are: *Klebsiella pneumoniae* and *Serratia marcescens*. Selected microbiological hazards of interest are bacteria (e.g. *Campylobacter, Salmonella, E. coli (O157:H7), Bacillus spp., Clostridium spp., Listeria spp*.; viruses, e.g.: Norovirus, Rotavirus, Hepatitis (A and E) viruses, Turkey Coronavirus, African swine fever virus; parasites, e.g.: *Eimeria, Cryptosporidium, Giardia.*

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Annex 1 Search strings used

The following search strings were used for the literature study

- TITLE-ABS-KEY (“Black soldier fl*” OR BSF OR “Hermetia illucens” OR mealworm* OR “tenebrio molitor” OR “insect*”) AND TITLE-ABS-KEY (production OR feeding OR substrate)
- TITLE-ABS-KEY (“Black soldier fl*” OR BSF OR “Hermetia illucens” OR mealworm* OR “tenebrio molitor” OR “insect*”) AND TITLE-ABS-KEY (“manure” OR food waste)
- TITLE-ABS-KEY (“Black soldier fl*” OR BSF OR “Hermetia illucens” OR mealworm* OR “tenebrio molitor” OR “insect*”) AND TITLE-ABS-KEY (“Food safety” OR “feed safety”)
- TITLE-ABS-KEY (“Black soldier fl*” OR BSF OR “Hermetia illucens” OR mealworm* OR “tenebrio molitor” OR “insect*”) AND ALL-FIELDS (“supermarket mix” OR “supermarket waste*” OR “vegetable waste*” OR “fruit waste*” OR “food waste*” OR substrate*) AND TITLE-ABS-KEY (“Food safety” OR “feed safety” OR “food hazard*” OR “feed hazard*” OR “chemical safety” OR “physical safety”)
- TITLE-ABS-KEY ("Clostridium perfringens" OR "Campylobacter jejuni" OR "Salmonella" OR "Listeria monocytogenes" OR "Vibrio vulnificus" OR "Vibrio parahaemolyticus" OR "Yersinia" OR "Escherichia coli" OR "0157:H7" OR "0157" OR "BVDV" OR "Bovine Viral Diarrhea" OR "HEV" OR "Hepatitis E" OR "PRSSV" OR "Porcine Reproductive and Respiratory Syndrome" OR "AI" OR "Avian Influenza" OR "COV" OR "Corona" OR "MERS" OR "SARS" OR "COVID" OR "ASF" OR "African Swine Fever" OR "CSF" OR "Classical Swine Fever" OR "FMD" OR "Foot and Mouth Disease" OR "norovirus" OR "Rotavirus" OR "HAV" OR "Hepatitis A" OR "prion") AND TITLE-ABS-KEY ("black soldier fly" OR "hermetic illudens" OR bsf OR mealworm OR "tenebrio molitor")
- TITLE-ABS-KEY ("Clostridium perfringens" OR "Campylobacter jejuni" OR "Salmonella" OR "Listeria monocytogenes" OR "Vibrio vulnificus" OR "Vibrio parahaemolyticus" OR "Yersinia" OR "Escherichia coli" OR "0157:H7" OR "0157" OR "BVDV" OR "Bovine Viral Diarrhea" OR "HEV" OR "Hepatitis E" OR "PRSSV" OR "Porcine Reproductive and Respiratory Syndrome" OR "AI" OR "Avian Influenza" OR "COV" OR "Corona" OR "MERS" OR "SARS" OR "COVID" OR "ASF" OR "African Swine Fever" OR "CSF" OR "Classical Swine Fever" OR "FMD" OR "Foot and Mouth Disease" OR "norovirus" OR "Rotavirus" OR "HAV" OR "Hepatitis A" OR "prion") AND TITLE-ABS-KEY (((supermarket OR household OR kitchen) AND (waste OR byproduct)) OR swill)
- TITLE-ABS-KEY ("Clostridium perfringens" OR "Campylobacter jejuni" OR "Salmonella" OR "Listeria monocytogenes" OR "Vibrio vulnificus" OR "Vibrio parahaemolyticus" OR "Yersinia" OR "Escherichia coli" OR "0157:H7" OR "0157" OR "BVDV" OR "Bovine Viral Diarrhea" OR "HEV" OR "Hepatitis E" OR "prrsv" OR "Porcine Reproductive and Respiratory Syndrome" OR "air" OR "Avian Influenza" OR "COV" OR "Corona" OR "myers" OR "SARS" OR "cvid" OR "ASF" OR "African Swine Fever" OR "CSF" OR "Classical Swine Fever" OR "FMD" OR "Foot and Mouth Disease" OR "norovirus" OR "Rotavirus" OR "HAV" OR "Hepatitis A" OR "prion") AND ALL (manure) AND TITLE-ABS-KEY ("black soldier fly" OR "hermetic illudens" OR bsf OR mealworm OR "tenebrio molitor" OR insect)
- TITLE-ABS-KEY ((resistance AND (antibiotic OR antimicrobial OR genes)) OR AMR) AND TITLE-ABS-KEY ("black soldier fly" OR "hermetic illudens" OR bsf OR mealworm OR "tenebrio molitor")
- TITLE-ABS-KEY ((resistance AND (antibiotic OR antimicrobial OR genes)) OR amr) AND TITLE-ABS-KEY (((supermarket OR household OR kitchen) AND (waste OR byproduct)) OR swill)

- TITLE-ABS-KEY ((resistance AND (antibiotic OR antimicrobial OR genes)) OR AMR) AND TITLE-ABS-
  KEY (manure OR ((supermarket OR household OR kitchen) AND (waste OR byproduct)) OR sw ill) AND ALL ("black soldier fly" OR "hermetic illudens" OR bsf OR mealworm OR "tenebrio molitor" OR insect)

Annex 2 List of pathogens of edible insects described in literature

DIPTERA

Diseases and pathogens of Diptera are described in the review of Maciel-Vergara et al. (2021).

