

Optimization of establishing and growing an axenic black soldier fly larvae (*Hermetia illucens*) model

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PREFACE

As the global population increases, so does the amount of food waste and the need for alternative foods to ensure food supply for future generations. As a result, there is an increasing demand for more sustainable agriculture and waste management. The use of insects could provide an answer to this, which has led to the emergence of industrial insect rearing facilities. One of the promising insects is the black soldier fly (*Hermetia illucens*), as they can efficiently convert organic waste into valuable biomass such as proteins and chitin. However, scientific knowledge on optimizing the productivity of this insect is limited. With this research, I would like to contribute to elucidating the nutritional requirements of black soldier fly larvae as well as the impact of the microbial community on their growth.

Through this way, I would like to thank my supervisors and colleague lab technicians for the support throughout this research. Their knowledge and experience contributed to the positive result of this study. In particular, I would like to thank my supervisors Nate Sibinga, Jeroen De Smet and Bert Verbinnen for their guidance, advice and confidence. I would like to thank Dario Lachi for his support during the chemical analyses. I am very grateful that I was able to learn so many different techniques during this period. Therefore, I look back on this time with satisfaction.

SUMMARY

The ever-increasing amount of food waste and demand for alternative protein sources is causing additional interest in the industrialization of black soldier fly larvae (BSFL) rearing. BSFL are increasingly recognized for their potential in sustainable waste management and as a high-protein feed source due to their ability to convert organic waste into valuable biomass. However, the influence of gut microbiota on their growth and development remains insufficiently understood.

This study focuses on the development and optimization of an axenic (germ-free) model for BSFL, *Hermetia illucens*, to better understand the role of microbiota in larval development and to enhance the efficiency of BSFL-based bioconversion systems. To explore these microbial functions in a controlled way, a reproducible method for producing an axenic BSFL model was established and suitable sterile substrates that could support larval growth in the absence of microbial assistance were identified. This would enable future studies to investigate how specific bacteria influence traits such as larval development, metabolism and resistance. The sterilization of BSFL eggs was achieved using alternating treatments of ethanol and sodium hypochlorite to remove the chorion, which is the primary source of initial microbial colonization. Sterility tests using nutrient broths and agar plates confirmed the effectiveness of the sterilization protocol, which achieved high sterility (93,75%) and viability (88,54%) rates, validating its overall reliability. For the initial eight to nine days, larvae were first reared on a sterile nursery diet consisting of chicken start mash (CSM) before being introduced to various test substrates.

The study compared axenic larvae grown on several treatments, including sterile, non-sterile and pre-incubated forms of CSM and wheat bran (WB) to evaluate the impact of microbial activity in different rearing substrates on larval growth. Pre-incubation allowed microbial activity to produce potentially beneficial metabolites prior to sterilization, enabling assessment of whether these residual compounds could support growth without live microbes. While CSM and WB offer limited flexibility in adjusting their nutritional composition, broth-based diets provide a promising solution. Additional experiments introduced broth-based diets combined with inert matrices like paper bedding and flax, which provided a three-dimensional structure that supports their natural crawling and feeding behavior. These diets allow precise control of nutrient composition, which is essential for future diet optimization studies.

The results showed that microbial activity positively influenced the growth of axenic BSFL for WB as a rearing substrate. While growth on sterile WB was significantly reduced, pre-incubation of WB prior to sterilization restored larval performance, suggesting that microbial metabolites formed during incubation can promote larval development even in the absence of live microbes. No significant differences were observed among the CSM treatments, likely due to its status as a nutritionally rich and well-balanced substrate. Nutritional analyses of these substrates confirmed that microbial activity altered their composition, increasing the availability of key nutrients such as proteins, fats and ash. Furthermore, broth-based diets paired with inert matrices were shown to be a promising approach for axenic rearing, providing the physical structure needed for larval development while maintaining sterility. Flax combined with a protein-rich broth supported the best growth, offering a promising platform for future research on defined diets.

In conclusion, the study successfully established a protocol for rearing axenic BSFL. The axenic model offers a powerful tool for dissecting the functional roles of microbiota in insect growth and metabolism. This research not only enhances the understanding of host-microbiota interactions but also provides practical insight for optimizing BSFL rearing systems for applications in sustainable waste management and animal feed production. The model revealed that microbial activity before sterilization can significantly enhance larval growth, particularly in low-nutrient substrates such as wheat bran. Furthermore, the study identified a broth-based diet that enables larval growth and adjustments in nutritional composition. Flax combined with a protein-rich broth yielded the best growth. This basal broth-based diet is very valuable for further studies on the nutritional requirements of BSFL.

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LIST OF USED ABBREVIATIONS

AMP	Antimicrobial peptide
BSF	Black soldier fly
BSFL	Black soldier fly larvae
CSM	Chicken start mash
GIT	Gastrointestinal tract
PBS	Phosphate buffered saline
PCA	Plate count agar
RT	Room temperature
SD	Standard deviation
SFA	Saturated fatty acids
TSB	Tryptone soy broth
WB	Wheat bran
WHC	Water holding capacity

INTRODUCTION

Hermetia illucens or the black soldier fly (BSF) is a saprophytic insect that shows promise in sustainable waste management due to its larval ability to efficiently convert organic waste into valuable biomass. The use of the black soldier fly larvae (BSFL) for bioconversion has as additional advantages that it is an insect with a short life cycle, does not serve as a vector for disease transmission and contains high quality nutrients allowing it to serve as an alternative protein source (Makkar et al., 2014). Due to the presence of nutrients such as amino acids and fatty acids, it has great potential as animal feed. In addition, the fatty acids can be used to produce biodiesel or incorporated into cosmetic products. The chitin present in the exoskeleton can be converted into chitosan that can be used for medicinal, cosmetic and biotechnological applications as a chelating and antimicrobial reagent. As part of the innate immune system, BSFL produce antimicrobial peptides (AMP) that may have potential as an alternative form of antibiotics. Finally, larval frass can serve as fertilizer (Siddiqui et al., 2022). All of this contributes to the sustainability of the use of the BSFL for agricultural and waste management purposes.

To serve in waste management, efficient mass production of the BSFL is necessary. Research on accelerating the growth and development of BSFL and mainly the role of the gut microbiota is very valuable. The gastrointestinal tract (GIT) of BSFL contains a bacterial flora consisting mainly of Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria. Li et al. (2022) emphasized that the presence of the bacteria *Providencia*, *Citrobacter*, *Klebsiella*, *Ochrobactrum* and *Dysgonomonas* significantly promoted the mass gain of BSFL, prepupae and pupae as well as the eclosion rate. Since the gut microbiota can influence the development of BSF, investing in research on the effect of this gut microbial community on BSF growth and development is extremely valuable. Over time, this will provide more insight into the use of these beneficial bacteria in the mass production of BSFL. Sterile larvae are essential for studying the role of microbiota on larval development (Gold et al., 2020). Axenic larvae are completely free of microorganisms, making this model crucial for testing previously established hypotheses. The effects of the microbiome can be examined by colonizing axenic larvae with microorganisms of interest. The comparison of an axenic model with inoculated axenic larvae will allow the functions of these bacteria to be ascertained (Wu et al., 2023). The aim of this research is to develop an axenic BSFL model which can be used in future studies to further investigate the effects of the microbiome on the growth, development, behavior, immune system, etc. of BSFL.

The axenic model was established by treating the BSF eggs with hypochlorite and ethanol (Gold et al., 2020). These chemicals remove the egg chorion from which the newly hatched larvae obtain their initial bacteria (Wu et al., 2023). The sterilized eggs must be applied to sterilized substrate as the larvae will incorporate external microorganisms from food (Pei et al., 2022). Since many sterilized substrates are not suitable for larval rearing (De Smet et al., 2018), it may be necessary to modify these substrates in a way that preserves sterility and optimizes larval growth. This was part of the focus during the experiments. This research contributes to larger studies on the impact of bacteria on larval growth and development which is valuable for the emerging insect industry.

Since the chorion mechanically protects the insect eggs and maintains moisture levels (Urban-Duarte et al., 2022), axenic larvae require a high-quality nutrient source during the first three to ten days. As a result, a nursery diet is used instead of raw waste materials (Zhang et al., 2023). However, axenic larvae have been shown to develop more slowly than non-sterile larvae. This emphasizes that the microbiome has an important function in larval development. The current research therefore searches for a nursery diet that minimizes the differences in growth rates between axenic and non-axenic larvae, especially over the first seven days. As larvae begin to store fats from the diet during the first instar, it is extremely important to use a suitable nursery diet that is beneficial to the further growth and development of the larvae (Zhang et al., 2023). To accomplish this, research on nutritional requirements will be conducted.

1 THEORETICAL BACKGROUND.

1.1 *Hermetia illucens* and its potential

1.1.1 *Hermetia illucens*

Hermetia illucens or the black soldier fly (BSF) is a fly (Diptera) of the Stratiomyidae family. Natively, it occurs in the tropical, subtropical and warm temperature zones of the Americas (Makkar et al., 2014). The life cycle of the BSF averages up to 45 days (depending on the conditions) and has five stages, namely egg, larva, pre-pupa, pupa and adult as shown in Figure 1. After approximately four days, the eggs hatch and start feeding on organic waste. The larva undergoes five instars where they increase in size and weight, as well as change from a whitish color to brown. The five instars take normally 13 to 18 days (De Smet et al., 2018). The prepupal stage is the last larval stage of the BSF. At this stage the larva empties its digestive tract and stops feeding and moving. The prepupae then migrate in search of a cool, dry, shaded and protected environment for pupation. The duration of pupation can vary from 10 days under optimal conditions to a month. An adult fly emerges after successful pupation by breaking the pupae exoskeleton. They mate as adult flies after which the females finally oviposit 500 to 900 oval-shaped eggs of about 1 mm in length into dry cracks and cervices adjacent to a feed source (Makkar et al., 2014).

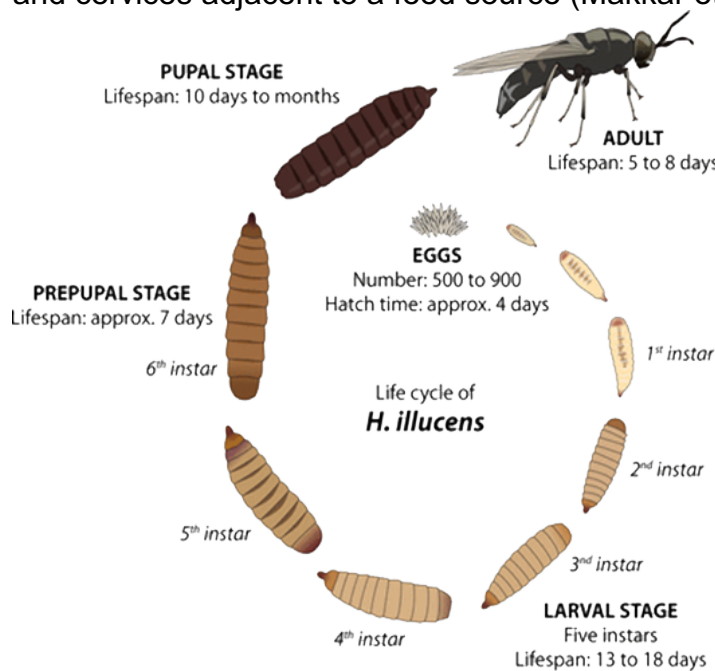


Figure 1. Life cycle of *Hermetia illucens*

Note: Acquired from De Smet, J., Wynants, E., Cos, P., Van Campenhout, L. (2018). Microbial community dynamics during rearing of black soldier fly larvae (*Hermetia illucens*) and impact on exploitation potential. *Applied and environmental microbiology*, 84(9), 2722-17. <https://doi.org/10.1128/AEM.02722-17>.

Optimum conditions for rearing BSF include a temperature between 24 and 40°C and relative humidity between 50 and 70%. Higher relative humidity results in substrate that is too moist, causing loss of structure and complicating the larvae's ability to crawl on it, consume it and get adequate oxygen supply. This also makes the texture, viscosity and moisture content of the substrate important for rearing BSF.

In the adult stage, the fly is black, wasp-like and has a length of 15-20 mm. It consists of three segments, namely the head, thorax and abdomen. BSFL primarily feed on organic wastes such as plant residues, decomposing materials, animal manure, etc. The larvae have a dull, whitish color. They can reach a length up to 27 mm and a width up to 6 mm. In their last larval stage, they can weigh up to 220 mg (Makkar et al., 2014). This insect is an excellent candidate as an alternative protein source. BSFL has the capacity to convert organic waste into chitin, amino-acids, peptides, proteins, lipids, vitamins etc. Since the adult larva has a hook-shaped mouth structure, no sting and no affinity for the human body, they do not serve as a vector for disease transmission (Lu et al., 2022).

1.1.2 Nutritional value

BSFL are rich in nutrients such as amino acids, fatty acids, proteins and minerals. This makes them considered as a more sustainable protein source. The most abundant essential amino acids observed by Zulkifli et al. (2022) were leucine, lysine and valine. Aspartic and glutamic acids are the most abundant non-essential amino acids. The most abundant saturated fatty acids (SFA) in BSFL are lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0). Oleic acid (C18:1 - cis n9) and palmitoleic acid (C16:1) are, according to Lu et al. (2022), the most abundant monounsaturated fatty acids. In contrast, linoleic acid (C18:2n6) and linolenic acid (C18:3n3) are the most abundant polyunsaturated fatty acids (Lu et al., 2022). Calcium (Ca) was the highest amount of mineral noticed in BSFL by Zulkifli et al. (2022). Besides calcium, other minerals present in BSFL are potassium (K), phosphorus (P), sodium (Na), magnesium (Mg), zinc (Zn), iron (Fe), manganese (Mn) and copper (Cu).

1.1.3 Products of BSFL

BSFL have an important role in organic waste management as well as high economic value as a commercial product. For instance, BSFL have great potential to be used as animal feed. This is possible in both processed form and living form. In processed form, the dried larvae or extracted oil/protein meal is used (Siddiqui et al., 2022). BSFL contain arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine of which leucine, lysine and valine, are the most abundant (Lu et al., 2022). These essential amino acids are required in animal feed. The presence of fatty acids is valuable as well since it can be used as a feedstock to produce biodiesel as well as cosmetic products. In addition to its high protein and lipid content, BSFL contain chitin. Chitin is a polysaccharide found mainly in the cuticle and exoskeleton of arthropods, including insects. Chitosan is a polysaccharide obtained by alkaline deacetylation of chitin. It is used as a surgical suture material, edible film, binder and as a chelating and antimicrobial agent in products for medicine, cosmetics and in biotechnology (Siddiqui et al., 2022). Verma et al. (2021) stated that chitosan has a strong ability to form chelates with proteins, amino acids, fats and cholesterol. The final product, larval frass, can serve as a fertilizer and soil enhancer as it is rich in organic nutrients (Siddiqui et al., 2022).

1.1.4 Waste management

Insects like BSFL can be used in organic waste conversion and could serve as alternative protein source for animal feed. The larvae feed on decaying organic matter, allowing them to be used to reduce waste streams. In addition, its capacity as a bio-converter to turn low-value biomass into high-quality products is especially interesting for waste management. The bioconversion rate can be affected by the feed composition (pH, moisture content and digestible nutrient content) and the feeding rates. The protein content of BSFL is comparable to that of traditional animal feed sources such as soybean and fish meal. This is interesting because of the reduced availability of fish due to overfishing and the decrease in available land and water resources. Therefore, BSFL can provide a solution in the evolution toward sustainable agriculture. The insect water footprint is reported to be more efficient compared to farmed animals. Namely, insects are considered 100% edible which makes the water footprint based on nutritional value in L/g protein lower. However, the conversion efficiency of feed substrate to insect protein should be considered on this result. This emphasizes the importance of waste streams as substrate rather than, for example, chicken feed that has high water depletion (Siddiqui et al., 2022).

Additionally, it is suggested that BSFL has antimicrobial activity due to the larvae's ability to modify the microflora of manure, reducing harmful bacteria such as *Escherichia coli* and *Salmonella enterica* (Makkar et al., 2014). BSFL produce antimicrobial peptides (AMPs) as part of the innate immune system. AMPs have the ability to either directly kill microbial pathogens or modify the host defense system (Siddiqui et al, 2022). AMPs are short, positively charged molecules of 10 to 100 amino acids, and are produced by all living organisms. The positive amino acid residues can interact with microorganisms by electrostatic forces with the negative charges on cell membranes.

BSFL are rich in AMPs due to their exposure to many microbial colonies when feeding on decaying substrates. This alternative form of antimicrobial agent could provide a potential solution to the ever-increasing threats of antibiotic resistance in the medical field (Moretta et al, 2020). BSF larval AMPs and the pathogen reducing effect are mostly interesting for waste management with as main purpose the use of BSFL as bioconversion and possibly also as sanitizing step. If BSFL could be used to reduce the presence of pathogens in food waste, this would lead to compost as the end product that could be used as a soil improver with a reduced risk of disease spread and agricultural contamination (Siddiqui et al., 2022).

Whenever BSFL is permitted to be processed into food or animal feed in the future, it is necessary to understand how foodborne pathogens behave in the larvae. By inoculating the rearing substrate with a specific microorganism, the colonization of both the substrate and the insects by this zoonotic pathogen can be monitored throughout the rearing cycle. De Smet et al. (2021) conducted several rearing trials in which chicken feed was inoculated with different levels of *Salmonella*. In contrast to previous studies with manure or aquaculture waste, no significant reducing effect of larvae on *Salmonella* counts in chicken feed as substrate was observed. This indicates a substrate dependence of the food pathogen reducing effect of BSFL. A possible explanation for this difference is that *Salmonella* cells in manure or aquaculture waste are confronted with a less favorable nutritional profile and a larger background microbiota making them more vulnerable to the antimicrobial mechanisms of the BSFL. Further research with other zoonotic pathogens as well as other substrates is necessary to estimate the risks for using BSFL reared on waste streams as animal feed.