Viruses:
Musca domestica - **viruses:** Lietze et al. (2011), Moussa (1978).

Bacteria:
- Shakoori et al. (1999)
- Zimmer et al. (2013), (Malik et al., 2007), Padmanabhan et al. (2005)
- *Lysinibacillus sphaericus* in mosquitos: potential for future insect control (Berry, 2021)

Fungi:
Hasaballah et al. (2017), Carswell et al. (1998), Barson et al. (1994), Bellini et al. (1992), Steinkraus et al. (1990), Burhan and Annon (2020), Mwamburi et al. (2010). *Hermetia illucens* – Lecocq et al., 2021.

Black Soldier Fly (larvae) *Hermetia illucens*

Viruses:
None (review Maciel-Vergara et al., 2021).

Bacteria:
- *Bacillus thuringiensis* (Joosten et al., 2020)
- *Lysinibacillus sphaericus* (Joosten et al., 2020)
- BSF are able to reduce the bacterial load of food-safety related bacteria such as *Escherichia coli, Salmonella* spp., and *Enterococcus* spp. (in Joosten et al, 2020: Erickson et al., 2004; Liu et al., 2008; Lalander et al., 2013)
- Bact food safety (VandeWeyer, 2018 thesis; Wynants thesis, 2019): *Bacillus cereus* and *Salmonella* sp.
- *Lysobacter* spp., Burhholderiales, *Bacteroides* spp., *Clostridium*, and *Bacillus* spp. (in Joosten et al., 2020: Zheng et al., 2013b)

Fungi:
- *Beauvaria bassiana* expt. pathogenic to BSF (picture disease card Eilenberg 2019, LeCocq et al. 2021)
- None (Joosten et al., 2020)
- *Pichia* (fed on vegetable waste), *Trichosporon, Rhodotorula* and *Geotrichum* (fed on chicken feed)(Varotto et al., 2017)

Parasites:
- To our knowledge, protozoan infections in BSF have not yet been documented (Joosten et al., 2020)
- Entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae): black soldier fly larvae produce only 10% of the nematodes produced on the standard rearing host *Galleria mellonella* (Tourtois, 2014 thesis)
- *Heterorhabditis bacteriophora* (Tourtois et al., 2017)
- *Steinernema carpocapsae* (Tourtois et al., 2017)
- *Steinernema riobrave* (Tourtois et al., 2017)
- *Steinernema feltiae* (Tourtois et al., 2017)

House fly (larvae) *Musca domesticus*

Viruses:
- Lietze et al. (2011), Moussa (1978)
- Hytrosaviruses
- Musca domestica hytrosavirus (**MdHV**)(dsDNA) Loss of the entire colony (Lietze et al. (2007), Prompiboon et al. (2010) in Maciel-Vergara & Ros, 2017)
- Reoviruses
- Idnoreovirus 3 (dsRNA) High mortality (Moussa (1978) in Maciel-Vergara & Ros, 2017)
- housefly salivary gland hyperplasia (SGH) virus (double stranded DNA virus), horizontal transmission, Florida (Coler et al., 1993)
- Muscavirus (MdSGHV) disease (Hytrosavirus)(double stranded DNA virus) cause characteristic salivary gland hypertrophy and suppress gonad development in their hosts (horizontal transmission) (Abd-Alla et al., 2011; Lietze et al., 2013)

Bacteria:
- *Bacillus thuringiensis* (Zimmer, 2013 in Eilenberg 2015; Joosten et al., 2020)
- *Lysinibacillus sphaericus* (Zimmer, 2013 in Eilenberg 2015; Joosten et al., 2020)
- *Pseudomonas fluorescens* (Zimmer, 2013 in Eilenberg 2015; Joosten et al., 2020)
- *Pseudomonas entomophila* (Zimmer, 2013 in Eilenberg 2015; Joosten et al., 2020)
- *Pseudomonas protegens* (Johnson et al., 2018)
- Sensitive to toxins produced by *B. thuringiensis* var. *israelensis, B. thuringiensis* var. *kurstaki* and *B. thuringiensis* var. *kyushuensis* (Zimmer, de Castro et al., 2013), (Pereira, Ferreira et al., 2019))
- *Brevibacillus laterosporus* (Zimmer, de Castro et al. 2013)
- *Pseudomonas aeruginosa* and *Pseudomonas protegens* can cause disease in *Z. morio* and *M. domestica* respectively (Maciel-Vergara, Jensen et al., 2018, Johnson, Weeks et al., 2019)
- *Brevibacillus laterosporus* (Zimmer, 2013 in Eilenberg 2015)
- *B. thuringiensis* var. *Israelensis* (Zimmer et al., 2013)
- *B. thuringiensis* var. *Kurstaki* (Zimmer et al., 2013)
- *Bacillus thuringiensis* (Maciel-Vergara et al., 2021)
- *Bacillus megaterium* (Maciel-Vergara et al., 2021)