1.2 Production of axenic black soldier fly larvae

1.2.1 Bacteria in the gut microbiota and their effect on the larval development

The gastrointestinal tract (GIT) of BSFL consists of 3 parts, namely the foregut, midgut and hindgut. The gut is the main site for digestion in BSFL. Therefore, this is where most enzymatic activity takes place. The bacterial flora in the gut of BSFL also produce hydrolytic enzymes such as cellulases, proteases and lipases that contribute to the digestion of organic waste and other nutrients. The BSFL contains a variety of digestive enzymes with high activity in its gut because it's a saprotrophic insect that lives in environments with high microbial loads. The gut microbes have a supportive function towards their host during the digestion of complex substrates by providing the insect's GIT with metabolic features that are not encoded by its own genome (De Smet et al., 2018). The microbiota contributes not only to host nutrients, but also to protection against parasites and pathogens and modulation of the immune system. Li et al. (2022) identified Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria as the most prevalent bacterial phyla in the intestines of all larval instars. The intestinal microbial communities vary depending on the larval instar.

A summary of the most abundant bacterial genus identified in the BSFL intestines per larval instar in the study by Li et al. (2022) is shown in Table 1. 1. The genera are grouped under their respective phyla.

Table 1. 1. The intestinal microbial community per larval instar at the genus level, as observed in Li et al. (2022)

Phylum	Genus					
	First larval instar	Second larval instar	Third larval instar	Fourth larval instar	Fifth larval instar	Sixth larval instar
Actinobacteria	/	/	/	/	<i>Actinomyces</i>	/
Bacteroidetes	/	/	<i>Dysgonomonas</i>	<i>Dysgonomonas</i> and <i>Chryseobacterium</i>	<i>Dysgonomonas</i>	<i>Dysgonomonas</i>
Firmicutes	/	<i>Clostridium</i>	/	<i>Enterococcus</i>	<i>Enterococcus</i>	<i>Enterococcus</i>
Proteobacteria	<i>Klebsiella</i>	<i>Acinetobacter</i> , <i>Pseudomonas</i> and <i>Klebsiella</i>	<i>Providencia</i> , <i>Morganella</i> and <i>Klebsiella</i>	<i>Acetobacter</i> and <i>Providencia</i>	<i>Providencia</i> and <i>Klebsiella</i>	<i>Providencia</i> and <i>Acetobacter</i>

Li et al. (2022) also investigated the influence of *Providencia*, *Citrobacter*, *Klebsiella*, *Proteus*, *Ochrobactrum* and *Dysgonomonas* on BSF growth and development. *Providencia*, *Klebsiella* and *Dysgonomonas* were found to be abundant in the gut of almost all larval instars and under different rearing conditions. *Citrobacter*, *Proteus* and *Ochrobactrum* were found in the intestines of the adult BSF and less in the different larval instars. The study demonstrated that *Providencia*, *Citrobacter*, *Klebsiella*, *Ochrobactrum* and *Dysgonomonas* significantly promoted the mass gain of BSFL prepupae and pupae, as well as increased the eclosion rate. These findings suggests that certain gut microbiota influence BSF development by promoting eclosion and shortening the larval stage. *Proteus*, however, did not promote the growth and development of BSFL. This may be attributed to its limited metabolic capabilities, as carbon utilization and enzymatic activity analyses revealed that *Proteus* utilizes fewer carbon sources and lacks the enzymes necessary for cellulose degradation, unlike the other bacteria studied. Research on the intestinal microbial community of BSFL and their effect on the growth and development of BSF provides further insight into the use of these beneficial bacteria in the mass production of BSFL and to advance applications in the waste management industry (Li et al., 2022).

1.2.2 Sterilization of the BSF eggs

To study the role of the microbiota on larval development, axenic BSFL are established. Newly hatched larvae typically obtain their initial gut bacteria by consuming the chorion of the eggs, which is naturally seeded with bacteria from the mother (Wu et al., 2023). Therefore, the entire surface area of the egg must be sterilized without destroying the inner layer that contains the larval embryo.

The chemical sodium hypochlorite (the active ingredient in bleach) in combination with ethanol can be used to sterilize the eggs by removing the egg chorion (Gold et al., 2020). The chorion and lipid layer on the surface of the vitelline membrane protect the insect eggs and provide mechanical support. In addition, they have effective functions such as enabling the exchange of respiratory gases, preventing dehydration and flooding by maintaining moisture levels and preventing the loss of valuable solutes (Urban-Duarte et al., 2022). Since the microorganisms of the first instar larvae are mainly from the surface of the eggshells, it is very important to let them make good contact with the chemicals (Li et al., 2022). The BSF lays its eggs in clumps requiring the eggs to be separated before sterilization.

Neonates are vulnerable at this early stage and need high-quality nutritional sources. Because of this, a nursery diet is used to rear newly hatched BSF neonates instead of raw waste materials. The larvae remain on the nursery diet (for which chicken feed is commonly used) for three to 10 days. First instar larvae initiate fat storage from their dietary intake making suitable nursery meals extremely important to have beneficial effects on further larval growth and development (Zhang et al., 2023).

1.2.3 Use of axenic BSFL in experimental research

Axenic BSFL are valuable in studying the functions of the microbiota and their applications in mass rearing for industrial BSF production. By studying the interactions between the microorganisms and the host as well as determining the nutritional requirements of the larvae, process performance and reliability in large-scale rearing can be optimized which is valuable for the emerging insect industry (Gold et al., 2020). The gut microbiota is important in the conversion of organic waste to proteins present in the BSFL. However, the insect gut also allows external microorganisms to colonize and interact with the initial gut microbiota. To examine the role of the whole gut microbiota, a germ-free animal model is often used. In contrast, the monobacterial intestinal animal model is used to study the function of a specific bacterial strain (Pei et al., 2022). The structure of the microbial community can vary depending on the substrate. The bacteria better adapted to digesting a specific substrate have an advantage in the GIT over the less adapted bacteria (De Smet et al., 2018). Additional research on bacteria that promote the bioconversion of certain substrates and the growth and development of BSFL is necessary to reduce the cost of industrial BSF production and increase the benefits.

The aim of this research is to obtain an axenic BSFL model that can contribute to discovering the effects of the microbiome on traits such as growth, development, behavior and immune responses. Axenic insects are insects that are completely free of microbes while the term gnotobiotic describes the association of axenic insects with one or more fully known microbial species. By colonizing axenic larvae with specific microbes assumed to have a positive influence on growth and development, hypotheses can be substantiated or broken down. This allows the effect of the microbiota on insect metabolism to be examined under controlled conditions. By comparing the axenic model with a gnotobiotic model (axenic larvae inoculated with live bacteria), it is feasible to investigate the functions of these bacteria on food digestion. Axenic or gnotobiotic insects can also be applied in research on conferring host resistance of symbiotic microbes to chemical insecticides. Removing or restoring the association of microbes with the insects could affect the insect resistance of the host. The reason for this is possibly because the microbes could provide the host with nutrients to improve adaptation to insecticides. By identifying these specific microorganisms using axenic and gnotobiotic models, insecticide resistance can potentially be countered. In the future, by revealing the bacteria involved in host resistance and associated mechanisms, this could provide, for example, an environmentally friendly pest control strategy.

Further research on beneficial microbial metabolites or the host digestive process can also be performed by using an axenic and gnotobiotic model (Wu et al., 2023). The gut microbiome not only contributes to the digestion of food, but also affects health and fitness. This increases interest in microbiome research. The axenic model can be used to study the effects of gut microbiota on host nutrition and metabolism. This will allow fundamental biological mechanisms to be further elucidated. As previously mentioned, there is hypothesized to be a link between gut microbiota and immunity as well as behavior. For instance, antimicrobial peptides could affect the composition and abundance of the gut microbiota. The gut microbiota could influence host behavior based on appetite, locomotor activity, oviposition choice and so on (Douglas., 2018). Establishing an axenic BSFL model offers many advantages in investigating interactions between the microbiota and the host. This is also a method of confirming previously proposed hypotheses about microorganism functions.

1.3 Impact of different substrates on the growth

1.3.1 Bacteria in substrates and their effect on the larval development

BSFL can grow on a wide range of different substrates ranging from food waste to agricultural by-products to animal and human manure. Previous research has shown that larval growth is stimulated by the presence of more complex or larger microbiota in the substrates. As a result, most sterilized substrates are not well-suited for larval rearing. This poses a challenge when developing an axenic model.

The bacteria present in substrates can improve the digestibility of the substrate, thereby supporting better larval development. Studies with *Bacillus* strains, for example, resulted in increased larval weight and a shortened development time (Yu et al., 2011). This makes research on probiotics, live microorganisms that are beneficial to the host when administered in appropriate and regular levels, promising for insect culture (De Smet et al., 2018).

Probiotics can promote substrate conversion by BSFL and increase insect protein content (Pei et al., 2022). Bacteria isolated from the gut microbiota of BSFL shorten the number of hatching days, enhance prepupae and pupae weight and improve substrate conversion.

Pei et al. (2022) specifically examined the effect of *Bacillus velezensis* EEAM 10B isolated from the gut of BSFL as a probiotic on the conversion performance of BSFL. During this study, a germ-free and a monobacterial BSFL model was used and compared to verify the specific function of *Bacillus velezensis* EEAM 10B. They confirmed that *Bacillus velezensis* EEAM 10B possesses high extracellular enzyme activity and beneficially affects the substrate conversion efficiency of BSFL. The study concluded that *Bacillus velezensis* EEAM 10B is important for BSFL survival by improving substrate conversion rate, average dry weight and protein content by regulating amino acid synthesis and metabolic process.

The addition of bacteria to the gut microbial community can cause structural changes by interacting with other functional bacteria rather than directly on their own. In addition, the gut microbiota also contributes to the health of its host by producing essential nutrients such as vitamins.

Quan et al. (2023) investigated the impact of fermented food waste on the growth and intestinal microorganisms of BSFL. Due to its high moisture content and rich organic material, food waste is highly susceptible to fermentation and decay by microorganisms. Microorganisms such as *Lactobacillus* and *Enterobacteria* hydrolyze macronutrients into smaller molecules for further metabolization. Their study showed that feeding fermented food waste slightly improved BSFL yield. Extended fermentation has also been shown to have a negative impact on the growth of BSFL through proliferation of growth-impeding microorganisms (Quan et al., 2023).

The addition of bacterial supplements in the substrate enables the formation of a specific microbial community structure through interactions between the gut microbiota of BSFL and the feeding environment which may be beneficial for BSFL culture. This new microbial community stimulates BSFL growth and development as well as substrate biotransformation (Li et al., 2022). Environmental bacteria affect not only growth and development, but also the oviposition. For instance, the presence of sterile eggs reduces oviposition of other female BSF (Zheng et al., 2013). Bacteria affect egg deposition as well. The presence of bacteria isolated from competitors of the BSF such as *Cochliomyia macellaria* and *A. diaperinus* leads to repellency and reduced egg deposition. In contrast, bacteria from non-competitive insects such as *Chrysomya rufifacies* enhance egg deposition (De Smet et al., 2018).

Obtaining optimal conditions for egg deposition as well as ensuring reduced development time by accelerating growth promotes BSF productivity for mass production and provides an economic advantage (Siddiqui et al., 2022).

1.3.2 Nutrient requirements of black soldier fly larvae

Larvae are reared better on a balanced diet of cereal processing waste than on a protein-rich or oil-rich substrate (De Smet et al., 2018). Liu et al. (2024) demonstrated that substrate quality was associated with BSFL growth performance and immunity. Optimizing feeding strategies that maximize biomass production is a key challenge for industrial BSFL rearing. The growth, development and waste reduction efficiency of BSFL can be maximized with a nutritionally balanced diet. Substrates composed of many essential macronutrients such as proteins, carbohydrates and fats provide the larvae with the necessary energy for biomass accumulation. For instance, the proteins contribute to the synthesis of enzymes, structural proteins and tissues essential for larval development.

Albalawneh et al. (2024) emphasized that nutrient-rich substrates such as chicken feed promote larval growth and bioconversion rate by containing the essential macronutrients. Combining cost-effective waste materials with nutrient-rich additives can improve the sustainability and efficiency of BSFL utilization. The balance between crude protein and carbohydrate concentrations in the substrate affects optimal growth, but also the compositional traits of the BSFL. However, the effect of the protein-carbohydrate ratio on life cycle traits is modulated by the moisture content of the diet. The BSFL composition can be modulated by the composition of the culture substrate as well. Differences in substrate composition can therefore cause changes in fat accumulation in BSF prepupae, at the expense of proteins and other constituents. The development of diets with the optimal nutritional requirements to meet the nutritional needs of larvae and guarantee excellent rearing performance is one of the challenges for future studies. The final goal of these studies is to initiate mass rearing at an affordable price (Danieli et al., 2019).

The microbiome is also important as symbiotic interactions help host insects adapt to the environment. Many symbionts provide the synthesis of essential amino acids, vitamins and nutrients not present in the substrate and unable to be synthesized by the insect host. In addition, the symbionts can break down indigestible substances for the insects. By systematically adding or removing specific nutrients from chemically defined media, the nutritional requirements of insects can be investigated. Making sterile food from large amounts of autoclaved bacteria for this purpose is innovative because of the possible beneficial role of microbial metabolites in insect development (Wu et al., 2023).

Since BSFL as a product can be used as a protein source for animal feed, it is beneficial to optimize protein meal yield. However, this requires understanding of the amino acid requirements of BSFL to improve the substrate for efficient utilization. Lemme et al. (2023) conducted an amino acid reduction method to determine the ideal ratios between amino acids for optimal performance. This revealed that substantially reducing individual amino acids in the substrate, does not negatively affect the growth of BSFL. However, balance calculations for individual amino acids in the diet indicated the ability of the microbiome to synthesize and balance the reduced amino acids for microbial protein synthesis. Quantification of these effects suggested that the microbiome is capable of adding up to 35% of total amino acid supply to prevent amino acid deficiency. Hence, it can be concluded that the interactions between substrate composition and microbial activity should be taken into account when investigating nutritional requirements (Lemme et al., 2024).

1.4 Aims of the research

Since scientific knowledge on optimizing BSFL productivity is currently limited, this research seeks to initiate further investigation into the influence of the microbial community on larval growth as well as nutritional requirements.

1.4.1 Establishing an axenic model for BSFL

1.4.1.1 Problem statement

The functions of specific microorganisms in the GIT of BSFL are challenging to determine. Indeed, the composition of the microbiota can be heavily influenced by external factors such as substrate and other environmental conditions. Over the years, however, several hypotheses have been stated. These hypotheses are often based on observational research that is not supported by experimental evidence for the stated correlations. An axenic model is valuable to support these hypotheses.

However, several difficulties are encountered while establishing an axenic BSFL model. Axenic BSFL, for example, are difficult to grow up resulting in reduced to no growth. Even the hatching of sterilized eggs is not occurring optimally. Even more difficult is obtaining a complete developmental cycle with axenic flies.

1.4.1.2 Research goals

The establishment of an axenic BSFL model that can serve for further investigation of interactions between the microbiota and the host. To accomplish this, a method should be established that maintains sterility throughout the entire rearing cycle as well as identifying a suitable sterile substrate on which the axenic larvae can grow.

1.4.1.3 Hypothesis

The microbiota is derived both from the chorion of the egg and from the substrate. Sodium hypochlorite alternated with ethanol are promising chemicals to induce dechoriation. These allow the surface area of the eggs to be sterilized without destroying the inner layer containing the larval embryo. This would ensure that the larval viability should remain sufficiently high.

A sterile nursery diet consisting of chicken start mash is used as substrate. This is a high-quality nutritional source commonly used for rearing BSF neonates. After future research on the nutrient requirements of BSFL, a new chemically defined composition can be used as nursery diet.

1.4.2 The effect of the microbiome in different rearing substrates on the growth of axenic BSFL

1.4.2.1 Problem statement

Currently, there is little to no information on the functional role of certain microorganisms on the growth and development of BSFL. As well, the mechanisms of action of these microorganisms and metabolism reactions are not well understood. However, previous studies have shown that the microbial community influences growth, but how and why is still unknown. To optimize production efficiency, it is important to better understand the microbiome and how it can be optimized to improve performance.

1.4.2.2 Research goals

Investigating whether live microorganisms must be present to affect the growth of BSFL. Finding a sterile nursery diet that enables the axenic BSFL model to develop as similarly as possible to non-sterile larvae. Compare the effect of the microbiome in different rearing substrates.

1.4.2.3 Hypothesis

In some substrates the microbiome is essential and in others it is less important for larval growth. As an example, the use of wheat bran as a sterile diet is improved during this research. By pre-incubating substrates before sterilization and subsequently adding the axenic larvae, growth-promoting metabolites produced by microorganisms may remain present even after sterilization. This makes the presence of live growth-promoting microorganisms less important for BSFL rearing. It is the metabolites formed by these microorganisms that are sufficient to promote larval growth.

1.4.3 Developing broth-based substrates for axenic BSFL rearing

1.4.3.1 Problem statement

Chicken feed and wheat bran cannot be easily adjusted in their nutritional composition. A broth diet can provide a solution here as it has the advantages of being easily sterilized and the concentrations of individual components can be easily adjusted for future research on nutritional requirements. However, broth-based diets have not been successful so far because BSFL like a three-dimensional substrate which they can crawl through.

1.4.3.2 Research goals

The determination of a basic diet that can grow axenic BSFL and be adjusted in its nutritional composition in the future.

1.4.3.3 Hypothesis

The problem with broths is not that they don't have enough nutrients but that they don't have the right shape or structure. Creating a three-dimensional structure for the larvae to crawl around in will improve the performance of broth-based diets. Therefore, the correct basal broth diet should be combined with the optimal inert substrate.

2 MATERIALS AND METHODS

2.1 Establishing an axenic model for BSFL

2.1.1 *Hermetia illucens*

The eggs of *Hermetia illucens* were collected weekly from a continuously maintained colony at the insect pilot plant by the Centre of Expertise Sustainable Biomass and Chemistry (Thomas More University of Applied Sciences, Geel, Belgium) on Mondays and Wednesdays. The days of incubation are counted from the day on which the eggs were collected.