Fungi:
- *Entomophthora muscae* (Six and Mullens 1995; Bellini et al., 1992; Lecocq et al., 2018; picture Eilenberg & Jensen, 2018; Kleespies et al., 2008 review) (Maciel-Vergara et al., 2021)
- *Entomophthora schizophone* (Six and Mullens 1995)
- *Entomophthora ferdinandii* (Lopez Lastra et al., 2006)
- *Erynia sensu lato* (Erynia, Pandora, Furia) (Joosten et al., 2020)
- *Strongwellsea* spp. (Joosten et al., 2020)
- *Beauveria bassiana* and spp (natural) (Joosten 2020; Maciel-Vergara et al., 2021)
- *Lecanicillium* spp (natural) (Maciel-Vergara et al., 2021)
- *Ophiocordyceps* spp. (natural) (Maciel-Vergara et al., 2021)
- *Isaria fumosoroseus* (Maciel-Vergara et al., 2021) (Farooq & Freed 2014)
- *Paecilomyces lilacinus* (Maciel-Vergara et al., 2021)
- *Paecilomyces farinosus* in adults (Barson et al., 1994)
- *Tolypocladium cylindrosporum* (M-V, 2021; Barson et al., 1994, Weeks et al., 2017)
- *Lecanicillium lecanii* (M-V, 2021) Barson et al., 1994, Steenberg & Humber 1999)
- *Lecanicillium psalliotae* (Steenberg & Humber 1999)
- *Beauvaria bassiana* ((Barson et al., 1994, Farooq & Freed 2014, Weeks et al., 2017; Eilenberg & Jensen, 2018)
- *Beauvaria bassiana* effective against house fly (Lecuona et al., 2005)
- *Beauveria bassiana, Metarhizium anisopliae* and *M. anisopliae* var. *acridum* growth in BSF: quantity determines effect (Anderson et al., 2011)
- *Beauveria bassiana* and *Metarhizium anisopliae* are horizontally transmitted, is effective (Carcamo et al., 2015)

- *Beauveria bassiana, Metarhizium anisopliae* and *Isaria fumosorosea* on *M. domestica* infectivity: responses were concentration-dependent, with mortality percentages ranging from 53.00% to 96.00% (Farooq and Freed, 2016)
- *Beauveria bassiana* and *Metarhizium anisopliae* effectivity to kill house fly (larvae): better performance of *M. anisopliae* as an adulticidal and larvicidal agent over *B. bassiana* (Mishra et al., 2011)
- *Beauveria bassiana* and *Metarhizium anisopliae* Effects on house fly (larvae): *M. anisopliae* most effective (Sharififar et al., 2011)
- *Geotrichum* sp. (Kleespies et al., 2008 review)
- *Metarhizium* spp. (natural) (Kleespies et al., 2008 review, Eilenberg & Jensen, 2018)
- *Metarhizium anisopliae* in larvae (Barson et al., 1994, Farooq & Freed 2014, Weeks et al., 2017)
- *Metarhizium brunneum* (Machtinger et al., 2016)
- *Acremonium* (Steenberg & Humber 1999)

Parasites:
- None (Maciel-Vergara et al., 2021)
- *Heterorhabditis georgiana* (Shapiro-Ilan et al., 2009 in Eilenberg 2015)
- *Heterorhabditis indica* (Archana et al., 2017)
- *Heterorhabditis heliothidis* (Geden et al., 1986)
- entomogenous nematodes *Steinernema feltiae, S. glaseri* (Steinernematidae), and *Heterorhabditis heliothidis* (Heterorhabditidae) and susceptibility of house fly: Both *S. feltiae* and *H. heliothidis* were more infective for 3rd-instar larvae (21-29%) than for 2nd-instar larvae (2- 6%) at this dosage in poultry manure (Geden et al., 1986)
- *Steinerma carpocapsae* (Archana et al., 2017)
- *Steinerma glaseri* (Archana et al., 2017)
- *Steinernema feltiae* (Geden et al., 1986, Archana et al., 2017)

Mealworms: *Tenebrio molitor, Alphitobius diaperinus, Zophobias morio*

Further reading: In: Review Maciel-Vergara et al., 2021:
- *Tenebrio molitor* - **viruses:** Kelly et al. (1979); **bacteria:** Du R and Laing (2011), Grimont et al. (1979); **fungi:** Barnes and Siva-Jothy (2000), Haine et al. (2008), Bhattarai et al. (2018), Oliveira and Rangel(2018), Rodríguez-Gómez et al. (2009); **microsporidia**: Fisher and Sanborn (1962)
- *Zoophobas morio* - **viruses:** Tokarev et al. (2019); **bacteria:** Maciel-Vergara and Ros (2017); **fungi:** Srygley et al. (2009), Gołębiowski et al. (2020), Rangel et al. (2015)