2.1.2 Sterilization Black Soldier Fly eggs

The sterilization protocol was modified from experiments performed by Gold et al. (2020). Approximately 0,1 g of eggs were transferred to a pre-weighed 1,5 mL Eppendorf tube using tweezers. The sterilization procedure is performed using sterile technique under a class II biosafety cabinet (Cleanair by Baker, BioVanguard Greenline). Sterilization is accomplished by adding first 1,2 mL 70% ethanol and separation of the eggs through gentle stirring. After discarding the supernatant, 1,2 mL of 1/5x diluted 8° sodium hypochlorite (final concentration: 0,5% NaClO) was added. Both steps are performed three times. After completion of three immersion cycles in ethanol and sodium hypochlorite, the eggs were rinsed three times with sterile demi-water. Another 600 µL of sterile demi-water is then added to the tube to allow the eggs to be transferred in suspension. Then, a setup was made with a sterile cotton pad covered with a sterile square of black construction paper pre-wetted with 70% ethanol as shown in Figure 2. 1. The purpose of pre-wetting the paper is to break the surface tension so that the liquid absorbs through the paper and into the cotton pad underneath. After evaporation of the ethanol, the eggs were pipetted onto the piece of paper using a p1000 pipet tip modified to have an enlarged opening by removal of the bottom 2 mm. After evaporation of the water (approximately 30 minutes), the paper with the eggs on was placed in an autoclaved glass pot (250 mL) with a nursery diet containing 22,46 g of chicken start mash (CSM) mixed with 33,80 g of demineralized water. This was incubated in a climate cabinet (PharmaEvent C/600, Weissttechnik) on 27°C and 65% relative humidity for eight to nine days.

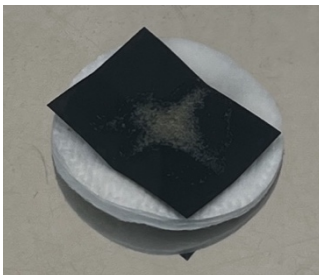


Figure 2. 1. Setup to dry the sterilized eggs

This sterilization protocol was validated by examining larval viability and sterility. For this trial, a 96 well plate (Sarstedt) was filled with 100 µL of plate count agar (PCA) per well as general nutrient medium. One sterile egg per well was then transferred with an autoclaved toothpick. The 96 well plate was then incubated in the climate cabinet. After 48 hours, the wells were checked for both egg hatching and microbial growth.

2.1.3 Sterility tests

Sterility tests were performed to verify the accuracy of the sterilization process. Two different tests that were conducted, namely incubating the sample in Tryptone Soy Broth (TSB) and homogenizing the sample followed by plating using a pour plate method.

Incubation in TSB has the advantage of being a very easy, cheap and sensitive method. Plating has the additional advantage of giving a more precise idea of the amount of contamination based on the number of colonies on the plate.

2.1.3.1 Incubation in Tryptone Soy Broth

Tryptone Soy Broth (TSB, Biokar) is a general nutrient medium. It has excellent nutritional value which makes it conducive to the growth of most microorganisms (Biokar diagnostics, 2022). This allows it to be used for performing sterility tests.

A larva or a small amount of substrate is added into an autoclaved glass test tube (160 mm, VWR) containing 9 mL of TSB using sterile techniques under a class II biosafety cabinet. Sampling is done with a spatula or tweezers sterilized with 99% ethanol and a bead sterilizer (STERI 350, Simon Keller). Finally, the test tubes are incubated shaking at 150 RPM in an incubator (Ecotron, Infors HT) at 30°C for two to three days and then inspected visually for microbial growth.

2.1.3.2 Plating after homogenization

Approximately 20 larvae are collected in a pre-weighed stomacher bag (190 mm x 300 mm – 400 mL, full filter blender bag, Interscience). TSB is added and the larvae are squeezed manually to ensure emptying of the gut content. Next, the stomacher bag is placed in the stomacher machine for three minutes at 8 strokes/second, to mix the sample with the nutrient medium (BagMixer 400W, Interscience). The filter in the stomacher bag keeps the frass from the BSFL on one side and allows 1 mL of suspension to be pipetted on the other side to plate out into a pour plate with Plate Count Agar (PCA, VWR). PCA is a general nutrient medium used for quantitative detection of total germ count. Finally, the petri dish is incubated at 30°C for two to three days and inspected visually for microbial growth.

2.1.4 Sterile transfer of axenic larvae to the substrates

The neonates were reared for the first eight to nine days on an autoclaved nursery diet consisting of 22,46 g of CSM mixed with 33,80 g of demineralized water. After this, the axenic larvae were transferred to the various substrates.

The transfer was performed using sterile techniques under a class II biosafety cabinet. Aluminum foil is sterilized by dry heat sterilization for two hours in an oven (U15, Memmert) at 150°C. A spoon and toothpicks are sterilized by autoclaving (VX-75, Systec) for 20 minutes at 121°C. Using an autoclaved spoon, substrate containing larvae was applied to a piece of sterile aluminum foil. Sterile toothpicks were used to pick up and transfer the individual larvae to the different substrates.

2.1.5 Water holding capacities

Frooninckx et al. (2024) investigated the importance of the substrate moisture content for *H. illucens* larval survival and growth. The substrate moisture content is positively correlated to larval growth but if the substrate moisture content is too high, survival decreases significantly. However, the optimum moisture content depends on the substrate. For example, wheat bran requires much more water than chicken feed before it is a good diet for BSFL. The highest survival and growth are achieved when the substrates are hydrated with an amount of liquid that can be fully absorbed by the substrate. Therefore, a standardized method for determining the optimal dry matter content was developed based on the water holding capacity (WHC). Approximately 0,1 g of substrate is put in a pre-weighted Eppendorf tube. To this, demi-water is added until visually oversaturated. The tube is mixed and incubated for 60 min at RT. After the incubation, the tube is centrifuged (Micro star 17R, VWR) at 10 000g for 30 min at 20°C. The excess water is removed by pipetting and the tube is then turned upside down for 30 min to allow free water to leak out. The tube with the substrate and retained water is weighed. Finally, the water holding capacity is calculated as the percentage of grams of retained water per grams of sample.

$$WHC (\%) = \frac{m_{1,5 \text{ mL tube} + \text{moist substrate after centrifugation}} - m_{1,5 \text{ mL tube} + \text{dry substrate}}}{m_{\text{dry substrate}}} \cdot 100\%$$

To prepare BSFL rearing substrate, the maximum amount of liquid is added to the dry matter to reach the WHC.

2.2 The effect of the microbiome in different rearing substrates on the growth of axenic BSFL

The effect of microbial activity on the growth and development of BSFL was investigated. Chicken start mash (CSM) and wheat bran (WB) were used during this setup, both purchased from AVEVE, Belgium. The effect of the microbiome was expected to be larger on WB than on CSM because CSM is richer in nutrients than WB and the microbiome would compensate for the nutritional deficiencies in WB. Thus, it was hypothesized that pre-incubating WB followed by autoclaving and applying the larvae would promote larval growth and the quality of WB as a substrate. During this pre-incubation, the substrate mixed with demineralized water was incubated for several days in a climate chamber (PharmaEvent C/600, Weisstechnik) at 27°C and 65% relative humidity. The microbial activity during this decaying process could potentially introduce microbially produced metabolites into the substrate that are still present after autoclaving and could positively influence larval development.

2.2.1 Pre-incubated substrates

To investigate these hypotheses, an experimental setup was created with eight treatments each performed in triplicate. The different treatments are presented in Table 2. 1. Each treatment contains 1 g of dry matter mixed with 1,505 g of demi-water for CSM and 3,353 g of demi-water for WB. The amount of demineralized water depends on the water holding capacity (Table 3. 2) of the used substrate. After adding water, the pre-incubated substrates were incubated in a climate cabinet at 27°C and 65% relative humidity for either three or six days, after which they were sterilized by autoclaving (VX-75, Systec) for 20 minutes at 121°C. All treatments were made in glass test tubes (15,30x100x0,80 mm, NAF-VSM). Finally, ten eight- to nine-day-old larvae were transferred from the nursery diet per glass test tube (2.1.4) and incubated in a climate cabinet at 27°C and 65% relative humidity. Once applied to the substrates, larval lengths were measured per incubation day. This was accomplished by using a ruler, without opening the test tubes to ensure sterility. After transferring and rearing the larvae for 21 days, sterility tests with TSB (2.1.3.1) were performed on both the larvae and substrates as well as measuring the lengths and masses of all the larvae.

Table 2. 1. Different substrates examined

Nr substrate	Name substrate
1a,b,c	Sterile CSM (no pre-incubation)
2a,b,c	Sterile WB (no pre-incubation)
3a,b,c	Fresh-made non-sterile CSM
4a,b,c	Fresh-made non-sterile WB
5a,b,c	3-day pre-incubated sterile CSM
6a,b,c	3-day pre-incubated sterile WB
7a,b,c	6-day pre-incubated sterile CSM
8a,b,c	6-day pre-incubated sterile WB

2.2.2 Nutrient determination

During these analyses, the dry matter, ash, fat and protein contents of both dry (fresh) and pre-incubated chicken start mash and wheat bran are determined. This enables these nutrient contents to be compared between CSM and WB as well as between the fresh and pre-incubated samples. Mineral, fat and protein contents are determined to reveal the effect of the microbiome in the different rearing substrates and its correlation to larval growth.

The dry matter, ash, fat and protein contents were analyzed for 35 g dry CSM; 35 g dry WB; 6 days pre-incubated 35 g CSM initially mixed with 52,68 g demineralized water and 6 days pre-incubated 35 g WB initially mixed with 117,36 g demineralized water. Before performing the nutrient analyses, all samples were freeze-dried using a freeze dryer (Lyovapor L-200, BUCHI) at -52,8°C and 0,900 mbar pressure.

2.2.2.1 Determination of dry matter content

The dry matter content is determined to assess the effectiveness of the lyophilization process. Three aluminum trays per sample are cleaned and dried in a ventilated oven (UF 110, Memmert) at 105°C for a minimum of one hour. From now on, the trays will not be touched with hands and only with tongs. After drying, the trays are removed from the oven and cooled to RT by placing them in a desiccator for at least an hour. They are then pre-weighed to the nearest 0,0001 g and weighed 3 g of freeze-dried sample per tray. The sample is spread over the bottom of the tray and placed in a ventilated oven at 105°C for at least 17 hours. The trays are removed from the oven and cooled to room temperature by placing them in a desiccator for at least an hour. Finally, the trays containing the samples are weighed to the nearest 0,0001 g and the dry matter content and moisture content are calculated using the following formulas.

$$\text{Dry matter content (\%)} = \frac{m_{\text{after drying}} - m_{\text{tray}}}{m_{\text{before drying}} - m_{\text{tray}}} \cdot 100\%$$

$$\text{Moisture content (\%)} = \left(1 - \frac{m_{\text{after drying}} - m_{\text{tray}}}{m_{\text{before drying}} - m_{\text{tray}}} \right) \cdot 100\%$$

2.2.2.2 Determination of ash content

The ash content is then determined gravimetrically. Ash is the inorganic residue left after burning organic matter in the presence of oxygen to form carbon dioxide and nitrogen oxides. This inorganic residue consists mainly of minerals present in the sample (Ismail, 2024).

First, the porcelain crucibles (VWR type 459-0211) are labeled. The crucibles are heated on a tripod over a Bunsen burner. Using a syringe filled with an arabic gum solution (10 g of cobalt dinitrate hexahydrate and 10 g of arabic gum in 100 mL of water), the crucibles are labeled with a unique sign per crucible. The labeled crucible is heated in the flame and cooled to room temperature after which the excess cobalt oxide is removed with a dry cloth.

Next, the crucibles are placed overnight in a muffle furnace (Nabertherm) at 550°C. The crucibles are removed from the muffle furnace using tongs and placed on a ceramic plate for 10 minutes to cool. To cool further to room temperature, they are placed in a desiccator for at least an hour. After cooling, the crucibles are accurately weighed to the nearest 0,0001 g.

Then about 5 g of grinded, freeze-dried sample is weighed into a crucible to the nearest 0,0001 g. The crucible is then placed in an angled position on a tripod over a Bunsen burner to ash the sample as shown in Figure 2. 2. The temperature is gradually increased to remove the moisture and the sample begins to ash. When the sample catches fire, it will be covered with a porcelain lid.

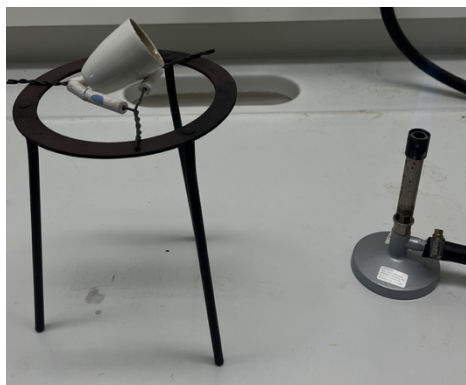


Figure 2. 2. Setup to ash the samples

After this, the sample is heated until no more smoke is visible. Finally, the crucibles are placed in a muffle furnace at 550°C until the sample is brought to constant weight and the ash content can be calculated using the following formula.

$$\text{Ash content (\%)} = \frac{m_{\text{crucible with ash}} - m_{\text{crucible}}}{m_{\text{sample}}} \cdot 100\%$$

2.2.2.3 Determination of fat content with Soxhlet extraction

The fat content is determined with a Soxhlet extraction. This is a liquid-solid extraction in which the liquid solvent is evaporated and condenses so that the droplets enter the extraction thimble with solid sample. Fat present in the sample will then slowly dissolve in the solvent. Finally, by evaporating the solvent, the fat content can be determined (Ismail, 2024).

A Soxhlet extraction tube and 250 mL round-bottom flask are cleaned with acetone, dried at 105°C for several hours and cooled to room temperature. Then 5 g of grinded, freeze-dried sample is weighed into an extraction thimble (30 x 100 mm, Macherey-Nagel) to the nearest 0,0001 g. The sample is covered with a layer of fat-free glass wool to prevent the sample from leaking out of the extraction thimble and the thimble is placed in the extraction tube. The round-bottom flask is then weighed to the nearest 0,0001 g and 120 mL of petroleum ether (40-65°C, VWR) and a stirring rod are added. The Soxhlet setup is made with a heating plate, round-bottom flask, extraction tube and cooler as shown in Figure 2. 3. The extraction is performed for 17 hours at 100°C with a stirring speed of 250 rpm.

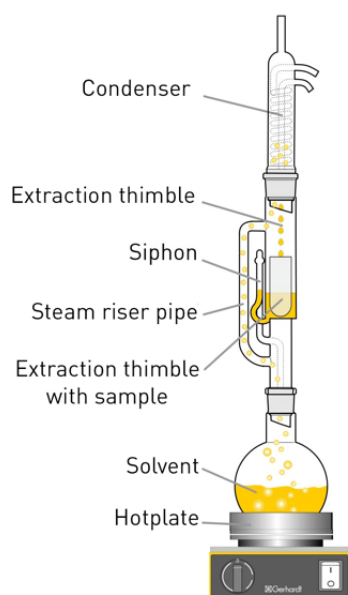


Figure 2. 3. Soxhlet extraction setup

Note: Acquired from C. Gerhardt Analytical Systems. (s.a.). *Extraction methods in fat analysis: solid-liquid extraction*. Gerhardt.de [Website]. Accessed on 8 April 2025 via <https://www.gerhardt.de/en/know-how/analytical-methods/extraction-methods-in-fat-analysis/>

After 17 hours, the setup is cooled to RT. Then the extraction thimble is removed with tweezers, the remaining solvent from the extraction tube is poured into the flask and the stirring rod is removed. The solvent in the round-bottom flask is evaporated with a rotary evaporator (Rotavapor R-300, BUCHI) in a hot water bath, first for 15 minutes at 400 mbar and then for 15 minutes at 260 mbar. After evaporation, the flask is placed in the oven at 105°C for one hour. Afterwards, the flask is cooled to room temperature by placing it in a desiccator for at least an hour. Finally, the flask containing the fat is weighed to the nearest 0,0001 g and the fat content is calculated using the following formula.

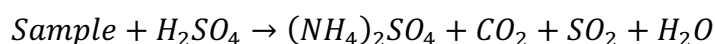
$$\text{Fat content (\%)} = \frac{m_{\text{flask with fat}} - m_{\text{flask}}}{m_{\text{sample}}} \cdot 100\%$$

2.2.2.4 Determination of protein content through Kjeldahl

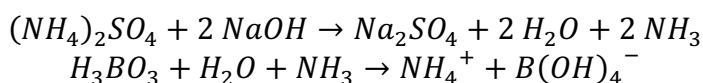
The Kjeldahl method measures the total organic nitrogen content. Three parts are performed, namely destruction, distillation and titration (Ismail, 2024).

For the destruction, 8,0 g of potassium sulfate (VWR), a spatula tip of copper sulfate (VWR) and 20,0 mL of sulfuric acid (95%, VWR) are added to six destruction tubes. The blank consists of only these components. The reference destruction tube additionally contains 0,2000 g of pure acetanilide (min. 98%, UCB) weighed to the nearest 0,0001 g. An additional 0,7000 g of freeze-dried sample, weighed to the nearest 0,0001 g, is added to the destruction tube for the sample.

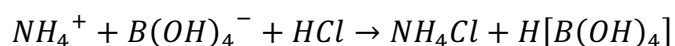
Destruction is performed under a fume hood. The destruction tubes are placed in the destructor (Kjeldatherm, Gerhardt) with the suction on the tubes. It is then heated at 380°C for 2 hours and 45 minutes. After this, the tubes are cooled by placing them at a higher level in the device for about an hour. During destruction, the organic nitrogen present in the sample is degraded to ammonium by heating in an acidic environment in the presence of copper sulfate as a catalyst. The following reaction takes place during destruction:



After destruction, the samples are distilled. Demineralized water is added to generate steam during distillation. The suction hose is first rinsed with a 33% sodium hydroxide solution and the distillation apparatus (Vapodest 200) is cleaned with water before use. To distill the samples, 100 mL of a 4% boric acid solution and 5 drops of bromocresol green indicator are added to a 500 mL Erlenmeyer flask and placed under the condenser of the apparatus. A destruction tube is then inserted into the device. This is going to react with an excess of the 33% sodium hydroxide for 20 seconds to convert the ammonium to ammonia. During the following 9 minutes, distillation is performed at 100% steam capacity to ensure that the ammonia formed is collected in the boric acid solution. The destruction tube is emptied into a waste vessel after cooling and the Erlenmeyer flask is kept for titration. Distillation is repeated for all samples. After distillation of all samples, the suction hose and device are rinsed with demineralized water. During distillation, the following reactions take place:



After distillation, the solutions are titrated with 0,1000 M hydrochloric acid solution to the equivalence point. The bromocresol green indicator will cause a color change from blue (pH >5,4) to yellow (pH <3,8). Finally, the nitrogen and protein content can be calculated from the titrated hydrochloric acid volume. The following reaction takes place during the titration:



The following formula is used to calculate the corrected volume of 0,1000 M hydrochloric acid:

$$V_{HCl, \text{ corrected}} = V_{HCl \text{ titrated}} - V_{HCl, \text{ blank}}$$

The recovery of the acetanilide standard is calculated by the following formulas:

$$m_{\text{acetanilide, calculated}} = V_{HCl, \text{ corrected}} \cdot c_{HCl} \cdot M_{\text{acetanilide}}$$

$$\text{Recovery (\%)} = \frac{m_{\text{acetanilide, calculated}}}{m_{\text{acetanilide, weighed}}} \cdot 100\%$$

The recovery is the percentage of reference compound remaining after the analysis process. In this manner, the accuracy of the method can be assessed.

The nitrogen and protein content are calculated through the following formulas:

$$\text{Nitrogen content (\%)} = \frac{V_{HCl, \text{ corrected}} \cdot c_{HCl} \cdot M_N}{m_{\text{sample}}} \cdot 100\%$$

$$\text{Protein content (\%)} = \text{nitrogen content (\%)} \cdot \text{protein factor}$$

The protein factor is 6,25.

2.3 Developing broth-based substrates for axenic BSFL rearing

To determine whether broth-based diets could be used for axenic rearing of BSFL if given sufficient three-dimensional structure, two non-digestible substrates to which a broth was added were used for the experiments, namely paper bedding (Kowloon) and flax (Jopack horsebedding), all purchased from AVEVE, Belgium.

2.3.1 Nutritional value of non-digestible substrates

To determine differences in nutritional values of the non-digestible substrates, BSFL growth is studied in these substrates mixed with demineralized water. Paper bedding, flax and agar were tested as solid matrix. These three treatments were each created in triplicate. The paper bedding and flax treatments consist of 0,5 g of dry matter mixed with 2,150 g and 2,225 g of demi-water, respectively. The amount of demineralized water depends on the water holding capacity (Table 3. 2) of the used substrate. The third treatment consists of 1,2% agar (VWR) as a control. All treatments were made in glass test tubes (15.30x100x0.80 mm, NAF-VSM) and autoclaved before ten eight-day old larvae per test tube were transferred from the nursery diet (2.1.4). Finally, all test tubes were incubated in a climate chamber at 27°C and 65% relative humidity for 20 days. The lengths were measured per incubation day by using a ruler and without opening the test tubes to ensure sterility. 20 days after the egg collection, sterility tests with TSB (2.1.3.1) were performed on both the larvae and substrates as well as measuring the lengths and masses of all the larvae.

2.3.2 Protein-rich diet

The protein-rich broth diet was used to investigate the effect of different concentrations of yeast and dextrose on the growth and development of BSFL. During this experimental design, 0,5 g of sterile paper bedding was used as dry matter to which 2,150 mL of liquid media consisting of different contents of autoclaved yeast extract (for microbiology, VWR), filter-sterilized dextrose (D(+)-Glucose anhydrous, VWR) and filter-sterilized vitamins (Vanderzant vitamin mixture for insects, Sigma-Aldrich) was added as presented in Table 2. 2. Ten different treatments were setup, each one performed in triplicate.

In addition, another setup was made in which the effect of protein-rich diet in the different non-digestible substrates on BSFL growth and development was examined. Two different treatments were each performed in triplicate. For the first treatment, 0,5 g of sterile paper bedding was used as a solid matrix. This was 0,5 g of sterile flax for the second treatment. The liquid media that was added consists of autoclaved 10% yeast extract (for microbiology, VWR), filter-sterilized 5% dextrose (D(+)-Glucose anhydrous, VWR) and 0,50% filter-sterilized vitamins (Vanderzant vitamin mixture for insects, Sigma-Aldrich). The amount of liquid medium added depended on the water holding capacity of the substrate (Table 3. 2).

Ten eight- to nine-day-old axenic larvae were added to all treatments of both experimental designs. All test tubes containing larvae and substrate were incubated in a climate chamber at 27°C and with 65% relative humidity. Once applied to the substrates, larval lengths were measured per incubation day. This was accomplished by using a ruler, without opening the test tubes to ensure sterility. For the second setup, sterility tests with TSB (2.1.3.1) were performed on both the larvae and substrates after 20 days and the lengths and masses of all larvae were measured.

Table 2. 2. Contents of yeast extract, dextrose and vitamins in the different liquid mediums

Nr	$\omega_{\text{yeast extract}} (\%)$	$\omega_{\text{dextrose}} (\%)$	$\omega_{\text{vitamin}} (\%)$
1	9,30	4,98	0,47
2	4,65	4,98	0,47
3	9,30	2,49	0,47
4	4,65	2,49	0,47
5	6,98	3,74	0,47
6	6,98	2,49	0,47
7	6,98	4,98	0,47
8	9,30	3,74	0,47
9	4,65	3,74	0,47
10	0,00	0,00	0,47

2.3.3 Sugar-rich diet

The sugar-rich broth diet was used to investigate the effect of different non-digestible substrates on BSFL growth and development. Three different treatments were each performed in triplicate. The applied liquid medium is based on the food composition used by Dodge et al. (2023) for *Drosophila melanogaster*. The liquid media consists of autoclaved 5% yeast extract (for microbiology, VWR) and filter-sterilized 10% dextrose (D(+)-Glucose anhydrous, VWR) and is added depending on the water holding capacity of the substrates (Table 3. 2) to 0,5 g dry matter. Two non-digestible substrates, namely paper bedding and flax were used as dry matter. The third treatment consisted of the liquid medium and 1,2% autoclaved agar (VWR). This experimental design was conducted twice: first with axenic larvae reared for eight to nine days on a nursery diet of chicken mash before being transferred to the different diets (2.1.4), and second, with freshly sterilized eggs applied directly to the different diets (2.1.2).

In addition, another setup was made as a repetition of the previous experiment. Here, ten eight-day-old neonates were transferred to each test tube (2.1.4) to standardize the number of larvae present. Additionally, 0,50% filter-sterilized vitamins (Vanderzant vitamin mixture for insects, Sigma-Aldrich) was added to the liquid medium. Two different treatments were each performed in triplicate. For the first treatment, 0,5 g of sterile paper bedding was used as a solid matrix. This was 0,5 g of sterile flax for the second treatment. The amount of liquid medium added depended on the water holding capacity of the substrate (Table 3. 2).

Finally, all test tubes containing larvae and substrate were incubated in a climate chamber at 27°C and with 65% relative humidity after which the lengths were measured per incubation day by using a ruler and without opening the test tubes to ensure sterility. For the second setup, sterility tests with TSB (2.1.3.1) were performed after 20 days on both the larvae and substrates and the lengths and masses of all larvae were measured.

2.3.4 Impact of grinded paper bedding and flax on obtaining the nutrients

The difference in obtaining the nutrients absorbed by the non-digestible substrates between regular and grinded solid matrices was investigated in this experimental setup. The effectiveness of the flax sterilization process was also investigated during this setup.

Four different solid matrices were used as shown in Table 2. 3. The treatments with paper bedding were performed once and the one with flax was performed in duplicate. By this approach, more results are obtained for flax since there were doubts about the sterility of this substrate. For each test tube, 0,5 g of dry matter was weighed and mixed with an amount of liquid medium depending on the water holding capacity (Table 3. 2) of the substrate. The liquid medium used was the sugar-rich diet consisting of autoclaved 5% yeast extract (for microbiology, VWR), filter-sterilized 10% dextrose (D(+)-Glucose anhydrous, VWR) and 0,50% filter-sterilized vitamins (Vanderzant vitamin mixture for insects, Sigma-Aldrich). A drop of water with sterilized eggs on a small square of black construction paper was transferred to all treatments. All test tubes containing sterile eggs and substrate were incubated in a climate chamber at 27°C and with 65% relative humidity after which the lengths were measured per incubation day by using a ruler and without opening the test tubes to ensure sterility. The sterility of the black construction paper and egg debris was tested after hatching with TSB (2.1.3.1). This was three days after egg collection. After 22 days, samples were taken for each test tube to verify sterility in TSB (2.1.3.1). as well as measuring the lengths and masses of all the larvae.

Table 2. 3. Solid matrices

Nr	Solid matrix
1	Sterile paper bedding
2	Sterile grinded paper bedding
3a, b	Sterile flax
4a, b	Sterile grinded flax

2.4 Data statistical analysis

The differences between the mean individual masses of axenic BSFL in different substrates were subjected to a one-way ANOVA test at a significance level of 5%, followed by the Bonferroni post hoc test ($p < 0,05$). Prism 10 was used to create the graphs and perform the statistical analyses.

3 RESULTS

3.1 Establishing an axenic model for BSFL

The final achieved sterilization protocol can be found in Appendix 1.

3.1.1 Larval sterility and viability after sterilization

A 96 well plate containing 100 μL PCA as general nutrient medium and one sterilized egg per well was used to investigate larval sterility and viability. The results two days after collection and sterilization of the eggs are shown in Table 3. 1.

Table 3. 1. Results validation sterilization protocol after 2 days

	1	2	3	4	5	6	7	8	9	10	11	12
A	No growth Hatched	No growth Not hatched	No growth Not hatched	No growth Hatched	No growth Not hatched	Growth Hatched	No growth Hatched	No growth Not hatched	No growth Not hatched	No growth Hatched	No growth Hatched	No growth Hatched
B	No growth Not hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Not hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Not hatched
C	No growth Not hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched
D	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	Growth Hatched	Growth Hatched
E	No growth Hatched	Growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched
F	No growth Hatched	Growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched
G	No growth Hatched	No growth Hatched	No growth Hatched	Growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched
H	No growth Hatched	No growth Hatched	No growth Not hatched	No growth Not hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched	No growth Hatched

Calculations:

$$\text{Larval sterility (\%)} = \frac{\text{number of sterile wells}}{\text{total number of wells}} \cdot 100\% = \frac{90 \text{ wells}}{96 \text{ wells}} \cdot 100\% = 93,75\%$$

$$\text{Larval viability (\%)} = \frac{\text{number of eggs hatched}}{\text{total number of eggs}} \cdot 100\% = \frac{85 \text{ eggs}}{96 \text{ wells}} \cdot 100\% = 88,54\%$$

3.1.2 Water holding capacities

Table 3. 2 presents the results of the water holding capacities of the different substrates

Table 3. 2. Results water holding capacities

Substrate	WHC (%)
Chicken start mash	150,5
Wheat bran	335,3
Paper bedding	430,0
Wood shavings	445,0

3.2 The effect of the microbiome in different rearing substrates on the growth of axenic BSFL

3.2.1 Sterility of the larvae and the substrates

After transferring the neonates to the substrates, the test tubes are not opened again to ensure sterility. After 21 days, sterility tests with TSB (2.1.3.1) were performed on both the larvae and substrates. The results of these sterility tests are presented in Table 3. 3. Pictures of the sterility tests can be accessed in Appendix 2 in Table 1.

Table 3. 3. Sterility of larvae and substrates

Nr substrate	Name substrate	Sterility 1 larva	Sterility substrate
1a	Sterile CSM (no pre-incubation)	No growth	No growth
1b	Sterile CSM (no pre-incubation)	No growth	No growth
1c	Sterile CSM (no pre-incubation)	No growth	No growth
2a	Sterile WB (no pre-incubation)	No growth	No growth
2b	Sterile WB (no pre-incubation)	No growth	No growth
2c	Sterile WB (no pre-incubation)	No growth	No growth
3a	Fresh-made non-sterile CSM	Growth	Growth
3b	Fresh-made non-sterile CSM	Growth	Growth
3c	Fresh-made non-sterile CSM	Growth	Growth
4a	Fresh-made non-sterile WB	Growth	Growth
4b	Fresh-made non-sterile WB	Growth	Growth
4c	Fresh-made non-sterile WB	Growth	Growth
5a	3-day pre-incubated sterile CSM	No growth	No growth
5b	3-day pre-incubated sterile CSM	Growth	Growth
5c	3-day pre-incubated sterile CSM	No growth	No growth
6a	3-day pre-incubated sterile WB	No growth	No growth
6b	3-day pre-incubated sterile WB	No growth	No growth
6c	3-day pre-incubated sterile WB	No growth	No growth
7a	6-day pre-incubated sterile CSM	No growth	No growth
7b	6-day pre-incubated sterile CSM	No growth	No growth
7c	6-day pre-incubated sterile CSM	No growth	No growth
8a	6-day pre-incubated sterile WB	No growth	No growth
8b	6-day pre-incubated sterile WB	No growth	No growth
8c	6-day pre-incubated sterile WB	No growth	No growth

Microbial growth was expected in tubes 3a-c and 4a-c, as these treatments used non-sterilized feed substrates. The results of test tube 5b were discarded during the establishment of the following graphs since sterility was not preserved in it.

3.2.2 Larval growth

Figure 3. 1 and Figure 3. 2 present the growth curves of the axenic BSFL applied to different treatments with chicken start mash and wheat bran, respectively. Larval lengths were measured from day 10 to 21. The growth curves are based on different numbers of measured larvae depending on their visibility. The number of larvae measured per treatment per day is shown in Appendix 2 in Table 2.

The growth performance of the axenic BSFL model was relatively similar for the different CSM treatments.

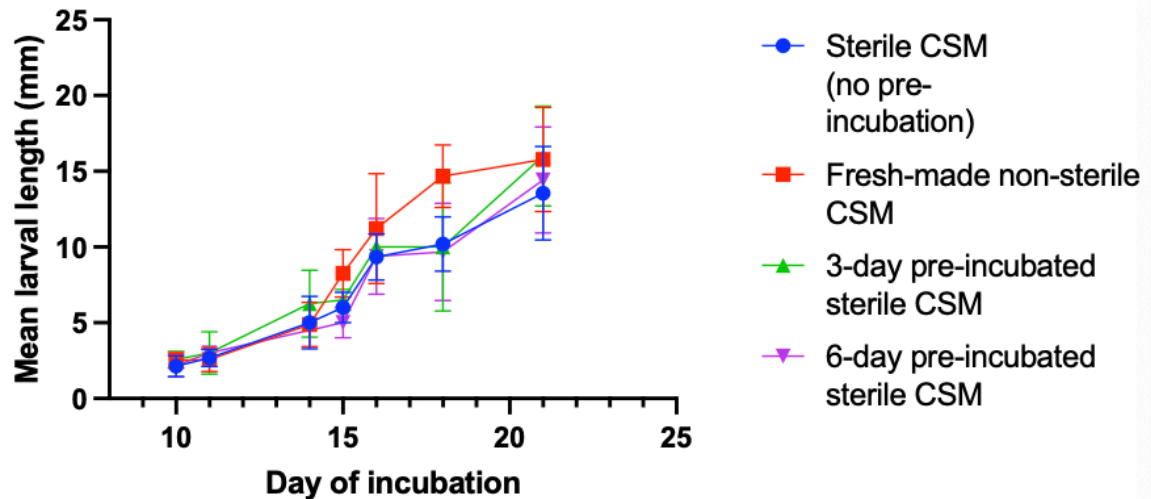


Figure 3. 1. Growth curve of axenic BSFL in the different chicken start mash treatments. The error bars represent the standard deviations (SD).

The larval growth of axenic BSFL on sterile WB was reduced compared to the pre-incubated and non-sterile WB.

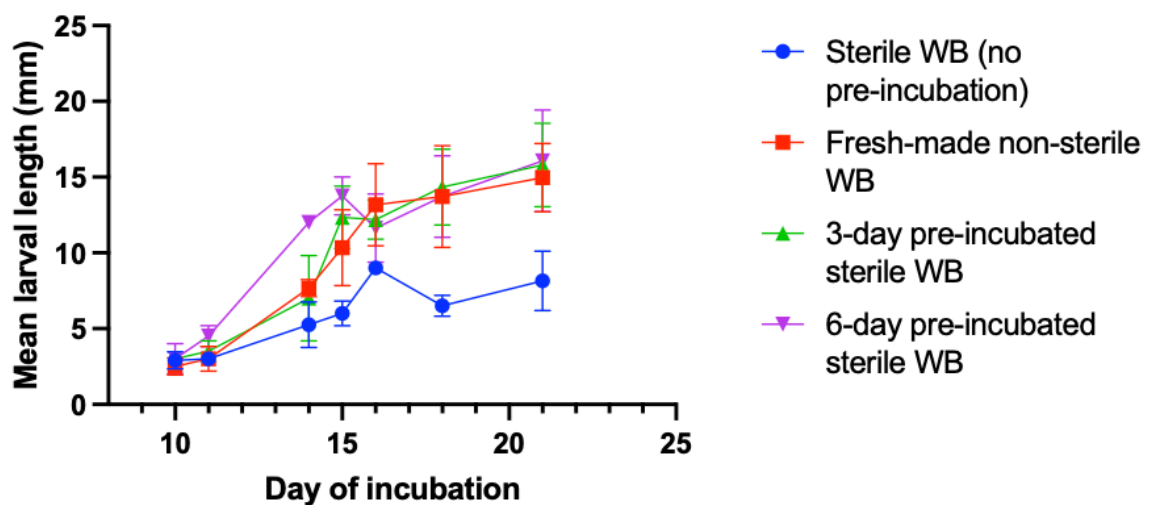


Figure 3. 2. Growth curve of axenic BSFL in the different wheat bran treatments. The error bars represent the SD.

Figure 3. 3 and Figure 3. 4 present the mean individual mass of axenic BSFL applied to different treatments with chicken start mash and wheat bran, respectively. Larval masses were measured on day 21.

For the CSM treatments, there is no significant difference between the mean values of the series (tested with $\alpha = 0,05$).