Viruses:
- Densoviruses (Parvoviridae)(T*. molitor, Z. morio*) Kelly et al. (1979)
- Iridoviridae:
  - Invertebrate iridescent virus 29 (IIV-29) (dsDNA) pupae and adults of *T. molitor* affected (Kelly et al. (1979) in Maciel-Vergara & Ros, 2017)
  - Iridovirus transmission in *Gryll. bimaculatus* (Papp and Marschang, 2019)
  - A Small Iridescent Virus (Type 29) from *T. molitor* (Kelly et al., 1979)(seems low pathogenic, Kelly, 1985)
- (poultry pathogen: Infectious bursal disease virus in *Alphitobius diaperinus* adult beetles (Panzer); McAllister et al., 1995)
- (poultry pathogens: Beetles vectors for birnavirus, infectious bursal disease, and rotavirus of chicken (Goodwin 1996 in Zabielska, 2008))

Bacteria:
- *Aeromonas hydrophila* pathogenic to *T.mol.* (Noonin et al., 2011 in Maciel-Vergara et al., 2021)
- *Pseudomonas aeruginosa* and *Pseudomonas protegens* can cause disease in *Z. morio* and *M. domestica* respectively (Maciel-Vergara, Jensen et al. 2018, Johnson, Weeks et al. 2019)
- *Pseudomonas aeruginosa* in *Z.morio* (LeCocq et al., 2018; Maciel-Vergara et al., 2018)

- *Pseudomonas* sp in *Z. morio* (picture Eilenberg & Jensen, 2018)
- *Serratia marcescens* (Maciel-Vergara et al., 2021)
- *Serratia marcescens* in pupa *T. mol.* (picture Eilenberg disease card 2019)
- *Bacillus subtilis* (Eilenberg disease card 2019)
- *Bacillus thuringiensis*: a new strain against coleoptera (Herrnstadt, C., 1986)
- *Rickettsiella popiliae* (idem)(Kleespies et al., 2018)
- Lezing van Campenhout, maart 2018, congres Ede: In *A. diaperinus*: *Acinetobacter baumannii, Lactobacillus antri, Lactococcus formosensis, Lactococcus formosensis, Aeromonas* spp., *Staphylococcus arlettae, Buttiauxella agrestis*
- Lezing van Campenhout, maart 2018, congres Ede*:* Substraat *A. diaperinus*: *Weissella* spp., *Acinetobacter baumannii, Corynebacterium* spp, *Acetobacter* spp., *Lactobacillus antri, Lactococcus formosensis, Lactobacillus amylolyticus, Lactococcus formosensis, Staphylococcus arlettae, Buttiauxella agrestis*
- *Salmonella typhimurium* in *A. diap:* (Casas, 1968 in Zabielska, 2008)
- *Salmonella enteriditis* (Davies, 1995 in Zabielska 2008)
- *S. saint paul, S. enterica*, thermophilic *Campylobacter* spp., *E.coli, E. intermediana, Micrococcus* sp., *Streptococcus* spp., *Bacillus subtilus* (Zabielska 2008)
- Human pathogens: *Proteus* sp., *Paracolobacterum intermedium* Borman, C. freundii, *Pseudomonas aeruginosa* (Schroeter) Migula, *Serratia marcescens* Bizio, *Klebsiella-Aerobacter* (Casas 1971 in & Zabielka, 2008)
- *Campylobacter jejuni* in adult beetles – however not important vector, because 2 weeks between cycles in poultry (Strother, 2005; Skov 2000, in and Zabielka, 2008)
- In *T. molitor*: *Bacillus thuringiensis* var. *tenebrionis, Bacillus cereus*, schizogregarines, *Rickettsia*, Amoeba, Eugregarines, *Mattesia* sp. (Kleespies et al. 2008 review)