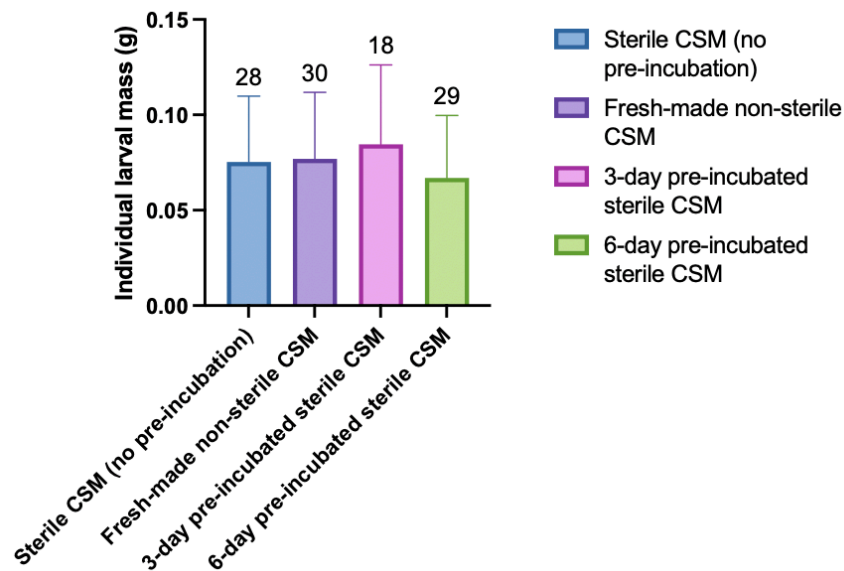


Figure 3. 3. Mean mass of an individual axenic BSFL in the different chicken start mash treatments at 21 days. The error bars represent the SD. The number of larvae measured per treatment is displayed above the error bar.

Significant differences between the sterile WB and the other three WB treatments were obtained. This is represented by the asterisks on the graph.

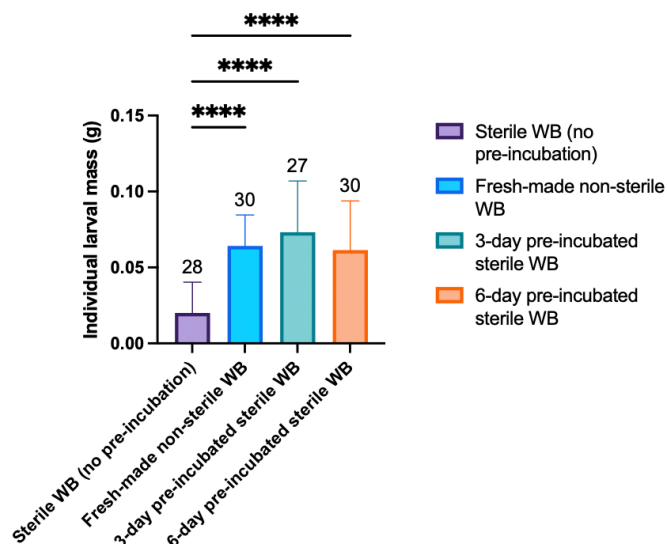


Figure 3. 4. Mean mass of an individual axenic BSFL in the different wheat bran treatments at 21 days. The error bars represent the SD. The number of larvae measured per treatment is displayed above the error bar. Significant differences are represented by the four asterisks ($p < 0,0001$).

Survival rates are determined for each test tube based on the number of larvae measured at the end of the experiment. The average survival rate per treatment is presented in Table 3. 4. Ten neonates were applied to each test tube.

Table 3. 4. Average survival rates of the larvae

Treatment	Average survival rate (%)
Sterile CSM (no pre-incubation)	93,3
Sterile WB (no pre-incubation)	93,3
Fresh-made non-sterile CSM	100
Fresh-made non-sterile WB	100
3-day pre-incubated sterile CSM	90
3-day pre-incubated sterile WB	90
6-day pre-incubated sterile CSM	96,7
6-day pre-incubated sterile WB	100

Formula for the larval survival rate in a test tube:

$$\text{Survival rate (\%)} = \frac{\text{Number of larvae measured at the end of the experiment}}{\text{Total amount of larvae added in the beginning of the experiment}} \cdot 100\%$$

3.2.3 Nutrient determination

The dry matter content, ash content, fat content and protein content of fresh and six-day pre-incubated chicken start mash and wheat bran was determined. The results are shown in Table 3. 5.

Table 3. 5. Results of nutrient analyses

Sample	Average dry matter content (%)	Ash content (%)	Fat content (%)	Crude protein content (%)
Fresh chicken start mash	96,208	6,5516	3,6611	19,47
Fresh wheat bran	97,840	6,1339	2,8344	18,17
6-day pre-incubated chicken start mash	96,168	8,6708	5,4058	32,16
6-day pre-incubated wheat bran	97,017	9,5226	2,7380	25,69

For the determination of protein content with Kjeldahl, the recovery of the acetanilide standard was calculated to evaluate the accuracy of the method. The recovery is 99,56 %.

3.3 Developing broth-based substrates for axenic BSFL rearing

3.3.1 Nutritional value of non-digestible substrates

After transferring the neonates to the non-digestible substrates, the test tubes are not opened again to ensure sterility. After 20 days, sterility tests with TSB (2.1.3.1) were performed on both the larvae and substrates. The results of these sterility tests are presented in Table 3. 6. Pictures of the sterility tests can be accessed in Appendix 3 in Table 3.

Table 3. 6. Sterility of larvae and substrates

Name substrate	Sterility 1 larva	Sterility substrate
Paper bedding mixed with demi-water 1	No growth	No growth
Paper bedding mixed with demi-water 2	No growth	No growth
Paper bedding mixed with demi-water 3	No growth	No growth
Flax mixed with demi-water 1	No growth	No growth
Flax mixed with demi-water 2	No growth	No growth
Flax mixed with demi-water 3	No growth	No growth
Agar mixed with demi-water 1	No growth	No growth
Agar mixed with demi-water 2	No growth	No growth
Agar mixed with demi-water 3	No growth	No growth

Figure 3. 5 presents the growth curve of the axenic BSFL applied to paper bedding, flax and agar mixed with demi-water. Larval lengths were measured from day 9 to 20. The growth curve is based on different numbers of measured larvae depending on their visibility. The number of larvae measured per treatment per day is shown in The larval growth of axenic BSFL on sterile flax mixed with demi-water was increased compared to paper bedding and agar as inert matrices.

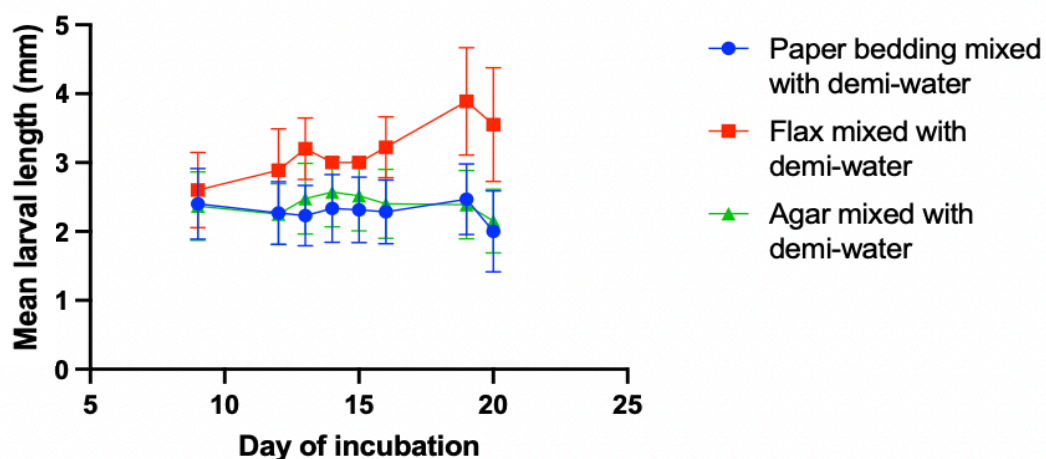


Figure 3. 5. Growth curve of axenic BSFL in paper bedding, flax and agar. The error bars represent the SD.

Figure 3. 6 present the mean individual mass of axenic BSFL applied to paper bedding, flax and agar mixed with demi-water. Larval masses were measured on day 20. Significant differences were obtained between the sterile flax and both the sterile paper bedding and the agar as inert matrices. This is represented by the asterisks on the graph.

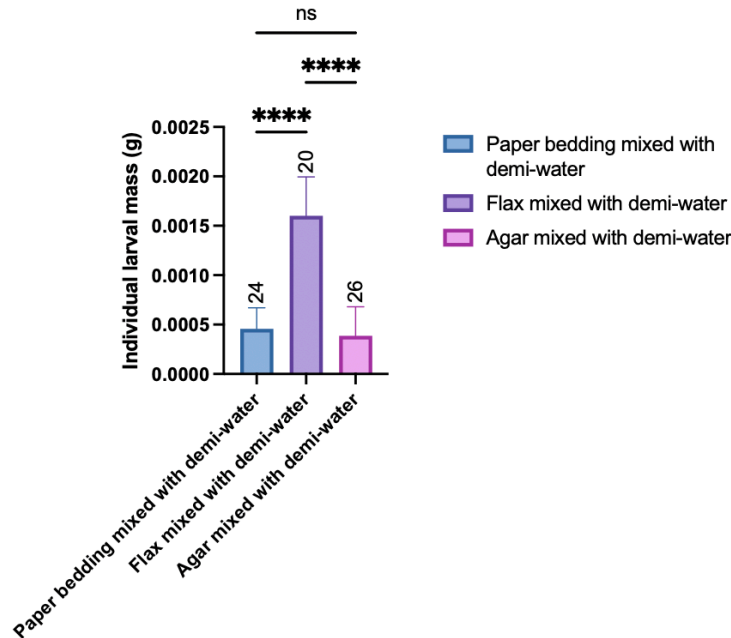


Figure 3. 6. Mean mass of an individual axenic BSFL in paper bedding, flax and agar at 20 days. The error bars represent the SD. The number of larvae measured per treatment is displayed above the error bar. Significant differences are represented by the four asterisks ($p < 0,0001$).

Survival rates are determined for each test tube based on the number of larvae measured at the end of the experiment. The average survival rate per treatment is presented in Table 3. 7. Ten neonates were applied to each test tube.

Table 3. 7. Average survival rates of the larvae in the non-digestable substrates

Treatment	Average survival rate (%)
Paper bedding mixed with demi-water	90
Flax mixed with demi-water	76,7
Agar mixed with demi-water	96,7

3.3.2 Protein-rich diet

Figure 3. 7 presents the growth curve of the axenic BSFL applied to paper bedding as inert matrix infused with the treatments with different concentrations of yeast and dextrose. Larval lengths were measured from day 12 to 26. The growth curve is based on different numbers of measured larvae, depending on their visibility since the test tubes were not opened after transferring the neonates to ensure sterility. The number of larvae measured per treatment per day is shown in Appendix 4 in Table 5. The growth performance of the axenic BSFL model was relatively similar for the different treatments, except for the treatment consisting of only vitamins and no yeast or proteins. This treatment showed reduced larval growth.

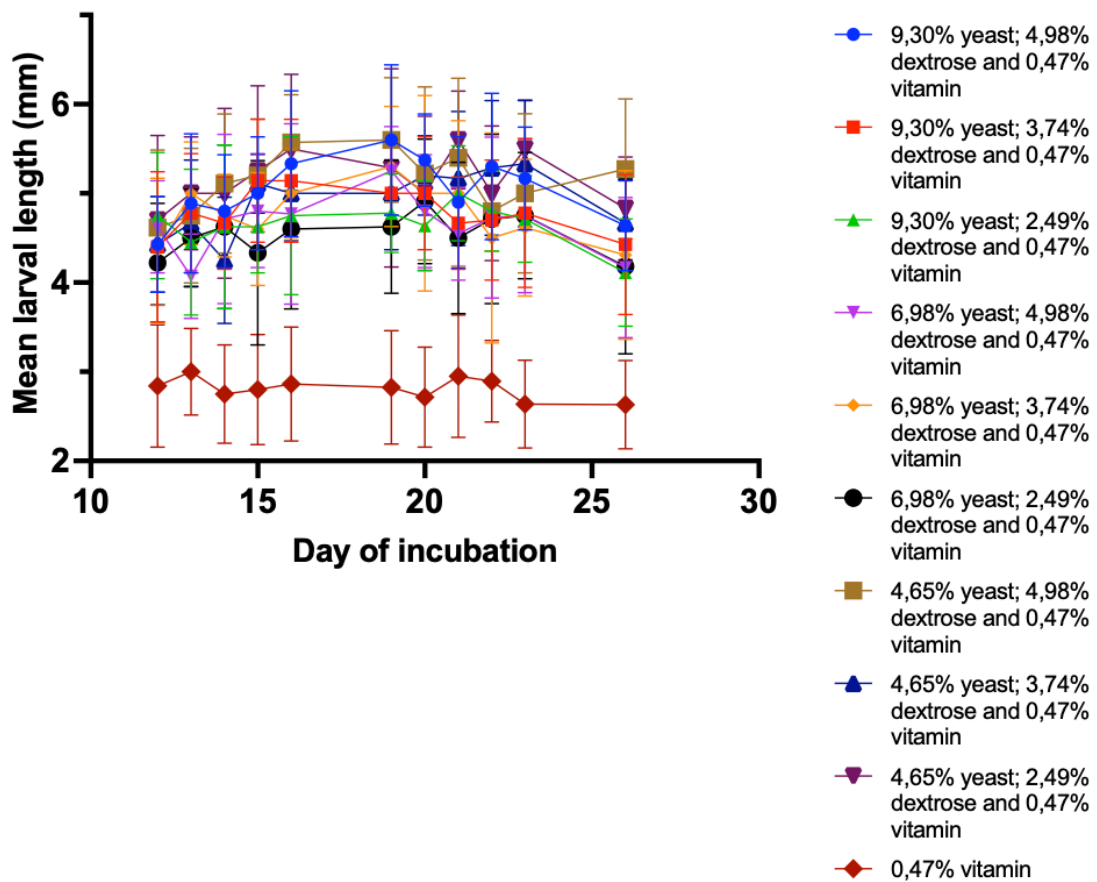


Figure 3. 7. Growth curve of axenic BSFL in the treatments with different concentrations of yeast and dextrose on paper bedding. The error bars represent the SD.

3.3.3 Sugar-rich diet

The growth curves of axenic BSFL, applied either as ten neonates per test tube or as sterilized eggs, on the sugar-rich diet added to the different non-digestible substrates are shown in Figure 3. 8 and Figure 3. 9, respectively. Larval lengths were measured from day 9 to 23 for the test tubes with transferred neonates and from 5 to 16 for the test tubes with sterilized eggs directly applied to the treatments. The growth curve is based on different numbers of measured larvae, depending on their visibility since the test tubes were not opened after transferring the neonates or sterilized eggs to ensure sterility. The number of larvae measured per treatment per day is shown for the tubes with 10 neonates in Appendix 5 in Table 6 and for the tubes with sterilized eggs in Table 7 in Appendix 5.

The larval growth of axenic BSFL on sterile flax mixed with a high-sugar media was increased compared to paper bedding and agar as inert matrices.

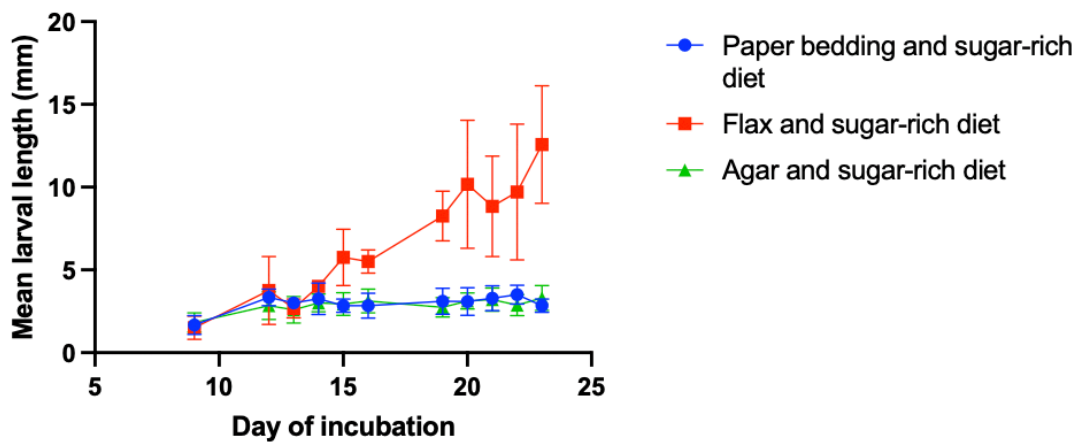


Figure 3. 8. Growth curve of axenic BSFL in the sugar-rich diet added to paper bedding, flax and agar. The error bars represent the SD.

The larval growth of axenic BSFL from sterilized eggs directly applied to the different substrates was increased for sterile flax infused with sugar-rich media compared to paper bedding and agar as inert matrices.

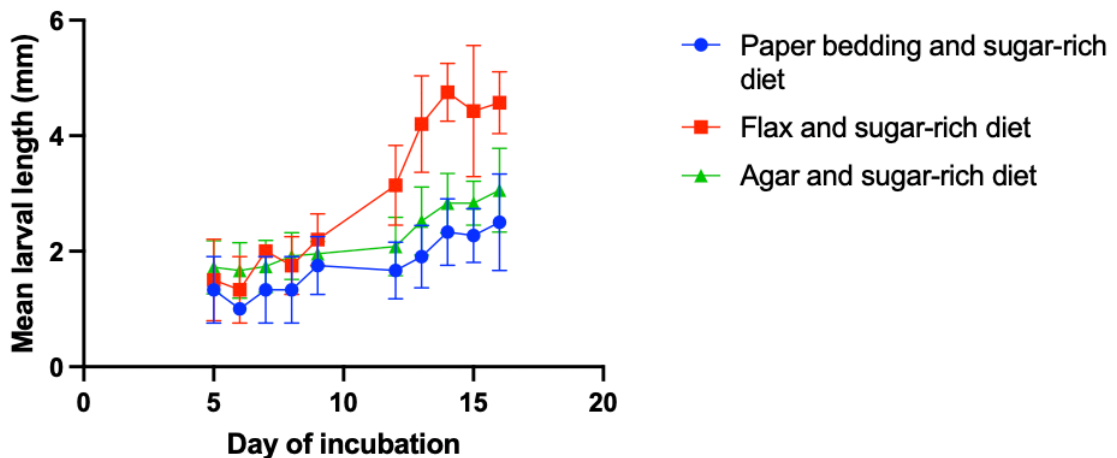


Figure 3. 9. Growth curve of axenic BSFL from sterilized eggs directly applied on the sugar-rich diets added to paper bedding, flax and agar. The error bars represent the SD.

3.3.4 Protein-rich diet compared to sugar-rich diet

After transferring the neonates to the non-digestible substrates, the test tubes are not opened again to ensure sterility. After 20 days, sterility tests with TSB (2.1.3.1) were performed on both the larvae and substrates. The results of these sterility tests are presented in Table 3. 8. Pictures of the sterility tests can be accessed in Table 8 in Appendix 6.

Table 3. 8. Sterility of larvae and substrates

Name substrate	Sterility 1 larva	Sterility substrate
High-protein media on paper bedding 1	No growth	No growth
High-protein media on paper bedding 2	Growth	Growth
High-protein media on paper bedding 3	No growth	No growth
High-protein media on flax 1	No growth	No growth
High-protein media on flax 2	No growth	No growth
High-protein media on flax 3	No growth	No growth
High-sugar media on paper bedding 1	No growth	No growth
High-sugar media on paper bedding 2	No growth	No growth
High-sugar media on paper bedding 3	No growth	No growth
High-sugar media on flax 1	No growth	No growth
High-sugar media on flax 2	No growth	No growth
High-sugar media on flax 3	No growth	No growth

The results of the second test tube with the high-protein media on paper bedding were discarded during the establishment of the following graphs since sterility was not preserved in it.

Figure 3. 10 presents the growth curve of axenic BSFL applied to paper bedding and flax infused with a protein-rich diet and a sugar-rich diet. Larval lengths were measured from day 9 to 20. The growth curve is based on different numbers of measured larvae, depending on their visibility since the test tubes were not opened after transferring the neonates or sterilized eggs to ensure sterility. The number of larvae measured per treatment per day is shown in Appendix 6 in Table 9.

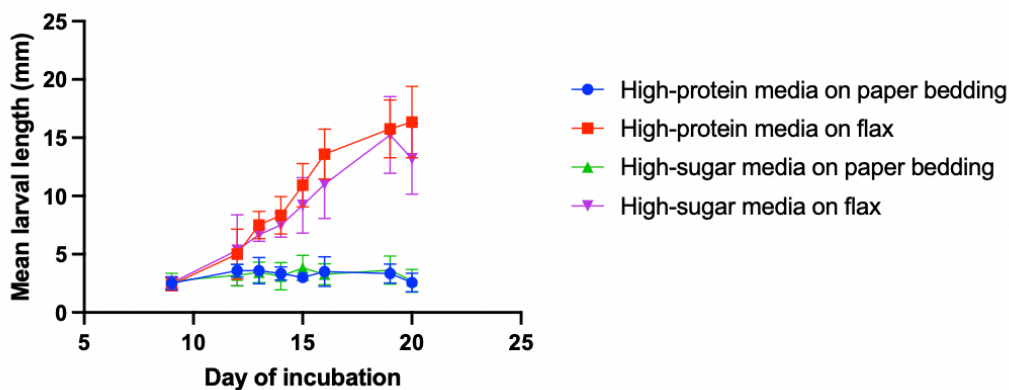


Figure 3. 10. Growth curve of axenic BSFL in the protein-rich diet compared to BSFL in the sugar-rich diet. The error bars represent the SD.

Figure 3. 11 present the mean individual mass of axenic BSFL applied to paper bedding and flax mixed with a protein-rich and sugar-rich diet. Larval masses were measured on day 20.

The growth performance of the axenic BSFL model was increased for the two flax treatments compared to the paper bedding treatments. This was both in larval length and individual mass.

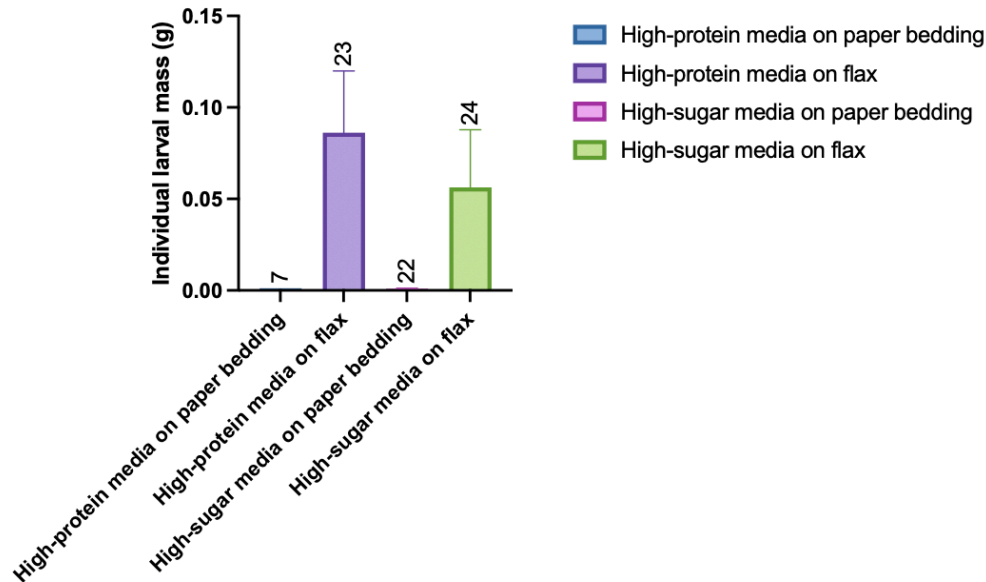


Figure 3. 11. Mean mass of an individual axenic BSFL in the protein-rich diet compared to axenic BSFL in the sugar-rich diet. The error bars represent the SD. The number of larvae measured per treatment is displayed above the error bar.

The mean individual mass of axenic BSFL applied to high-protein and high-sugar diet is shown in Figure 3. 12 and Figure 3. 13 for paper bedding and flax, respectively. The values of these non-digestible substrates mixed with demineralized water is also shown as blank. Significant differences were obtained only between the different flax treatments.

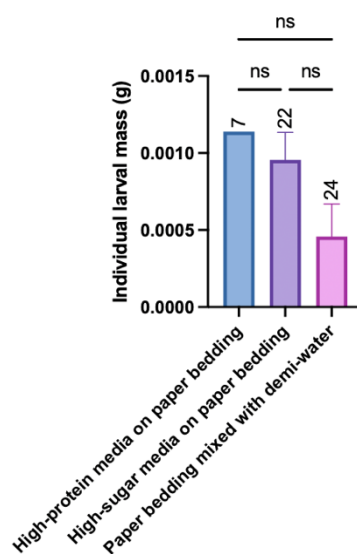


Figure 3. 12. Comparison of the mean mass of an individual axenic BSFL on paper bedding infused with the protein-rich diet and the sugar rich diet. The error bars represent the SD. The number of larvae measured per treatment is displayed above the error bar.

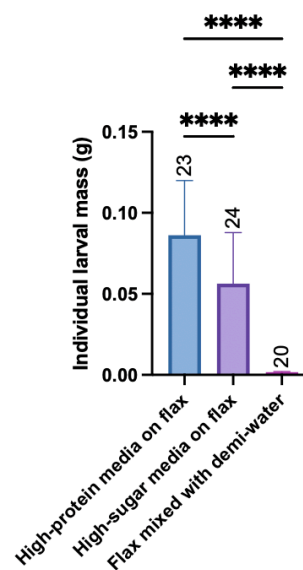


Figure 3. 13. Comparison of the mean mass of an individual axenic BSFL on flax infused with the protein-rich diet and the sugar-rich diet. The error bars represent the SD. The number of larvae measured per treatment is displayed above the error bar. Significant differences are represented by the four asterisks ($p < 0,0001$).

Survival rates are determined for each test tube based on the number of larvae measured at the end of the experiment. The average survival rate per treatment is presented in Table 3. 9. Ten neonates were applied to each test tube.

Table 3. 9. Average survival rates of the larvae

Treatment	Average survival rate (%)
High-protein media on paper bedding	40
High-protein media on flax	76,7
High-sugar media on paper bedding	83,3
High-sugar media on flax	80

3.3.5 Impact of grinded paper bedding and flax on obtaining the nutrients

The black construction paper and the egg debris were sterile as well as all samples taken from the test tubes on day 22.

Figure 3. 14 presents the growth curve of axenic BSFL applied to regular and grinded paper bedding and flax infused with a sugar-rich diet. Larval lengths were measured from day 8 to 22. The growth curve is based on different numbers of measured larvae, depending on their visibility since the test tubes were not opened after transferring the neonates to ensure sterility. The number of larvae measured per treatment per day is shown in Appendix 7 in Table 10. The larval growth of axenic BSFL on the two flax treatments was increased compared to the paper bedding treatments.

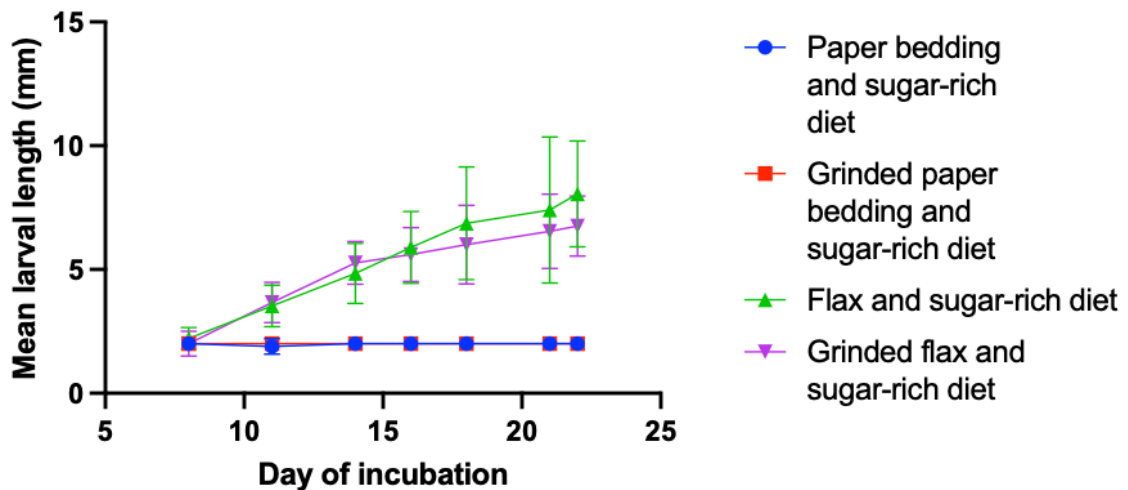


Figure 3. 14. Growth curve of axenic BSFL in the sugar-rich diet added to regular and grinded paper bedding and flax. The error bars represent the SD.

4 DISCUSSION

4.1 Establishing an axenic model for BSFL

4.1.1 Larval sterility and viability after sterilization

The first larval bacteria are mainly derived from the mother as the neonates consume the egg chorion (Wu et al., 2023). Therefore, dechoriation was performed during the sterilization process. Since the chorion and lipid layer on the surface of the vitelline membrane contribute to maintaining the moisture levels, it is important to bring the eggs into an ideal environment for rearing after sterilization (Urban-Duarte et al., 2022). As a result, it is opted to transfer the sterilized eggs on a piece of black paper. This allows the eggs to dry faster, and once transferred, the paper is able to absorb small levels of moisture from the substrate. This ensures that the eggs do not dry out completely during incubation. Previous experimental trials have shown that ensuring certain moisture levels is important for egg hatching. The eggs did not hatch when they were pipetted directly onto the nursery substrate. When using a black plastic weighing boat, a 1,5 mL Eppendorf tube or a cap of an Eppendorf tube to transfer the eggs, the eggs did not sufficiently dry after the sterilization process and viability was severely reduced. Once applied to the substrate, these plastic materials do not allow moisture to pass through from the substrate, which could also cause the eggs to dry out completely. These are possible reasons for failure to hatch eggs in these experimental trials.

Two days after collecting, sterilizing and applying the eggs in a 96 well plate filled with 100 μ L and one egg per well, the results shown in Table 3. 1 were obtained. The wells were observed for possible visible colonies on the PCA and larval crawling lines (Gold et al., 2020). A sterility of 93,75% and a viability of 88,54% were obtained. This is a relatively high percentage for sterility indicating high reliability of the sterilization process. The discrepancies could be from undetected clumps of eggs that may have reduced the contact between the chemicals and the eggs, leaving microorganisms on the eggshells. Another possibility is that contaminations occurred during the transfer of the eggs after sterilization into the wells. This was done with autoclaved toothpicks since the eggs are very small. The percentage obtained for viability is also relatively high. For conventional BSFL, it is stated that eggs hatch after about 4 days (De Smet et al., 2018). However, for the axenic model, hatching was expected to be delayed since the eggs could be more vulnerable after undergoing the chemical treatments that induce the removal of the egg chorion (Urban-Duarte et al., 2022). Remarkably, this was not the case since the majority of eggs hatch just two days after collection. The eggs for this experimental setup were collected and sterilized on Wednesday. Eggs were also collected on the previous Monday so the used eggs could be up to two days old at the time of harvesting. As a result, at the time of the acquired results, the eggs could be two to four days old. This could explain the large amount of eggs already hatched. Due to this high sterility and viability, it can be concluded that the established sterilization protocol for BSF eggs is successful.

Remarkably, the complete development circle can be obtained with this established protocol. This was not expected, but the larvae are able to pupate into axenic flies in the nursery pots containing CSM.

4.1.2 Water holding capacities

The WHC is determined in order to use the amount of liquid that can be completely absorbed by the substrate for further experimental purposes. Substrates hydrated according to their WHC achieve the highest survival and growth for BSFL (Frooninckx et al., 2024). The WHC was determined using a standardized method established by Frooninckx et al. (2024). Different from this method is that 1,5 mL Eppendorf tubes were used instead of 50 mL Falcon tubes. This is because a centrifuge for 50 mL Falcon tubes with a centrifugal force of 10 000 g was not available. Eppendorf tubes have a smaller opening than 50 mL Falcon tubes, which increases the surface tension and causes water to leak out more difficult from these. As a result, the excess water is pipetted off before being placed upside down for 30 minutes to allow further leaking. For chicken start mash and wheat bran, a WHC of 150,5% and 335,3% is obtained by this method, respectively. The WHC for the non-digestible substrates paper bedding, wood shavings and flax were 430,0%, 401,3% and 445,0%, respectively. By visual inspection, no excess water was present after 30 minutes of leaking and the substrates appeared hydrated. As for the used flax, it is stated from the manufacturer that it has a moisture absorption up to 450 % (Jopack, 2025). The experimental WHC of the flax used is in accordance with the value obtained from the manufacturer. Because of this, it can be assumed that the adjusted protocol is relatively accurate. For assurance of this, the original protocol can be performed on these substrates after which the WHC values can be compared.

4.2 The effect of the microbiome in different rearing substrates on the growth of axenic BSFL

The neonates are transferred to the different substrates to be examined because applying the sterilized eggs directly to the substrates causes some difficulties. The eggs are in fact very small to count with the bare eye and sterility cannot be guaranteed when counting under the microscope. A bigger issue is that it is unknown how many eggs would hatch so that when applying a standardized number of sterilized eggs to the treatments, there could still end up with a different number of larvae. As a result, the amount of sterilized eggs per substrate would not be standardized.

In contrast, transferring the neonates in a sterile manner is labor intensive and critical for ensuring sterility. This makes it necessary to perform sterility tests on both larvae and substrates. There is also a lot of variation in length of the eight- or nine-day-old larvae. Therefore, the ten larvae per substrate are randomly picked. The small differences in initial weight are probably less likely to influence the final outcome since the larvae grow so much from the beginning to the end of the experiment.

4.2.1 Sterility of the larvae and the substrates

It was expected that only the larvae and substrates from the test tubes of treatments 3 and 4 cause growth after incubation in TSB since that the axenic neonates here are transferred onto non-sterile substrates. However, growth was also observed in treatment 5b as shown in the picture in Appendix 2 in Table 1. Remarkably, mainly fungal growth is suspected and no bacterial growth since that the TSB remains clear. This contamination most probably came from transferring the neonates to the substrate since mold was already observed in test tube 5b on day 16.

4.2.2 Larval growth

Once applied to the substrates, larval lengths were measured per incubation day. This was accomplished by using a ruler, without opening the test tubes to ensure sterility. As a result, each measurement had a different number of larvae per test tube depending on the number of visible larvae. This number of measured larvae used to establish the growth curves are presented in Table 2 in Appendix 2. As the growth curves were established with different numbers of larvae measured per treatment per day, these curves represent an estimate of the growth performance. Therefore, the variation in the lengths of the different BSFL applied to the same treatment is only included in the results of the final measurements. Length and mass are two important indicators to measure the biomass of BSFL.

The growth curves for the chicken start mash and wheat bran treatments are presented in Figure 3. 1 and Figure 3. 2, respectively. From day 10 to day 21, larval length increases for all CSM and WB treatments. The days of incubation are counted from the day on which the eggs were collected. The eggs are routinely collected by the Centre of Expertise Sustainable Biomass and Chemistry (Thomas More University of Applied Sciences, Geel, Belgium) on Mondays and Wednesdays. For this experimental setup, eggs collected on Monday were used which gives additional variation since that the ages of the eggs can differ up to four days, depending on whether they were laid in the collector on Friday, Saturday, Sunday, or Monday.

For the CSM treatments, the growth curves are relatively similar. This could be because it is a source of high-quality nutrition which is important for neonate survival. For this reason, chicken feed is also the most commonly used nursery diet in BSF industrial rearing (Zhang et al., 2023). For the WB treatments, axenic larvae did not grow as fast on the sterile non pre-incubated substrate as on the other substrates. This is similarly for the CSM treatments, but the difference here is much smaller. As a result, it can be concluded that the presence of sterile microbial metabolites promotes the growth of BSFL. The axenic BSFL applied to the pre-incubated substrates grow about as fast as those applied to non-sterile substrates which is very valuable for further investigation of the microbiological functions and metabolism reactions. When comparing the growth curves of the CSM treatments with those of the WB treatments, it can be concluded that axenic larvae grow faster on sterile CSM than on sterile WB. This is probably because CSM is richer in nutrients than WB. Performing nutrient analyses on these substrates is very valuable to validate this hypothesis.