Fungi:
- *Beauveria* and Metarhizium in T. mol. (pictures in disease card Eilenberg, 2019)
- *Beauvaria bassiana* (in *Alphitobius diaperinus*: Castrillo and Brooks (1998), Gindin et al. (2009), Chernaki-Leffer et al. (2007) in Maciel-Vergara et al., 2021; in *T.molitor*: Eilenberg & Jensen, 2018; Lecocq et al., 2018; Kim et al., 2018)
- *B. bassiana* in *T. mol*. (picture, Eilenberg & Jensen, 2018)
- *Beauveria brongniartii* in *T.mol*. (Kim et al., 2018)
- *Beauveria caledonica* in *T.mol*. larvae (Glare et al., 2007)
- *Beauveria australis* in *T.mol*. larvae (Korosi et al., 2019)
- *Beauveria pseudobassiana* in *T.mol*. larvae (Korosi et al., 2019)
- *Beauveria medogensis* in *T.mol.* larvae (Imoulan et al., 2016)
- *Beauveria* and *Metarhizium* in *T. mol*. (pictures Eilenberg disease card, 2019)
- *Isaria fumosoroseus* in *T.mol*. larvae (idem) (Kim et al., 2018)
- *Isaria javanicus* in *T.mol*. larvae (Kim et al., 2018)
- *Heterorhabditis georgiana* in *T. molitor* (Shapiro-Ilan et al., 2009 in Eilenberg 2015)
- *Aspergillus, Penicillium* and *Candida* in *A. diap*. (Casas 1971, Goodwin 1996 in Zabielska 2008).
- *Aspergillis flavus* dominates and produces aflatoxins, harmful to humans (Casas, 1971 in Zabielska 2008)
- *Clonostachys rogersoniana* in *T.mol.* larvae (Kim et al., 2018)
- *Clonostachys rossmaniae* in *T.mol.* larvae (Kim et al., 2018)
- *Cordyceps confragosa* in *T.mol*. larvae (Kim et al., 2018)
- *Paecilomyces* spp. in *T.mol*. larvae (Kim et al., 2018)
- *Metarhizium anisopliae* (Barnes and Siva-Jothy, 2000 in Eilenberg 2015)
- *Metarhizium anisopliae* in *T.mol.* larvae (Kim et al., 2018)
- *Metarhizium anisopliae* in *Z.morio* larvae (Rangel et al., 2004)
- *Metarhizium flavoviridae* (Maciel-Vergara et al., 2021)
- *Metarhizium brunneum* (picture in disease card *T. mol*., Eilenberg, 2019)
- *Metarhizium brunneum* in *T.mol*. larvae (Kim et al., 2018; Korosi et al., 2019)
- *Metarhizium lepidiotae* in *T.mol*. larvae (Kim et al., 2018)
- *Metarhizium robertsii* (Kim et al., 2018, Korosi et al., 2019)
- *Metarhizium flavoviride* (Kim et al., 2018, Korosi et al., 2019)
- *Metarhizium guizhouense* in *T.mol.* larvae (Korosi et al., 2019)
- *Metarhizium pingshaense* in *T.mol*. larvae (Korosi et al., 2019)

- *Pochonia bulbillosa* in *T.mol*. larvae (Kim et al., 2018)
- *Lecanicillium attenuatum* in *T.mol*. larvae (Kim et al., 2018)
- *Purpureocillium lilacinum* in *T.mol*. larvae (Kim et al., 2018)

Parasites:
- *Nosema whitei* in *T. molitor* (Fisher and Sanborn (1962)
- *Heterorhabditis bacteriophagae* in *T. mol*. Larvae (Caroli et al., 1996; Eilenberg, 2015)
- *Heterorhabditis georgiana* in *T. mol*. (Eilenberg, 2015)
- *Steinernema affine* in *T. mol*. (Eilenberg, 2015)
- *Steinernema bibionis* in *T. mol*. (Eilenberg, 2015)
- *Steinernema feltiae* in *T. mol*. (Eilenberg, 2015, 2018)
- Schizogregarines in *T.mol*. (Kleespies et al., 2008)
- Schizogregarines in *Z. morio* (Kleespies et al. 2008 review)
- Eugregarines in *T. mol*. (Kleespies et al., 2008)
- *Gregarina polymorpha* in *T.mol*. and in *Z. morio* (Nocciolini et al. 2018)
- *Eimeria* in *A. diap.* Kleespies 2008, causing coccidiosis in poultry (Goodwin 1996 in Zabielska review, 2008)
- Entomopathogenic nematodes (Rhabditida: Heterorhabditidae and Steinernematidae) screening in *A. diap*.: *Steinernema arenarium* was the most effective causing 99% mortality (Alves et al., 2012)
- Susceptibility *A. diap*. to Entomogenous Nematodes *Steinernema feltiae, Steinernema glaseri* (Steinernematidae) and *Heterorhabditis-Heliothidis* (Heterorhabditidae): Pupal mortality was higher in sandy loam than in clay soil (Geden et al., 1985)
- *Steinernema innovationi* in *T. mol*. larvae: high mortality S-Africa, 100% larval mortality. Pupal mortality ranged from 47 to 68%. (Ramakuwela et al., 2018)
- *Steinernema feltiae* in *T.mol*. larvae (Caroli et al., 1996)
- *Steinernema riobravis* in *T.mol*. larvae (Caroli et al., 1996)
- *Steinernema carpocapsae* in *T.mol*. larvae (Caroli et al., 1996)
- Amoeba, Eugregarines, *Mattesia* sp. in *T.mol*. (Kleespies et al., 2008 review)
- *Hymenolepis diminuta* (Cestoda) 9barley, 1999) and transmission between beetles *T.molitor* (Pappas, 1999)
- *Alphitobius diaperinus* as intermediate host for chicken tapeworms *Choanotaenia-Infundibulum*, and others (Elowni, E. E. and Elbihari, 1979)
- *Mattesia* in *T. mol*. (Kleespies et al., 2008 review)

Crickets: Acheta domesticus, Gryllodes sigillatus

Further reading: Review Maciel-Vergara et al., 2021:
- *Acheta domesticus* - **viruses:** Weissman et al. (2012), Longworth (1978), Pham et al. (2013a), Pham et al. (2013b), Szelei et al. (2011), Christian and Scotti (1994), Plus and Scotti (1984), Eberle et al. (2012), Huger (1985); **bacteria:** Louis et al. (1986), Adamo (1998), Adamo (1999)
- *Gryllus bimaculatus* - **viruses:** Just and Essbauer (2001), Jakob et al. (2002), Wang et al. (2007); **bacteria:** Nai et al. (2014), Vago et al. (1966); **microsporidia:** (Sokolova et al. (2003)
- *Gryllodes sigilatus* - **viruses:** Kleespies et al. (1999); Weissman et al. (2012)
- *Gryllus campestris* - **viruses:** Jakob et al. (2002); Kleespies et al. (1999)
- *Gryllus assimilis* - **viruses:** Jakob et al. (2002); **bacteria:** (Vago and Martoja, 1963)