Some growth curves do not trend upward every day because the mean larval length is lower than the days before and after. This is not possible since the larval length would then decrease. These discrepancies are due to the different numbers of larvae measured per treatment per day. This results in different larvae being measured each day so that smaller or larger larvae that have a large impact on the mean are not included on some days. Another reason for these discrepancies is that the larvae sometimes crawl during measurement. This causes elongation and enlargement of their length. The error bars displayed on the growth curves represent the standard deviations (SD). These represent the spread of data around the mean value. Since that the error bars are relatively large, this indicates a wider spread of the different results obtained for the same sample under well-defined conditions. This large spread is probably from both natural variation and variation from less precise performance of measurements to maintain the sterility. This makes the repeatability of this experiment weaker since a different amount of larvae was measured on each day. However, given the possibility of contamination each time the vials are opened, this was determined to be the best way to measure intermediate growth.

The mean individual masses of BSFL on day 21 in chicken start mash and wheat bran treatments, respectively, are presented in Figure 3. 3 and Figure 3. 4. For the CSM treatments, there is also not much variation in individual larval mass between the different substrates. This is probably due to the presence of high-quality nutrition. The statistical analysis also revealed that there was no significant difference for the means of the four CSM treatments examined (tested with $\alpha = 0,05$).

The individual mass of larvae in sterile non-preincubated WB is much lower than in the other substrates. The difference in mean individual larval mass between the sterile WB and the other WB treatments is significant. This is in line with the growth curve of the WB treatments and indicates the presence of growth-promoting metabolites whose beneficial characteristics are retained after the sterilization process. Microorganisms present in the substrate are able to hydrolyze macronutrients into smaller molecules. This is beneficial as these products of microbial hydrolysis are more readily available for BSFL metabolism. The microbes can also provide molecules that the larvae themselves are unable to make. In addition, the microorganisms may be able to convert molecules into microbial biomass which, for example, increases the total amount of amino acids (Gold et al., 2018). This hypothesis can be examined by performing the nutrient analyses.

The SD is represented via the error bars on the graphs. These error bars are also relatively large for both graphs that represent individual masses. This indicates a wider spread of the results around the mean value. This large spread probably comes from natural variation in masses of equally aged larvae coming from the same colony. There is also variation in the number of weighted larvae. The number of larvae measured to obtain the mean individual larval mass is displayed above the error bars on the curves. The maximal number of larvae measured was 30 per treatment since each treatment was performed in triplicate and ten larvae were applied per test tube. For the three-day pre-incubated sterile CSM, the mean individual larval mass is only based on 18 measurements since the values from test tube 5b were omitted due to the presence of contamination. The average larval survival rates for each treatment were also calculated depending on the number of larvae measured at the end of the experimental setup. These results are shown in Table 3. 4. The average survival rate of this experimental setup is at least 93,3% which is relatively high. For both the fresh-made non-sterile CSM and WB, survival rates were 100% in all test tubes. This may be due to the introduction of microorganisms to the axenic BSFL. In contrast, the decrease in the survival rate may be due to various factors such as a deficiency of essential nutrients in the substrate, a reduction in vitality due to the sterilization process, the experiencing of stress due to the transfer etc. Competition may also be a factor in the reduction in survival since it is hypothesized that smaller larvae can sometimes be eaten by larger larvae.

4.2.3 Nutrient determination

The results of the nutrient analyses performed are shown in Table 3. 5. Four analyses were performed, namely the determination of dry matter content, ash content, fat content and protein content. These analyses were performed on fresh and six-day pre-incubated chicken start mash and wheat bran.

The dry matter content was determined after the lyophilization process. Therefore, this analysis does not provide information on the moisture content of the substrates but on the accuracy of the lyophilization process. For further analyses, it is important that the moisture contents are less than 10%. Indeed, complete combustion can be delayed by the moisture present when determining the ash content of a moist sample. The methods to determine ash, fat and protein content are also gravimetric which means that high moisture levels that can affect weight measurements can lead to incorrect results. The average dry matter content of the fresh CSM and WB was 96,208% and 97,840%, respectively. For the six-day pre-incubated CSM and WB, the average dry matter content was 96,168% and 97,017%, respectively. The average moisture content is then 3,792% and 2,160% for the fresh CSM and WB, respectively. For the six-day pre-incubated CSM and WB, the moisture contents after lyophilization were 3,832% and 2,983%, respectively. The moisture contents of all four samples to be analyzed are less than 10%, allowing them to be used in further analyses. To determine the dry matter content of the substrates before freeze-drying, this experiment should be performed again.

Ash contents of 6,5516% and 6,1336% were obtained for fresh CSM and WB, respectively. For the six-day pre-incubated CSM and WB, ash contents of 8,6708% and 9,5226%, respectively, were obtained. For the fresh CSM sample, the reference value from the manufacturer is 5,7% (Aveve, 2025). For the experimentally determined ash content compared to the manufacturer's value, a deviation of 14,9% is obtained. This deviation is relatively high and is possibly from differences between CSM batches and/or errors during the execution of the experiment. The ash contents of the fresh samples are relatively similar. For both the CSM and WB substrates, a higher ash content was obtained for the pre-incubated samples as for the fresh samples. Based on these results, it can be inferred that the microbial activity during the pre-incubation process, is capable of converting metal ions present in the sample into minerals. Bacteria would be able to interact with metal ions from the environment due to their small size and metabolic capacities. Their high surface-to-volume ratio along with the presence of charged chemical groups on the cell surface are responsible for the mineral-forming ability. Metal ions present in an aqueous environment can interact with the charged groups on the cell surface. These bonds will initiate further metal deposition and aggregate formation by lowering the total free energy (Douglas & Beveridge., 1998).

The fat content determined by Soxhlet extraction was 3,6611% and 2,8344% respectively for the fresh CSM and WB. For the six-day pre-incubated CSM and WB, the fat content was 5,4058% and 2,7380%, respectively. The fat content is the highest for the CSM samples. This confirms the hypothesis that CSM is richer in nutrients. The fat contents of the fresh WB sample and the pre-incubated one are quite similar. For CSM, however, there is more difference between the fresh and pre-incubated sample. For the fresh CSM sample, the reference value from the manufacturer is 4,00% (Aveve, 2025). The fat content, experimentally determined with Soxhlet extraction, shows a deviation of -8,47% from the manufacturer's value. The experimentally obtained value is lower than that of the manufacturer. Most probably, this deviation is from variation on different CSM batches. Remarkably, of the six-day pre-incubated samples, only the CSM one has a higher fat content than the fresh sample. However, it is suggested that metabolic capacities can be enhanced by conversion of substrate carbohydrates into short-chain fatty acids by fermentation (Gold et al., 2020). It is possible that CSM contains more carbohydrates than WB which allowed more short chain fatty acids to be formed by the microorganisms present. To confirm this hypothesis, the carbohydrate content of the samples must be determined.

The protein content determined by Kjeldahl was 19,47% and 18,17% for the fresh CSM and WB, respectively. For the six-day pre-incubated CSM and WB, the protein contents were 32,16 and 25,69%, respectively. For both fresh and pre-incubated substrates, CSM contains a higher protein content than WB. This also indicates CSM as a higher quality nutrient source than WB. For the fresh CSM sample, the reference value from the manufacturer is 18,00% (Aveve, 2025). A deviation of 8,14% is obtained for the experimentally determined protein content by Kjeldahl compared to the manufacturer's value. The experimentally obtained value is higher than that of the manufacturer. This deviation is most probably from variation between different CSM batches. For the reference, a recovery of 99,56% is obtained. This approaches 100% indicating a high accuracy of the method. The relatively large difference in protein levels between the fresh and pre-incubated substrates for both CSM and WB is remarkable. This confirms the previously established hypothesis that it is the microbial metabolites formed that promote growth. However, it is suggested that microorganisms can produce metabolites from non-protein molecules such as carbohydrates and increase amino acid levels in the diet (Gold et al., 2018). Further research on the identification of these microorganisms and the metabolization reactions is very valuable in scaling up BSFL rearing. BSFL have great potential as bioconverters in waste management. To obtain maximum yields, it is important to allow the larvae to grow up optimally in low-value substrates. Adding proteins to the substrates to make the larvae develop faster would decrease the sustainability. The use of microorganisms could provide a solution to this.

4.3 Developing broth-based substrates for axenic BSFL rearing

4.3.1 Nutritional value of non-digestible substrates

All test tubes in this setup are sterile for both larvae and substrates, tested with TSB as microbial growth medium. This makes all the results useful for constructing the curves. Since larval lengths were measured without opening the test tubes to maintain sterility, a different number of larvae per test tube was obtained during each measurement. This number of measured larvae used to establish the growth curve are presented in Appendix 3 in Table 4.

The growth curve of the axenic BSFL applied to paper bedding, flax and agar, all mixed with demi-water is shown in Figure 3. 5. This curve represents an estimate of the growth performance as it was established with different numbers of larvae measured per treatment per day. This curve revealed that axenic BSFL grow relatively better in flax as a solid matrix than in paper bedding or agar. These results were expected for agar since Auger et al. (2023) previously published that sterilized eggs hatch successfully on sterile culture media but do not exhibit the expected growth. This is probably due to the lack of a three-dimensional matrix for the larvae. In contrast, paper bedding also causes reduced growth. This is possibly due to a very low nutrient composition of paper bedding compared to flax. To validate this, nutrient analyses on both the flax and paper bedding should be performed. It is also possible that the reduced growth in paper bedding is due to the denser packing of the paper particles in the test tubes. The larval lengths were measured from day 9 to 20 and after the sterility tests on day 20, all larvae in the test tubes were weighed. The mean individual masses of axenic BSFL applied to paper bedding, flax and agar, all mixed with demi-water is shown in Figure 3. 6. The number of larvae measured is displayed above the error bar on the curve. The mean individual larval mass, as for the mean larval length, is the highest for flax as a solid matrix. The statistical analyses performed also revealed that the difference between individual larval masses on flax and those on paper bedding and agar was significant.

The SD is represented via the error bars on the graphs. These error bars are relatively large for both the growth curve and the bar chart representing the masses. This indicates a relatively large spread of the results around the mean value. Natural variation among different larvae of the same colony is a possible cause for this wider spread. Another possible cause is the age of the larvae. For this experimental setup, eggs collected on Wednesday were used, which creates additional variation because the eggs for this were collected on Monday and the age of the eggs may vary depending on the time of collection and whether they were placed in the collector on Monday, Tuesday or Wednesday. Advantageously, eggs collected on Wednesdays vary less in age than those collected on Mondays. The different number of larvae measured is also a factor that may be involved in the variation of the data.

The average larval survival rate was determined per treatment depending on the number of weighted larvae on day 20 and is shown in Table 3. 7. The highest survival rate was obtained for larvae in agar and the lowest for larvae in flax. This may be due to the fact that the larvae present in flax have the best growth performance and larger larvae may eat the smaller ones.

4.3.2 Protein-rich diet

Once 10 axenic neonates were applied to the different treatments, larval lengths were measured with a ruler per day of incubation. To maintain sterility, the test tubes were not opened once the neonates were applied. As a result, each measurement had a different number of larvae per test tube depending on the number of visible larvae. This number of measured larvae used to establish the growth curves are presented in Table 5 in Appendix 4. Therefore, the growth curve represents only an estimate of growth performance. The growth curve of axenic BSFL applied to treatments with different concentrations of yeast and dextrose shown in Figure 3. 7 revealed that from the concentrations tested, there is no particular ratio that improves BSFL growth markedly. Since this is a preliminary experiment and no clear difference was observed in growth performance between the different concentrations, no final sterility tests as well as final measurements were performed. The growth curve revealed mainly that axenic BSFL require proteins, sugars and a suitable three-dimensional matrix to grow. The previous trial has shown that flax is more suitable as a three-dimensional matrix than paper bedding. When a basal broth-based medium is identified that is optimal for BSFL growth, it can be opted to repeat this experiment with different concentrations of the nutrients present in that medium applied to flax as a three-dimensional matrix.

4.3.3 Sugar-rich diet

The growth curves for axenic neonates and sterilized eggs applied in sugar-rich diet, respectively, are shown in Figure 3. 8 and Figure 3. 9. This number of measured larvae used to establish the growth curves are presented in Appendix 5 in Table 6 for the tubes with the axenic neonates and in Table 7 for the tubes with the sterilized eggs. For both the axenic neonates and sterilized eggs applied to the different treatments, larval growth performance was highest in flax infused with sugar-rich media. As this is a preliminary experiment, no final sterility tests and final measurements were performed. This experimental trial revealed that applying sterilized eggs directly to the treatments presented several difficulties. For instance, the number of larvae per test tube is not standardised. This is due to the eggs being very small to count individually and apply to the substrates as well as because not all sterilized eggs hatch. The larval density per test tube also affects growth performance. During this experimental design, the number of applied sterilized eggs was very high which may negatively affect larval growth (Yakti et al., 2022). This preliminary trial also revealed that flax is a promising three-dimensional matrix for axenic BSFL rearing.

4.3.4 Protein-rich diet compared to sugar-rich diet

Growth was observed for both the test of a larva as well as the substrate of the second test tube of the high-protein media on paper bedding as shown in the picture in Appendix 6 in Table 8. Remarkably, mainly fungal growth is suspected and no bacterial growth since that the TSB remains clear. This contamination most probably came from transferring the neonates to the substrate since mold was already observed in the test tube on day 19. All other test tubes tested sterile for both a larva and substrate however. The graphs were thereby created using only the results from the sterile test tubes.

The growth curve and the curve showing the individual larval masses of axenic BSFL in high-protein and high-sugar medium on paper bedding and flax are shown in Figure 3. 10 and Figure 3. 11. The growth curve is based on different number of larvae measured per day. The number of larvae measured per treatment on each day is shown in Appendix 6 in Table 9. For the curves showing individual larval masses, the number of larvae are displayed above the error bars. The graphs show increased growth performance for axenic BSFL in flax compared to paper bedding. This both in larval length and individual mass. Previous research had also shown that flax is a suitable three-dimensional matrix for BSFL rearing.

Figure 3. 12 and Figure 3. 13 show the mean individual masses for BSFL on the paper bedding and flax treatments, respectively. There is no significant difference between the mean values of individual larval mass of the different paper bedding treatments. This is consistent with the growth curve as the lengths of the BSFL on paper bedding are relatively similar for the high-protein and high-sugar diets. However, there are significant differences for the different flax treatments. Also on the growth curve, there is a visible difference in larval length between BSFL in protein-rich diet applied on flax as in the sugar-rich diet. The statistical analysis also revealed that there was a significant difference between the mean larval mass of the three flax treatments examined (tested with $\alpha = 0,05$). Therefore, it can be concluded from the experimental trial that the protein-rich media combined with flax, is a promising substrate for axenic BSFL rearing.

The average larval survival rate was determined per treatment depending on the number of weighted larvae on day 20 and is shown in Table 3. 9. The lowest survival rate was obtained for larvae in the high-protein media on paper bedding. The average survival rates of axenic larvae in the flax treatments are acceptable. A repeat trial with these treatments is recommended to gain more insights into these survival rates.

4.3.5 Impact of grinded paper bedding and flax on obtaining the nutrients

All test tubes in this setup are sterile, tested with TSB as microbial growth medium. This makes all the results useful for constructing the growth curve, shown in Figure 3. 14. The number of measured larvae used to establish the growth curve are presented in Appendix 7 in Table 10. As in the previous experiments, the average larval length is higher for the flax treatments than for the paper bedding treatments. For both paper bedding and flax, little to no difference is obtained in larval length between the regular and grinded substrate. As this is a preliminary experiment, no final sterility tests and final measurements were performed. This experimental trial revealed that grinding the solid matrices, did not positively affect the growth performance of axenic BSFL.

CONCLUSION

This study successfully established and optimized an axenic model for black soldier fly larvae, providing a foundational tool for future investigations into the functions of the microbiome and individual microbes. The axenic model, achieved through a carefully refined sterilization protocol involving 0,5% sodium hypochlorite and 70% ethanol, demonstrated high sterility (93,75%) and viability (88,54%) rates. This confirms the reliability of the dechoriation process and the suitability of the environment post-sterilization, particularly the use of black construction paper to maintain optimal moisture levels during drying and application to the nursery diet.

The research further explored the impact of microbial presence and substrate composition on larval growth. It was discovered that axenic larvae reared on sterile, non pre-incubated wheat bran exhibited significantly reduced growth compared to those reared on pre-incubated or non-sterile substrates. In contrast, axenic larvae reared on chicken start mash exhibited no significant differences in growth performance between the sterile, pre-incubated or non-sterile substrates. This suggests that the microbiome has a larger effect on low-quality substrates, probably due to its ability to compensate for its deficiencies. The microbial metabolites formed during substrate pre-incubation play a crucial role in promoting larval development in these low-quality nutritional sources, even in the absence of live microorganisms. Nutrient analyses supported this hypothesis, revealing increased protein and ash content in pre-incubated wheat bran, likely due to microbial conversion of carbohydrates and mineralization processes. These findings underscore the importance of substrate selection and microbial conditioning in optimizing BSFL rearing systems.

In addition to solid substrates, the research investigated the feasibility of broth-based diets combined with non-digestible matrices such as paper bedding, flax and agar. A broth diet can be easily adjusted in nutritional composition which is advantageous for further research on the nutritional requirements of the BSFL. Flax emerged as a superior three-dimensional matrix, supporting better larval growth and survival compared to paper bedding and agar. The combination of flax with a protein-rich broth diet yielded the most promising results. This opens new avenues for developing customizable, sterile and nutritionally defined diets for axenic BSFL rearing.

Overall, this research provides a robust framework for studying host-microbiota interactions in BSFL and optimizing the rearing conditions. The axenic model enables controlled experimentation with specific microbial strains, facilitating the identification of beneficial microbe functions and their metabolic contributions. This has far-reaching implications for large-scale BSFL rearing for purposes such as sustainable waste management and animal feed production. Future research should focus on identifying and characterizing key microbial metabolites that drive larval development and refining chemically defined diets to determine the nutritional requirements of BSFL.