Viruses:
- Parvoviruses:
  - Acheta domesticus densovirus (**AdDV**) (Parvoviridae (ssDNA)) (densonucleosis virus) mortality up to 100% (Kleespies et al., 2008; (Liu et al. (2011), Szelei et al. (2011), Weissman et al. (2012) In) Maciel-Vergara & Ros, 2017) and only for *A. dom*. pathogenic, not for *Gryllus assimilis, Gryllus bimaculatus* (Szelei et al., 2011)
  - Acheta domesticus mini ambidensovirus (**AdMADV**) Parvoviridae (ssDNA) (Pham et al. (2013c) In: Maciel-Vergara & Ros, 2017)
- Acheta domesticus volvovirus (**AdVVV**) (not yet a virus family) and of *Gryll assimilis* (ssDNA), Pham et al. (2013a,b in Maciel-Vergara & Ros, 2017)

- Iridoviruses in *A. domesticus* en in *Gryll. sigg*. en *Gryll. bimaculatus* (Kleespies et al., 1999, 2008)
  - Gryllus bimaculatus iridovirus (Kleespies et al., 2008; Eilenberg & Jensen, 2018)
  - Invertebrate iridescent virus type 6 (IIV-6) (dsDNA) in young instars of *Gryll. bimacul*. (Just & Essbauer et al., 2001; Gupta et al. (2015), Williams (2008a,b, 2017), Marina et al. (2003), Kleespies et al. (1999) in Maciel-Vergara & Ros, 2017)
- Nudoviruses
  - Gryllus bimaculatus nudivirus (GbNV) (dsDNA), high larval mortality (Huger (1985), Wang et al., 2007; Wang and Jehle (2009) in Maciel-Vergara & Ros, 2017)
- Dicistroviruses:
  - Cricket Paralysis Virus (**CrPV**) (ssRNA) in
    - *domesticus* (Eilenberg & Jensen, 2018; Lecocq et al., 2018)
    - *Gryll. bimaculatus*: 95% mortality (Reingabum et al., 1970; Christian and Scotti (1994), Plus and Scotti (1984), Scotti et al. (1981) in Maciel-Vergara & Ros, 2017)
- Baculovirus in *G. bimaculatus* (Kleespies 2008)

Bacteria:
- *Bacillus cereus* in *Gryll. sigg*. (Kleespies et al., 2008)
- *Bacillus thuringiensis* in *A. diaperinus* larvae (Hua et al., 2014)
- *Pseudomonas aeruginosa* (Maciel-Vergara et al., 2021)
- *Serratia marcescens* (Adamo, 1998; Kleespies et al., 2008)
- *Serratia liquefaciens* experimentally (Eilenberg 2015, Gray 1997)
- *Rickettsiella grylli* is a known intracellular pathogen of *G. bimaculatus* (Vago and Martoja, 1963) but can also infect *A. domesticus* (Seureau 1968) (Adamo, 1998; Maciel-Vergara et al., 2021)
- *Spiroplasma* sp. in *G. bimaculatus* (Nai et al., 2014)
- *domesticus* is also susceptible to infection by *Serratia liquefaciens* and *Serratia marcescens* (Gray 1998), (Adamo 1998)

Fungi:
- *Metarhizium anisopliae* in *A. domesticus* (Ginsberg et al., 2002; Maciel-Vergara et al., 2021)
- *Metarhizium anisopliae* in *A. diaperinus* (Rhode et al., 2006)
- *Metarhizium brunneum* in *A. diaperinus* (Behele and Jackson 2014)
- *Beauveria bassiana* in *A. domesticus* (picture Eilenberg & Jensen, 2018)
- *Beauveria bassiana* in *A. diaperinus* (Santoro et al., 2008)
- *Heterorhabditis georgiana* (Shapiro-Ilan et al., 2009 in Eilenberg 2015)
- *Lecanicillium lecanii* in *A. diaperinus* (Steenberg & Humber 1999)
- *Acremonium* sp. in *A. diaperinus* (Steenberg & Humber 1999)
- *Isaria farinosa* in *A. diaperinus* (Humber and Hansen 2005, Zimmermann 2008)
- *Isaria fumosorosea* in *A. diaperinus* (Humber and Hansen 2005, Zimmermann 2008)
- *Entomophthora grylli* in *G. bimaculatus* (Mcdaniel and Bohls, 1984)
- *Paranosema grylli* in *G. bimaculatus* (Tokarev et al., 2007)

Parasites:
- Eugregarines in *A. domesticus* (Kleepsies et al., 2008)
- *Paranosema grylli* (microspore) in *G. bimacul*. (Maciel-Vergara et al., 2021)
- *Microsporidium grylli* sp nov. In the mediterranean cricket, *G. maculatus* (Tokarev et al., 2007; 2018)
- *Steinernema arenarium* in *A. diaperinus* (Alves et al., 2012)
- *Steinernema innovationi* in *A. domesticus*: low mortality, S-Africa (Ramakuwela et al., 2018)
- *Steinernema carpocapsae* in *A. domesticus* (Rueda 1993)
- *Steinernema carpocapsae* in *A. diaperinus* (Szallanski et al., 2004, Alves et al., 2012)
- *Steinernema feltiae* in *A. diaperinus* (Szallanski et al., 2004, Geden et al., 1985)
- *Steinernema scapterisci* in *A. diaperinus* (Szallanski et al., 2004)
- *Steinernema glaseri* in *A. diaperinus* (Geden et al., 1985)
- *Heterorhabditis bacteriophora* in *A. domesticus* (Rueda 1993)
- *Heterorhabditis bacteriophora* in *A. diaperinus* (Alves et al., 2012)
- *Heterorhabditis heliothidis* in *A. domesticus* (Geden et al., 1985)
- *Heterorhabditis amazonensis* in *A. diaperinus* (Alves et al., 2012)

- *Rhabditis* sp in *A. domesticus* (Rueda 1993)
- *Mermis nigriscens* in *G. bimaculatus* (Tanada and Kaya 1993)
- *Adelina grylli* in *G. bimaculatus* (Tokarev 2005)

Grasshoppers: Locusta migratoria

Further reading: Review Maciel-Vergara et al., 2021:
Bidochka and Khachatourians (1991) - **viruses**: King et al. (1998), Kleespies et al. (1999); Purrini et al. (1988); **bacteria:** Oulebsir-Mohandkaci et al. (2015), Seureau (1968); **fungi:** Maniania et al. (2008), Fu et al. (2010)

Viruses:
- Iridoviruses (Kleespies et al., 1999)
- Entomopox viruses

Bacteria:
- *Bacillus thuringiensis* (Song et al., 2008 in Eilenberg 2015)
- *Pseudomonas aeruginosa* (Maciel-Vergara et al., 2021)
- *Serratia marcescens* (Maciel-Vergara et al., 2021)
- *Serratia* sp. (Kleespies et al., 2008)
- *Serratia marcescens* for biological control of locusts (Tambong et al., 2013)

Fungi:
- *Beauveria bassiana* (Maniania et al. (2008), Fu et al. (2010)
- *Metarhizium anisopliae* (Maniania et al. (2008), Fu et al. (2010)
- *Metarhizium acridum* (Ouedraogo et al., 2004 in Eilenberg 2015)
- *Isaria fumosoroseus*
- *Nosema locustae* (Kleespies et al., 2008)
- *Aspergillus flavus* (Kleespies et al., 2008)

Parasites:
- *Paranosema locusta* (microspore)(Maciel-Vergara et al., 2021)
- *Johenrea locusta* (microspore)
- *Malamoeba locustae* (Kleespies et al., 2008)
- Eugregarines (Kleespies et al., 2008)
- *Malamoeba locustae* (Kleespies et al., 2008)

Annex 3 Description of categories of animal by-products as laid down in Regulation (EC) No 1069/2009

Category 1 material

Category 1 material shall comprise the following animal by-products:

(a) entire bodies and all body parts, including hides and skins, of the following animals:

(i) animals suspected of being infected by a TSE in accordance with Regulation (EC) No 999/2001 or in which the presence of a TSE has been officially confirmed;

(ii) animals killed in the context of TSE eradication measures;

(iii) animals other than farmed and wild animals, including in particular pet animals, zoo animals and circus animals;

(iv) animals used in a procedure or procedures defined in Article 3 of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes (2), in cases where the competent authority decides that such animals or any of their body parts have the potential to pose serious health risks to humans or to other animals, as a result of that procedure or those procedures without prejudice to Article 3(2) of Regulation (EC) No 1831/2003;

(v) wild animals, when suspected of being infected with diseases communicable to humans or animals;

(b) the following material:

(i) specified risk material;

(ii) entire bodies or parts of dead animals containing specified risk material at the time of disposal;

(c) animal by-products derived from animals which have been submitted to illegal treatment as defined in Article 1(2)(d) of Directive 96/22/EC or Article 2(b) of Directive 96/23/EC;

(d) animal by-products containing residues of other substances and environmental contaminants listed in Group B(3) of Annex I to Directive 96/23/EC, if such residues exceed the permitted level laid down by Community legislation or, in the absence thereof, by national legislation;

(e) animal by-products collected during the treatment of waste water required by implementing rules adopted under point (c) of the first paragraph of Article 27:

(i) from establishments or plants processing Category 1 material; or

(ii) from other establishments or plants where specified risk material is being removed;

(f) catering waste from means of transport operating internationally;

(g) mixtures of Category 1 material with either Category 2 material or Category 3 material or both.