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APPENDIX 1: STERILIZATION PROTOCOL

The sterilization protocol is adapted from the methods described by Gold et al. (2020).

Materials:

- Basal chicken feed diet (Chicken Start Mash) for BSF rearing
- Small autoclavable container with sealable lid (~250 mL glass pots with a plastic lid):



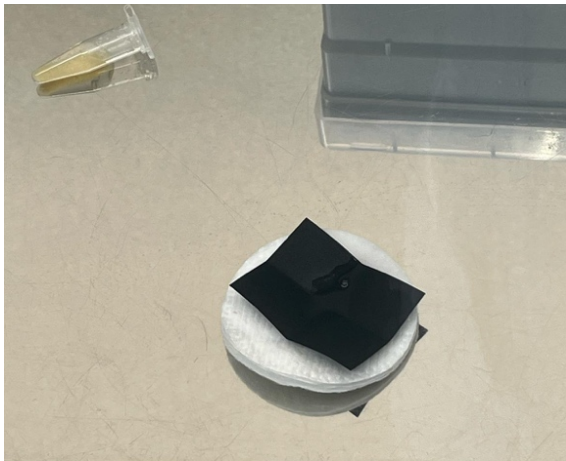
- p1000 pipette and disposable sterile tips
- Metal tweezers/spoon
- Aluminum foil
- Black construction paper
- Cotton pads
- Demineralized water
- Ethanol
- Bleach
- LB/TSB/BHI or other rich bacterial media
- 1,5 mL Eppendorf tubes and rack

Before beginning:

- Sterilize chicken feed or other nursery diet
 - Add 22,45 g chicken feed and 33,80 g water (ratio of 1,505)
 - Autoclave the moistened substrate
- As needed, prepare 5-10 wide-opening pipette tips by cutting off the bottom 2 mm of a p1000 tip with a razor blade, then sterilize tips by autoclaving
- Sterilize tweezer and spoon(s) by wrapping in aluminum foil and autoclaving
- Sterilize at least 20 mL demineralized water by autoclaving
- Sterilize squares of black paper and some cotton pads by autoclaving
- Prepare 70% ethanol and 0,5 – 0,6% NaClO solutions
 - Bleach concentrations are still commonly labelled in ° or °Cl
 - 3.16° is equivalent to 1% active Chlorine (NaClO)
 - Bleach (8°) is equivalent to ~2.5% active Chlorine
 - This can be diluted 1/5X to produce 0,5%
- To confirm sterility, it is useful to have a rack of vials containing 5-10 mL TSB.

When ready to sterilize eggs:

1. Working in a biosafety cabinet, sterilize the interior surfaces with 70% ethanol
2. Surface sterilize the following before placing in the biosafety cabinet:
 - Bottles containing ethanol, bleach and sterile demi-water
 - An additional beaker of 70% ethanol that can be used to sterilize the tweezers/spoon after unwrapping
 - Foil-wrapped sterile tweezer/spoons
 - Container with sterile chicken feed or other nursery diet
 - P1000 pipette and tips
 - Eppendorf tube rack
 - Container with sterile black paper and cotton pads
 - Waste beaker for discarded ethanol, bleach, water, and tips
3. Transfer approximately 0,1 g of eggs to a pre-weighed 1,5 mL Eppendorf tube (outside the biosafety cabinet). To sterilize more eggs, use multiple 1,5 mL tubes.
4. Sterilization of eggs consists of alternating ethanol and bleach treatments:
 - a. Add 1,2 mL 70% ethanol and disperse eggs by gently pipetting up and down
 - b. Let eggs settle to the bottom, then remove ethanol by pipette
 - c. Add 1,2 mL 0,5% NaClO and gently mix
 - d. Let eggs settle, then remove bleach by pipette
 - e. Repeat steps a-d 2x. The eggs should no longer be sticking to each other at all, and should move freely in the liquid. The color is also visibly whiter (less yellow).
5. Rinse eggs 3x with 1 mL sterile water (add water, mix gently, allow eggs to settle, then remove and discard water)
6. Using the sterile tweezer, arrange sterile paper filter(s) on top of a cotton pad. Tweezer should then be placed in the beaker of 70% ethanol rather than on the work surface.



7. Saturate the paper by adding 70% ethanol, 1 mL at a time. The goal is to break the surface tension of the paper so that liquid absorbs through the paper and into the cotton pads below. It works if the water does not form beads on the surface, but immediately soaks into the paper.
8. Using a wide-opening tip, resuspend the eggs in 600 μ L sterile demi-water. By pipetting up and down gently, it should be possible to get them (almost) all in suspension and then aspirated into the tip.

9. Moving quickly (the wide-opening tip may otherwise start to drip), transfer the water and eggs to the pre-wetted sterile paper. Most of the water should quickly absorb through the paper into the cotton pads below.



10. Let the eggs dry for ~30 minutes in the biosafety cabinet.
11. Using sterile tweezers, carefully transfer the paper with eggs into the pot of sterile chicken feed and close the container.



12. Larval growth should be monitored without opening the container. Early development may be somewhat delayed, but after 1 week there should be active 1st instar larvae visible in the substrate. If they seem crowded after 2 weeks, it is possible to thin them out by transferring a single scoop of larvae and substrate to an additional pot of sterilized feed. This should be done in the biosafety cabinet under the same conditions as described above using a metal spoon autoclaved in foil and only unwrapped in the biosafety cabinet.

APPENDIX 2: PRE-INCUBATION EXPERIMENT

Table 1. Sterility tests in TSB of both a larva and substrate of the experiment that investigated the impact of pre-incubated CSM and WB on the growth of axenic BSFL

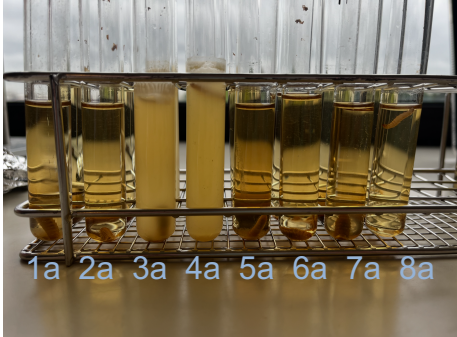
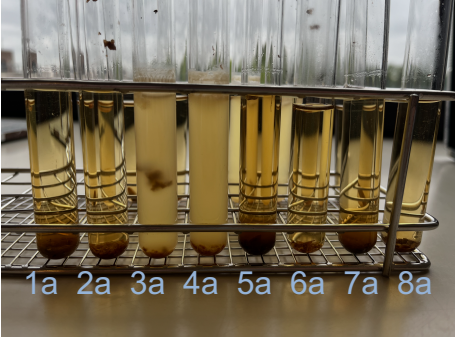
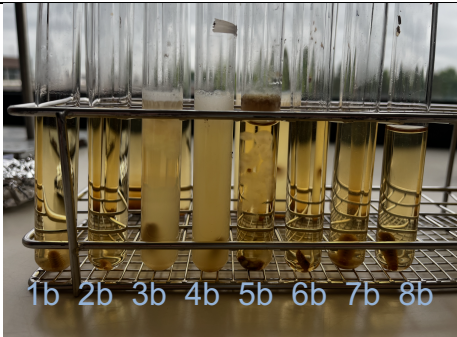
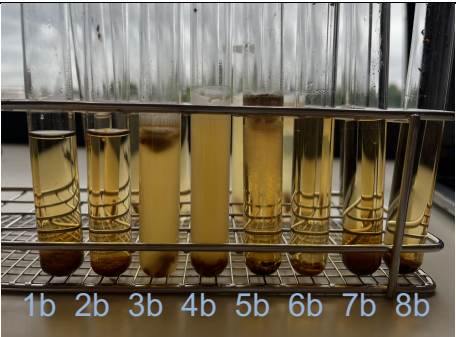
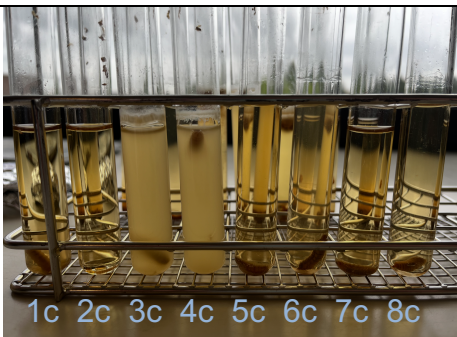
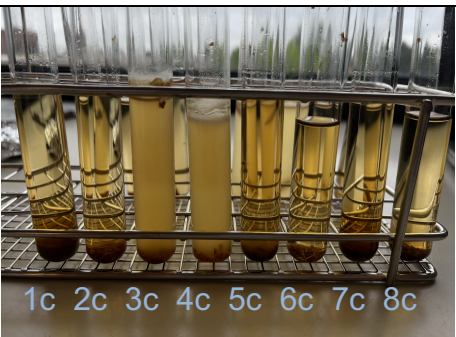
1 larva	Substrate
	
	
	

Table 2. Number of larvae measured for establishing a growth curve of the experiment investigating the impact of pre-incubated CSM and WB on the growth of axenic BSFL

Day of incubation	Number of larvae measured for the larval growth curve							
	Sterile CM (no pre-incubation)	Sterile WB (no pre-incubation)	Fresh-made non-sterile CM	Fresh-made non-sterile WB	3-day pre-incubated sterile CM	3-day pre-incubated sterile WB	6-day pre-incubated sterile CM	6-day pre-incubated sterile WB
10	7	10	10	4	7	1	6	3
11	3	3	7	4	2	2	2	2
14	3	4	9	3	4	2	0	1
15	3	4	8	6	2	3	3	4
16	3	1	9	6	3	5	8	8
18	5	2	6	7	5	3	3	7
21	25	25	27	27	16	24	26	27

APPENDIX 3: NUTRITIONAL VALUE OF THE NON-DIGESTIBLE SUBSTRATES

Table 3. Sterility tests in TSB of both a larva and substrate of the experiment that investigated the nutritional value of the non-digestible substrates

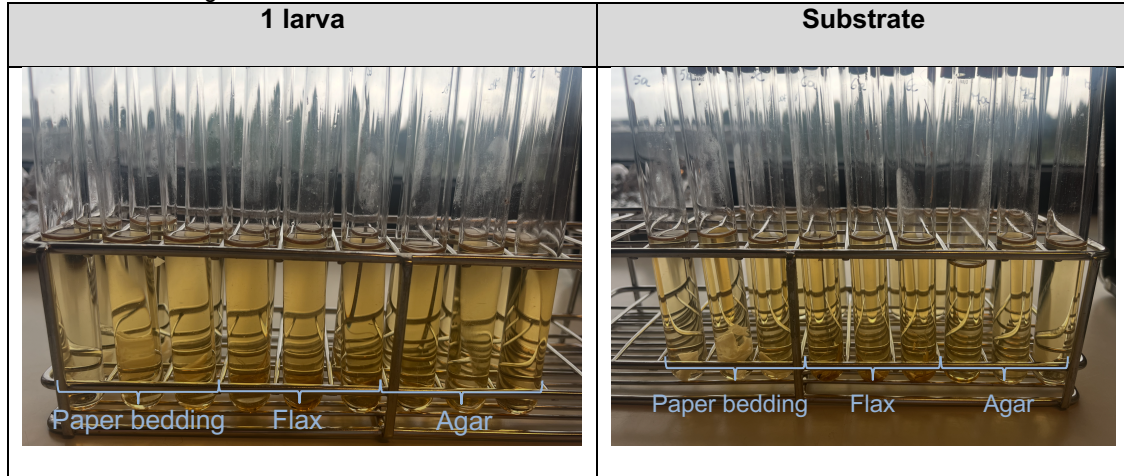


Table 4. Number of larvae measured for establishing a growth curve of the experiment investigating the nutritional value of the non-digestible substrates

Day of incubation	Number of larvae measured for the larval growth curve		
	Paper bedding with water	Flax with water	Agar with water
9	10	5	19
12	15	9	20
13	13	5	21
14	12	2	21
15	16	2	25
16	21	9	20
19	15	9	23
20	24	20	26

APPENDIX 4: PROTEIN-RICH DIET

Table 5. Number of larvae measured for establishing a growth curve of the experiment investigating the impact of different concentrations of yeast en dextrose on the growth of axenic BSFL

Day of incubation	Number of larvae measured for the larval growth curve									
	9,30% yeast; 4,98% dextrose and 0,47% vitamin	4,65% yeast; 4,98% dextrose and 0,47% vitamin	9,30% yeast; 2,49% dextrose and 0,47% vitamin	4,65% yeast; 2,49% dextrose and 0,47% vitamin	6,98% yeast; 3,74% dextrose and 0,47% vitamin	6,98% yeast; 2,49% dextrose and 0,47% vitamin	6,98% yeast; 4,98% dextrose and 0,47% vitamin	9,30% yeast; 3,74% dextrose and 0,47% vitamin	4,65% yeast; 3,74% dextrose and 0,47% vitamin	0,47% vitamin
12	7	13	8	10	11	9	8	10	7	19
13	9	12	11	6	13	6	14	9	9	18
14	10	9	8	12	8	8	7	6	8	20
15	11	10	8	4	15	6	10	7	9	20
16	6	7	8	6	9	5	13	7	8	22
19	10	10	9	7	10	8	4	6	6	17
20	8	9	11	9	6	11	10	6	5	21
21	10	5	8	5	13	10	11	6	6	20
22	10	5	5	8	10	7	11	10	7	19
23	12	6	7	6	13	8	12	9	9	22
26	11	11	9	12	13	11	18	7	12	19

APPENDIX 5: SUGAR-RICH DIET

Table 6. Number of larvae measured for establishing growth curve of the experiment investigating the impact of a sugar-rich diet applied to different non-digestible substrates on the growth of axenic BSFL

Day of incubation	Number of larvae measured for the larval growth curve		
	10 neonates on paper bedding with the sugar-rich diet	10 neonates on flax with the sugar-rich diet	10 neonates on agar with the sugar-rich diet
9	3	2	23
12	6	4	19
13	2	3	17
14	4	1	21
15	6	4	16
16	6	2	16
19	9	4	15
20	11	6	17
21	7	12	14
22	4	7	15
23	6	7	13

Table 7. Number of larvae measured for establishing growth curve of the experiment investigating the impact of a sugar-rich diet applied to different non-digestible substrates on the growth of axenic BSFL from sterilized eggs directly applied to the treatments

Day of incubation	Number of larvae measured for the larval growth curve		
	Sterilized eggs on paper bedding with the sugar-rich diet	Sterilized eggs on flax with the sugar-rich diet	Sterilized eggs on agar with the sugar-rich diet
5	3	2	18
6	2	3	24
7	3	2	19
8	3	4	24
9	4	5	23
12	12	7	24
13	11	5	23
14	3	4	18
15	11	7	24
16	6	7	18

APPENDIX 6: PROTEIN-RICH DIET COMPARED TO SUGAR-RICH DIET

Table 8. Sterility tests in TSB of both a larva and substrate of the experiment that investigated the most optimal broth-based substrate for BSFL rearing

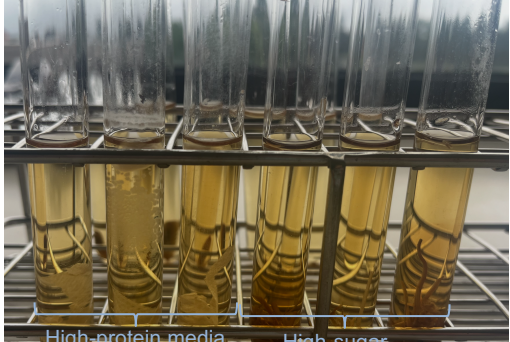
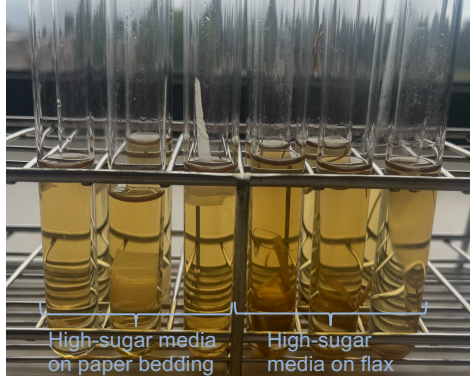
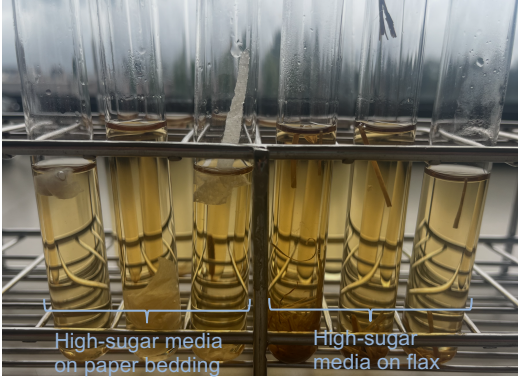
1 larva	Substrate
 High-protein media on paper bedding High-protein media on flax	 High-protein media on paper bedding High-sugar media on flax
 High-sugar media on paper bedding High-sugar media on flax	 High-sugar media on paper bedding High-sugar media on flax

Table 9. Number of larvae measured for establishing growth curve of the experiment investigating the most optimal broth-based substrate for BSFL rearing

Day of incubation	Number of larvae measured for the larval growth curve			
	High-protein media on paper bedding	High-protein media on flax	High-sugar media on paper bedding	High-sugar media on flax
9	6	5	10	9
12	5	4	10	3
13	5	10	9	3
14	3	12	9	6
15	6	14	11	5
16	4	12	11	9
19	6	13	11	13
20	7	20	22	21

APPENDIX 7: GRINDED PAPER BEDDING AND FLAX

Table 10. Number of larvae measured for establishing growth curve of the experiment investigating the impact of grinding the non-digestible substrates on obtaining the nutrients

Day of incubation	Number of larvae measured for the larval growth curve			
	Paper bedding and sugar-rich diet	Grinded paper bedding and sugar-rich diet	Flax and sugar-rich diet	Grinded flax and sugar-rich diet
8	5	7	9	17
11	10	11	15	6
14	16	19	19	19
16	30	39	19	18
18	42	57	22	24
21	44	48	17	24
22	53	58	20	21