Category 2 material

Category 2 material shall comprise the following animal by-products:

(a) manure, non-mineralised guano and digestive tract content;

(b) animal by-products collected during the treatment of waste water required by implementing rules adopted under point (c) of the first paragraph of Article 27:

(i) from establishments or plants processing Category 2 material; or

(ii) from slaughterhouses other than those covered by Article 8(e);

(c) animal by-products containing residues of authorised substances or contaminants exceeding the permitted levels as referred to in Article 15(3) of Directive 96/23/EC;

(d) products of animal origin which have been declared unfit for human consumption due to the presence of foreign bodies in those products;

(e) products of animal origin, other than Category 1 material, that are:

(i) imported or introduced from a third country and fail to comply with Community veterinary legislation for their import or introduction into the Community except where Community legislation allows their import or introduction subject to specific restrictions or their return to the third country; or

(ii) dispatched to another Member State and fail to comply with requirements laid down or authorised by Community legislation except where they are returned with the authorisation of the competent authority of the Member State of origin;

(f) animals and parts of animals, other than those referred to in Article 8 or Article 10,

(i) that died other than by being slaughtered or killed for human consumption, including animals killed for disease control purposes;

(ii) foetuses;

(iii) oocytes, embryos and semen which are not destined for breeding purposes; and

(iv) dead-in-shell poultry;

(g) mixtures of Category 2 material with Category 3 material;

(h) animal by-products other than Category 1 material or Category 3 material.

Category 3 material

Category 3 material shall comprise the following animal by-products:

(a) carcases and parts of animals slaughtered or, in the case of game, bodies or parts of animals killed, and which are fit for human consumption in accordance with Community legislation, but are not intended for human consumption for commercial reasons;

(b) carcases and the following parts originating either from animals that have been slaughtered in a slaughterhouse and were considered fit for slaughter for human consumption following an ante-mortem

inspection or bodies and the following parts of animals from game killed for human consumption in accordance with Community legislation:

(i) carcases or bodies and parts of animals which are rejected as unfit for human consumption in accordance with Community legislation, but which did not show any signs of disease communicable to humans or animals;

(ii) heads of poultry;

(iii) hides and skins, including trimmings and splitting thereof, horns and feet, including the phalanges and the carpus and metacarpus bones, tarsus and metatarsus bones, of:

— animals, other than ruminants requiring TSE testing, and

— ruminants which have been tested with a negative result in accordance with Article 6(1) of Regulation (EC) No 999/2001;

(iv) pig bristles;

(v) feathers;

(c) animal by-products from poultry and lagomorphs slaughtered on the farm as referred to in Article 1(3)(d) of Regulation (EC) No 853/2004, which did not show any signs of disease communicable to humans or animals;

(d) blood of animals which did not show any signs of disease communicable through blood to humans or animals obtained from the following animals that have been slaughtered in a slaughterhouse after having been considered fit for slaughter for human consumption following an ante-mortem inspection in accordance with Community legislation:

(i) animals other than ruminants requiring TSE testing; and

(ii) ruminants which have been tested with a negative result in accordance with Article 6(1) of Regulation (EC) No 999/2001;

(e) animal by-products arising from the production of products intended for human consumption, including degreased bones, greaves and centrifuge or separator sludge from milk processing;

(f) products of animal origin, or foodstuffs containing products of animal origin, which are no longer intended for human consumption for commercial reasons or due to problems of manufacturing or packaging defects or other defects from which no risk to public or animal health arise;

(g) petfood and feedingstuffs of animal origin, or feedingstuffs containing animal by-products or derived products, which are no longer intended for feeding for commercial reasons or due to problems of manufacturing or packaging defects or other defects from which no risk to public or animal health arises;

(h) blood, placenta, wool, feathers, hair, horns, hoof cuts and raw milk originating from live animals that did not show any signs of disease communicable through that product to humans or animals;

(i) aquatic animals, and parts of such animals, except sea mammals, which did not show any signs of disease communicable to humans or animals;

(j) animal by-products from aquatic animals originating from establishments or plants manufacturing products for human consumption;

(k) the following material originating from animals which did not show any signs of disease communicable through that material to humans or animals:

(i) shells from shellfish with soft tissue or flesh;

(ii) the following originating from terrestrial animals:

— hatchery by-products,

— eggs,

— egg by-products, including egg shells,

(iii) day-old chicks killed for commercial reasons;

(l) aquatic and terrestrial invertebrates other than species pathogenic to humans or animals;

(m) animals and parts thereof of the zoological orders of Rodentia and Lagomorpha, except Category 1 material as referred to in Article 8(a)(iii), (iv) and (v) and Category 2 material as referred to in Article 9(a) to (g);

(n) hides and skins, hooves, feathers, wool, horns, hair and fur originating from dead animals that did not show any signs of disease communicable through that product to humans or animals, other than those referred to in point (b) of this Article;

(o) adipose tissue from animals which did not show any signs of disease communicable through that material to humans or animals, which were slaughtered in a slaughterhouse and which were considered fit for slaughter for human consumption following an ante-mortem inspection in accordance with Community legislation;

(p) catering waste other than as referred to in Article 8(f).

Wageningen Food Safety Research
P.O. Box 230
6700 AE Wageningen
The Netherlands
T +31 (0)317 48 02 56
wur.eu/food-safety-research

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To explore the potential of nature to improve the quality of life

Wageningen Food Safety Research
P.O. Box 230
6700 AE Wageningen
The Netherlands
T +31 (0) 317 48 02 56
wur.eu/food-safety-research

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